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In this issue

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Foreword: non-coding RNAs as potential laboratory biomarkers

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FOREWORD

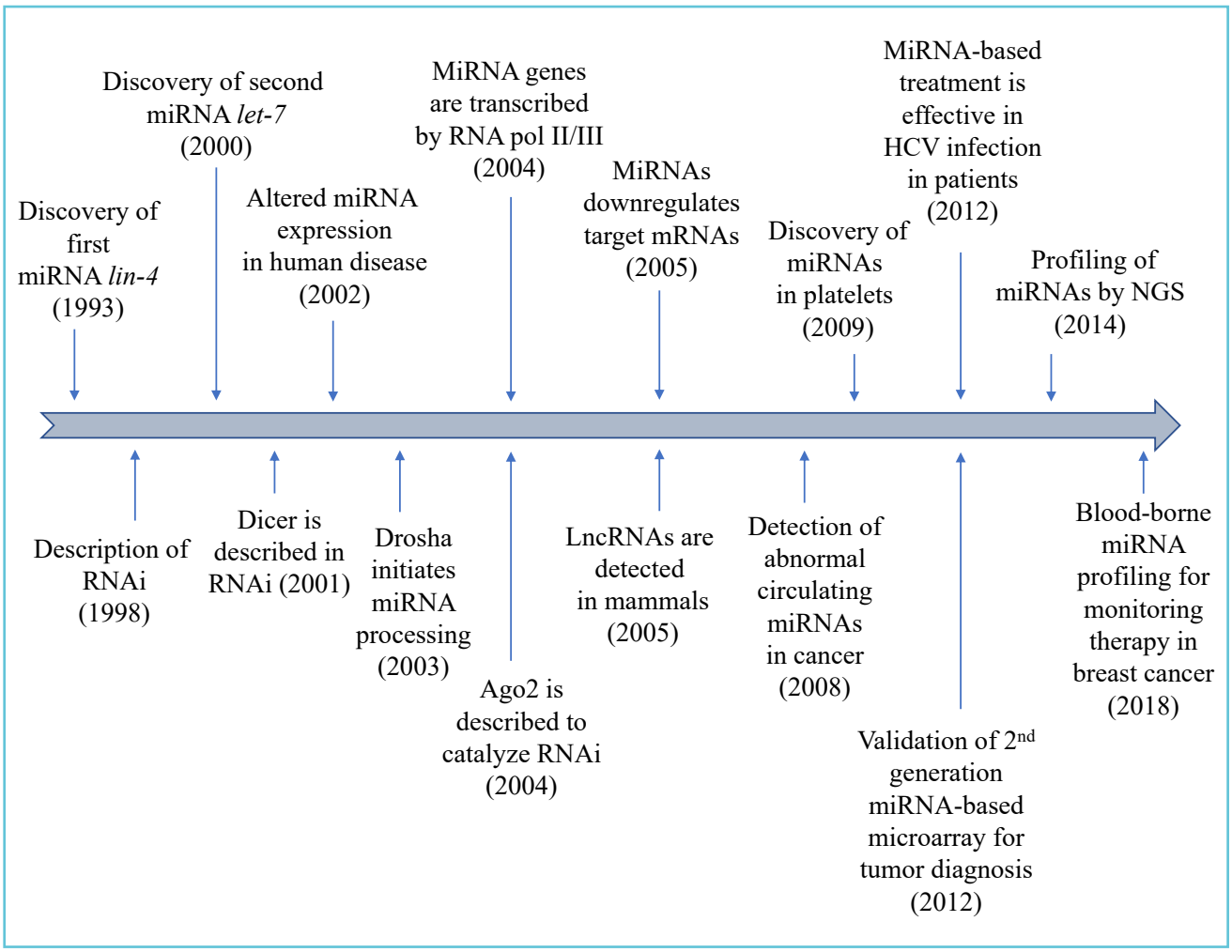
Since the discovery of non-coding RNAs, enormous information has been accumulated about the function of these molecules acting as fine-tuners of cellular processes in development, maintenance of homeostasis up to the generation of malignancies. The group of non-coding RNAs includes a large number of microRNAs (miRNAs), long non-coding RNAs (lncRNAs), small-nucleolar RNAs (snoRNAs) and circular RNAs (circRNAs), which are not translated into a protein, but regulate the translation of at least two-thirds of messenger RNAs (mRNAs) with chromatin modification and gene silencing. Furthermore, release of non-coding RNAs from donor cells and their uptake by recipient cells can provide additional intercellular signaling that may allow a direct regulation of gene expression in the recipient cells. Altered expression of non-coding RNAs have been implicated to the pathogenesis of diverse human diseases suggesting their potential to become diagnostic or prognostic molecular biomarkers in the near future.

Among non-coding RNAs, miRNAs have been the most intensively investigated generating thousands of publications in this field each year. Compared to mRNAs, miRNAs are stable in human body fluids, such as blood

plasma, serum, urine or saliva due to the association with RNA-binding proteins (e.g. high-density lipoproteins) and housing in shed microvesicles. In addition, they are fairly viable even after repeated cycles of freeze-thawing and long-term frozen storage. Despite these facts, there are still many pre-analytical and analytical challenges for accurate detection of extracellular miRNAs in body fluids. For instance, sample preparation and handling need to be minimized, and special attention is required to avoid contaminations with cellular miRNAs potentially released from erythrocytes or platelets. The concentration of cell-free miRNAs may be low and variable, thus equal volumes of specimens should be used for total RNA extraction. There is still no standardized methodology for the normalization of non-coding RNAs.

As a consequence, variable normalization methods are applied in different studies, such as small endogenous nucleolar RNAs (e.g. RNU-43) as reference genes, or external "spike-in" synthetic oligonucleotides (e.g. cel-miR-39), or one specific (mi)RNA, or global mean normalization when hundreds of miRNAs are simultaneously profiled. Sometimes these circumstances can

Figure 1 **Key findings in the non-coding RNA research with miRNA-based diagnostic and therapeutic implications from 1993 up to present time***



** lncRNA: long non-coding RNA; RNAi: RNA interference; NGS: new generation sequencing.*

make difficult the comparison of results. This is one reason why a wider miRNA profile prefers to be evaluated in patient samples in contrast to the analysis of individual miRNA. For such, new miRNA panels are now commercially available to observe "miRNA signatures". The milestones of 25-year-old evolution of non-coding RNA research are depicted in Figure 1.

This special issue of the eJIFCC incorporates a series of manuscripts that summarize the recent issues of non-coding RNAs as non-invasive biomarkers in various clinical conditions, especially focusing on cell-free miRNAs in different human diseases.

In the first manuscript, Bonneau *et al.* raised the question whether circulating miRNAs could be a reality in near clinical practice for diagnostic and therapeutic aspects. The authors reviewed the latest issues on miRNA-based laboratory diagnostics in malignancies, age-related diseases, and abnormal heart and liver function. In addition, some new therapeutic products are represented for liver disease, fibrotic disorders and cancers.

Sepsis is still a demanding clinical condition and early (differential) diagnosis and evaluation of prognosis are a must for these patients. Szilágyi *et al.* summarized the most important intracellular miRNAs with their function in Toll-like receptor mediated signaling in immune cells as well as platelets along with those circulating miRNAs, which have been recently reported to be valuable in sepsis.

In the last couple of years, a number of miRNA-related manuscripts have been published in the field of endocrine neoplasms, such as pituitary adenomas. Here, Butz and Patócs reviewed the current knowledge on circulating and tissue specific miRNAs in thyroid, adrenal, pituitary and neuroendocrine malignancies for diagnostic and prognostic implications.

Congenital heart diseases (CHD) are the most common type of birth anomalies, with high morbidity and mortality rates. Hence, a better understanding of the function of miRNAs in the pathomechanism of CHD may propagate their application for laboratory analysis to improve the diagnosis and prognosis of these patients. Nagy and her colleagues gave an overview about altered expression of miRNAs in different subtypes of CHD.

Coronary artery disease (CAD) is one of the leading cause of death worldwide. Several former studies reported that certain circulating miRNAs have substantial diagnostic and prognostic values for CAD. Melak *et al.* described the most suitable miRNAs as potential biomarkers in CAD with those circumstances that may limit utility or interfere with their levels.

The first evidence about abnormal miRNA expression in relation to a disease was reported in chronic lymphocytic leukemia in 2002. Since then, several hematological disorders have been investigated for profiling circulating miRNAs. In this issue, Getaneh *et al.* summarized the clinical values of miRNAs in the subtypes of B-cell non-Hodgkin lymphoma in terms of diagnostic and prognostic implications.

Among cell-free nucleic acids, circulating miRNAs have been considered promising tools for the diagnosis of pregnancy associated conditions like preeclampsia, fetal growth restriction and gestational diabetes. These prenatal tests, which are still in the experimental phase, were also reviewed by Dr. Nagy.

Beside circulating miRNAs, lncRNAs have been also confirmed to be involved in the pathogenesis of cancers and inflammatory diseases. Kelemen and her colleagues summarized the role of exosomal lncRNAs as potential biomarkers in chronic inflammatory diseases, such as rheumatoid arthritis and psoriasis as well as in different types of cancer.

In case of rare diseases, profiling of tissue specific miRNAs can also assist the differential di-

agnosis. Zilahi *et al*. described five specific tissue miRNAs (myomiR) that are associated with the development of polymyositis in an original article.

In conclusion, we are learning about more and more aspects of the diagnostic application of non-coding RNAs, but we should note that these results may be incorporated into the area of therapeutic implications. Based on preliminary data, the modulation of certain miRNA function by specific mimics or inhibitors may result in beneficial effects in hepatitis C infection, or in cancer. However, it is a far-reaching story, and is still under intensive (pre)clinical investigation.

In this issue: Non-coding RNAs as potential laboratory biomarkers

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How close are miRNAs from clinical practice? A perspective on the diagnostic and therapeutic market

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ABSTRACT

The discovery of miRNAs in the mid-90s has changed the dogma of gene expression regulation. Currently, miRNAs are the main theme of thousands of publications each year and their involvement in human diseases is everyday more deeply understood. With that being known, what are the actual clinical applications of miRNAs and how far are they truly from the patients? To address this question, we reviewed the miRNA diagnostic and therapeutic market. With many companies developing miRNA panels, the activity is high in the diagnostic area. Some products, notably for thyroid cancer (Interpace Diagnostic), are already available to clinician and covered by major insurance companies. In comparison, the therapeutic market, mainly driven by miRNA mimics and antagomiR products, is less advanced. Miravirsen (produced by Roche/Santaris) and RG-101 (produced by Regulus Therapeutics), designed to treat hepatitis C, are considered the flagship products of this class of future

drugs. All of the miRNA-based drugs are currently in clinical trials and none have yet reached the pharmaceutical breakthrough. However, acquisition of miRNA-based companies by major pharmas is sending a positive feedback on their potentials. With multiple initiatives on their way, the next years will definitely be determinant for the miRNA market that is still in his infancy.

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INTRODUCTION

For the last thirty years, the fundamental research into RNA biology has grown at an exponential rate. We are now better positioned than ever to understand the involvement of RNA in almost all critical cellular processes. Indeed, for many years, the number of non-coding RNA discovered has steadily increased. Hence, it is not surprising that several Nobel prizes were awarded for corner stone RNA discoveries, such as those won by Cech and Altman in 1989 (RNA catalytic activities; (1)), Ramakrishnan, Steitz and Yonath in 2009 (ribosome structure; (2)), and of most interest for this review, to Fire and Mello in 2006 (RNA interference; (3)).

Considering the increase in RNA-focused research, one can expect that the advancement of general and specific knowledge about RNA could result in direct clinical applications. For example, more than 45,000 studies were published in 2017 on RNA (Figure 1A). From these, a large proportion of the studies either considered that their work could contribute to the diagnosis or the treatment of disease (about 13,000 and 10,000, respectively; Figure 1B).

From the multitude of RNA discoveries, one of the most important was the discovery of RNA interference by Fire and Mello and miRNAs by Ambros and colleagues (4, 5). Thousands of these small RNAs of approximately 20 nucleotides in length have been identified in humans so far and are conserved across all species (6). Detectable in biopsies and body fluids, miRNAs are considered as very sensitive and specific circulating biomarker (7). The enthusiasm for miRNA in the diagnostics field is reflected by the number of related publications, reaching around 11,000 papers in 2018 (Figure 2A).

On the therapeutic side, polypharmacology is gaining a lot of interest in the pharmaceutical era (8). It is now clear that human diseases are complex and that deregulation of multiple genes is often needed to transform a normal cell into a pathological one (9). Furthermore, redundant cellular pathways can limit efficiency of monogenic targeting compounds (10). Conversely, the miRNA's function is by definition based on multitargeting (11). In fact, it is well established that these small RNAs recognize their mRNA targets mainly by the 2nd to the 8th nucleotides of their 5' end. Mismatches in the 3' sequence allow one miRNA to specifically bind to hundreds of different mRNAs simultaneously regulating their expression (11, 12). It is not surprising that these endogenous multitargeting molecules gained a lot of interest in the therapeutic field. In fact, nearly 3,500 studies were published in 2018 on miRNA-based therapeutics (Figure 2B).

Similarly, a multitude of clinical trials were conducted or are currently underway to test new miRNA based treatments. This effervescence is therefore expressing the evolution of this still young and relatively immature field of utilizing the miRNA as a therapeutic tool.

With thousands of academic publications each year, how far from patients are miRNAs? To answer this question and bring a different angle, we reviewed the market of the diagnostic and therapeutic applications of miRNAs. With a lot of companies offering miRNA-profiling services, our focus was to highlight the ones providing specific expression panels for a given clinical application. On the therapeutic side, multiple clinical trials

Figure 1 Number of studies published in PubMed per year for: (A) RNA studies; (B) RNA diagnostic and therapeutic studies

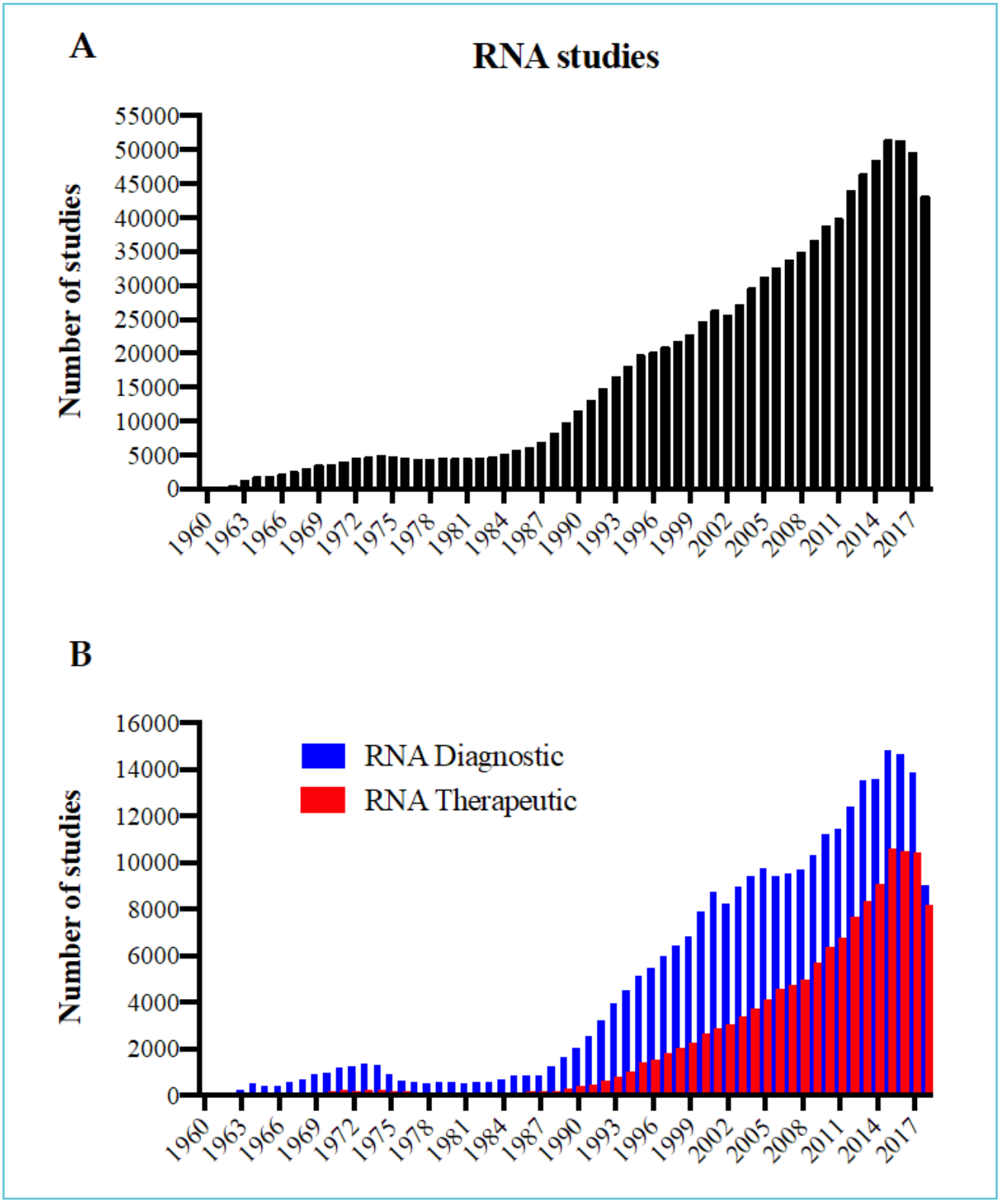
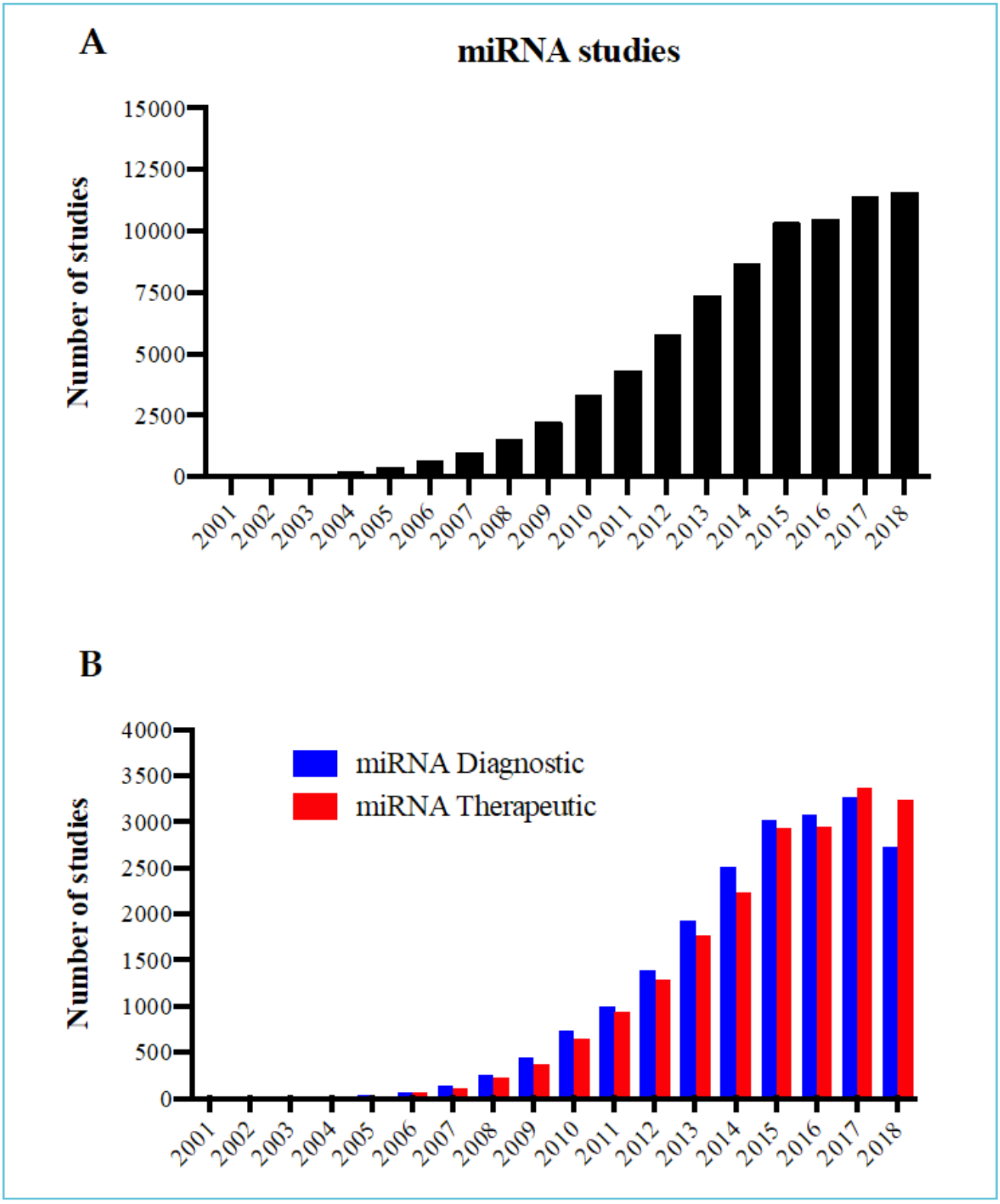


Figure 2 Number of studies published in PubMed per year for: (A) miRNA studies and (B) miRNA diagnostic and therapeutic studies



are currently ongoing and we focused on the products that are the most advanced in the field.

miRNA-BASED DIAGNOSTICS

Applications in the cancer field

Since their initial discovery in 1993, miRNA have showed a great diagnostic potential by being associated with various diseases (5, 7). Since then, the number of publications on the diagnostic potential of miRNA grew almost exponentially (Figure 2B), which attracted numerous companies to develop new miRNA-based diagnostic tools. To our knowledge, the first company focusing on miRNA-based diagnosis assays was Rosetta Genomics (NASDAQ: ROSG), an Israeli company incorporated in early 2000. In partnership with Precision Therapeutics, a personalized cancer therapy company, they launched in 2012 miRview™ mets a miRNA panel allowing the identification of cancers of unknown or uncertain primary origin (CUP) (Table 1).

These cancers account for up to 15% of newly diagnosed cancer in the U.S. every year (13, 14). This CUP classifier was able to identify 42 different tumor types using microarray that measures the expression levels of 64 miRNAs. The miRview™ mets panel was able to identify accurately 90% of the 509 validation sample set. The assay also showed 88% correspondence with the patient's clinicopathological evaluation (14). Based on this success, Rosetta Genomic introduced a new product called RossettaGX Reveal™ (Reveal) in 2016.

This new miRNA classifier relied on qRT-PCR to differentiate between benign or indeterminate thyroid nodules using FNA cytology smears. Reveal's performance was validated using a multicenter retrospective cohort of 189 FNA smears and achieved a negative predictive value of 91%, a sensitivity of 85% and a specificity of 72% (15). Unfortunately, the company declared bankruptcy in May 2018 after a $10 million acquisition deal by Genoptix failed. Interestingly, Interpace Diagnostics acquired most of the equipment through a bankruptcy auction and hired some of Rosetta Genomics employers.

Interpace Diagnostics (NASDAQ: IDXG), is based in New Jersey and is a molecular diagnostic testing company that is offering personalized medicine strategies for the diagnosis of thyroid and pancreatic cancer. Interpace acquired a solution developed by Asuragen combining ThyraMIR®, a miRNA classifier, and ThyGeNEXT®, an oncogene panel for thyroid cancer stratification (Table 1). Initial validation was completed by Asuragen in 2015, over 12 endocrinology centers across the U.S. and 638 surgical and fine needles aspirations (FNA) biopsies were analyzed (16). The combination of ThyraMIR® and ThyGeNEXT® offers an interesting alternative as 15-30% of standard cytological evaluations fail to discriminate benign from the malignant lesions (17, 18). The ThyraMIR® classifier includes the quantification of 10 miRNAs: miR-29b-1-5p, miR-31-5p, miR-138-1-3p, miR-139-5p, miR-146b-5p, miR-155, miR-204-5p, miR-222-3p, miR-375 and miR-551b-3p (Table 1).

This panel was trained using 240 well-characterized, surgically resected, benign or malignant thyroid lesions. A validation set of 54 independent resected thyroid tissues and 235 preoperative thyroid FNAs was then used for threshold optimization (16). Based on this study, Interpace Diagnostic claims a Negative Predictive Value of 94%, a Positive Predictive value of 74% and a reduction of 85% of unnecessary surgeries. Interpace Diagnostic is CLIA certified and CAP accredited, but both tests are not FDA approved. Availability of ThyraMIR® through Labcorp, was announced on January 12, 2016. Interpace also received Medicare coverage in 2016 covering over 50 million patients across the United States. Most recently, in November 2018, the Blue Cross Blue Shield and the U.S. Federal Employee Health Benefit Program have agreed to include

Table 1 Currently active companies working on miRNA-based diagnostics

Companies	Product	Targeted miR	Disease type	Development phase	Reference
Interpace Diagnostics/ Asuragen	ThyraMIR/ ThyGENX	miR-29b-1-5p, miR-31-5p, miR-138-1-3p, miR-139-5p miR-146b-5p, miR-155, miR-204-5p, miR-222-3p, miR-375 and miR-551b-3p	Thyroid and pancreatic cancer	Available	thygenext-thyramir.com
Rosetta Genomics/ Precision Therapeutics	miRview mets	miRNA library	Identify tumor origin of cancer	Available	rosettagenomic.com oncotest.co.il
Genoptix	Reveal	miRNA library	Thyroid	Available	genoptix.com
TAmiRNA	OsteomiR	Panel of 19 miRNAs	Osteoporosis	Available	tamirna.com
TAmiRNA	ThrombomiR	Panel of 11 miRNAs	Cardiovasc. Disease	Available	-
Hummingbird Diagnostics	-	Panels (unknown)	Multiple (cancers, brain, heart)	Pre-Clinical & Phase 1	hummingbird-diagnostics.com
DiamiR	CogniMIR Others	Panel (unknown) Panel (unknown)	Alzheimer Brain diseases	Phase 1	diamirbio.com
Mirnext		Panel with miR 423-5p	Heart failure	Development	hmirnext.com
Quanterix/ DestiNA Genomics	Simoa	miR-122	Liver toxicity	Pre-Clinical	quanterix.com

the ThyGeNEXT®/ThyraMIR® combined tests for their 5.3 million beneficiaries.

Hummingbird Diagnostics (formerly known as Comprehensive Biomarker Center) was founded in 1998 in Heindelberg, Germany, and has now extended to Boston, Massachusetts, in the United States. Operating as a subsidiary of Febit Holding, this company is hands-on in the development of novel miRNA signatures in liquid biopsies for early detection of various diseases, ranging from cancer (Non-small-cell lung carcinoma, melanoma, breast cancer), to neurodegenerative (multiple sclerosis, Alzheimer, Parkinson), cardiovascular (acute myocardial infarction and heart failure) and inflammatory bowel disease (19-21). With its DIN EN ISO/IEC 17025:2005 accreditations for RNA (including miRNA) extraction and microarray services (Agilent Certified Service Provider), the company profiled more than 7,000 disease-related body fluid samples so far. The bioinformatics and statistical processing of those large expression data led to the identification of multiple disease-related miRNA panels (Table 1). Although none of these are currently commercially available, Hummingbird Diagnostics has 17 granted patents in the field of whole blood expression profiling. The clinical validation of their miRNA signatures for early diagnostic use are ongoing with the funding received through their participation in three European FP7-funded consortia (BestAgeing, RiskyCAD and EURenOmics).

Applications in age-related diseases

DiamiR is located in Monmouth Junction, New Jersey and published their first article describing the use of miRNA biomarkers in mild cognitive impairment in 2012 (22). DiamiR have since published several articles with a similar scope, which is the use of miRNA as markers of neurodegenerative and neurodevelopmental disorders (23-25). This work was funded in part by the Michael J. Fox Foundation for Parkinson's Research and through a Small Business Innovation Research (SBIR) phase II grant of $1.5M from the National Institute on Aging (NIA) of the National Institutes of Health (NIH) in 2014 and 2015. More recently, in March 2017, the NIA of the NIH awarded DiamiR another SBIR Phase IIB grant of $2.75M over three years to further support the development of their branded lead product, CogniMIR™ (Table 1). CogniMIR™ is currently in clinical trial testing for early detection of Alzheimer's disease at the presymptomatic, mild cognitive impairment and dementia stages. Using different brain-derived miRNA signatures, DiamiR also expects to test for Parkinson's disease, frontotemporal degeneration, and amyotrophic lateral sclerosis. However, those products are still in the validation process.

TAmiRNA is another European leader in miRNA diagnostics that was founded in 2013 as a spinoff of two Austrian companies, BOKU and Evercyte. This R&D company develops and offers validated microRNAs panels as additional tools for the diagnostic of age-related disorders. Funded by AWS Seedfinancing and EU Horizon2020 programs, TAmiRNA demonstrated the clinical utility of their licensed miRNAs as biomarkers in osteoporosis (26). OsteomiR™ is their lead product intended to provide the risk of a first fracture in female patients with postmenopausal osteoporosis and type-2 diabetes (27-29). The integration of the expression level of 19 blood-circulating miRNAs gives a calculated fracture-risk index that could be used for preventive therapy and treatment follow-up. Similarly, TAmiRNA also proposes the ThrombomiR™ panel (11 miRNAs) to assess platelet function and the ToxomiR™ panel (19 miRNAs) to evaluate the toxicity occurring in various tissues (Table 1). Kits based on primer-coated qPCR plates can be either purchased (except ToxomiR™) or samples can be directly processed by TAmiRNA, from extraction to data analysis. By starting a partnership agreement with SimplicityBio in February 2017,

development of additional miRNA panels are expected. The Swiss-based bioinformatics company has a robust in silico biomarker identification pipeline that could accelerate TAmiRNA's main goal in offering advanced miRNA markers for the diagnosis and prognosis of age-related disorders.

Applications in cardiac function and liver toxicity

Mirnext is a Dutch biomedical company based in Amsterdam that is interested in the diagnostic potential of miRNAs. It was established in 2014 as a new entity of ACS Biomarker, which was built on the Galectin-3 biomarker of heart failure (30, 31), now out-licensed to BG Medicine and available in clinics. Mirnext is currently financed through Life Sciences Fund Amsterdam and Limburg Ventures, two venture capital investors of The Netherlands. Similar to ACS Biomarker, Mirnext's main goal is to identify and commercialize biomarkers in the cardiovascular field but with full dedication towards miRNAs. Their high-throughput, disease-based miRNA profiling identified, among others, miR423-5p as a useful marker of heart failure (32). Together with other clinically relevant miRNAs, Mirnext pursued their validation in large patient cohorts with different cardiovascular diseases including heart failure, coronary artery disease and myocardial infarction. Thus, their miRNA panel integrates many different disease mechanisms useful for the identification and stratification of those pathologies. In addition to the diagnosis of heart diseases, Mirnext is aiming to evaluate cardiovascular risk profiles (mortality, hospitalization) of the individuals tested as part of their multi-marker heart failure test. A single test is expected to provide the clinician extensive information on the patient's cardiovascular health to initiate targeted treatments. At the time of this writing, we were unable to access the company's website.

Quanterix (NASDAQ QTRX) is a biotech company founded in 2007 in Lexington, Massachusett. Through the development of their ultra-sensitive digital biomarker detection technology Simoa®, Quanterix provides healthcare researchers the ability to investigate the continuum of disease progression. In March 2018, they announced a collaborative effort with DestiNA Genomics to enhance RNA biomarker detection. DestiNA was founded in 2010 in Edinburgh, United Kingdom, where they developed and patented a unique PCR-free, chemical-based technology for the detection and quantification of nucleic acids such as miRNAs, without prior isolation from serum or plasma (33, 34). This highly-specific nucleic acid detection combined to the ultra-sensitive Simoa® system provides a solid support for disease-related miRNA biomarker testing. Accordingly, the collaboration's first initiative was focused on miR-122 as a liver toxicity marker (35, 36). They demonstrated that their assay detects miR-122 earlier and outperforms the current protein-based biomarkers in specifically detecting and quantifying liver toxicity.

miRNA-BASED THERAPEUTICS

Several pharmaceutical and biotech companies have launched miRNA projects in their development pipeline (Table 2). Companies are mainly working on two types of products; miRNA mimics and antagomiRs. The miRNA mimics are used to re-establish the concentration of a specific miRNA suppressed by the evolution of a given pathology (37, 38). Inversely, antagomiRs are used to suppress the function of specific miRNAs overexpressed and mechanistically involved in a disease (37, 38). In order to allow the development of miRNA therapeutics, scientists must address two main challenges: the stability and delivery. First, RNA molecules are quite unstable because of their 2'-OH chemical group (39). Therefore, several companies, such as Dharmacon, BioSyn and GenScript, can

Table 2 Currently active companies working on miRNA-based therapeutics

Companies	Product	Targeted miR	Disease type	Development phase	Reference
Roche/Santaris	Miravirsen	miR-122	HCV	Phase 2	roche.com
Regulus Therapeutics	RG-101	miR-122	HCV	Phase 2 (hold)	regulusrx.com
	RG-012	miR-21	Alport syndrome	Phase 1	
	RG-125	miR-103/107	NASH	Phase 1	
MiRagen Therapeutics	MRG-201	miR-29b	Fibrosis	Phase 2	miragentherapeutics.com
	MRG-106	miR-155	Lymphoma and leukemia	Phase 1 and Phase 2	
	MRG-107	miR-155	ALS	Pre-Clinical	
	MRG-110	miR-92	Ischemia	Phase 1	
ENGeneIC	Mesomir	miR-16	Mesothelioma	Phase 2	engeneic.com
Abivax	ABX464	miR-124	IBD	Phase 2	abivax.com
Synlogic	Screening				synlogictx.com
Opko	Screening				opko.com
Alnylam Pharmaceuticals	Screening				alnylam.com
Interna Technologies	Screening				interna-technologies.com
Mello Biotech	Screening				mellobiotech.com

produce natural and chemically modified RNA (2'-O-methyl; 2'-OMe, locked nucleic acid; LNA, of 2'-fluor; 2'-F, phosphorothioate; PS) to stabilize and reduce the high reactivity of RNA molecules. The other major challenge is the delivery of these RNAs to the desired site of action (39). Therapeutic application requires the correct delivery of the RNAs to the targeted organs in order to maintain adequate treatment specificity. When a treatment requires a systemic

delivery through intra-venous injection, the delivery strategies are either passive or active (40, 41). The passive strategy utilizes the tendency of several organs, like the liver, the spleen and the lymph nodes to internalize accumulated particles. Using this non-specific approach, designed nanoparticles or liposome-like particles incorporating RNAs can be targeted to these organs (40, 41). Inversely, the active strategies combine the RNA or the particle with a specific molecule that will bind to the cells of interest and will be endocytosed (40, 41). These structural and delivery challenges, albeit being constantly addressed by new design strategies, still complicate the development of miRNA therapeutics.

Applications in liver disease

Among the most advanced products, there is Miravirsen (or SPC3649), an antagomiR targeting miR-122. Santaris Pharma initially developed this drug candidate before Roche acquired the company in 2014 to expand its RNA therapeutic research and development department (Table 2). Miravirsen is a locked nucleic acid (LNA) containing phosphorothioate modifications. MiR-122, is known to be essential in the life cycle of hepatitis C virus (HCV) expressed in the liver (42, 43). Reducing the activity of miR-122 in the context of HCV infection is important. In fact, miR-122 is a host factor that binds to the 5'-UTR region of the HCV genome and enhances its transcription (43, 44). In phase 1 clinical trials, some patients who received high doses of Miravirsen in monotherapy resulted in undetectable HCV RNA levels (43, 44). Because Miravirsen is a modified RNA (LNA and phosphorothioate), it naturally accumulates in the liver and does not require special delivery strategy. Miravirsen is currently undergoing multiple phase 2 clinical trials.

Another product was developed to target miR-122, RG-101, and is produced by Regulus Therapeutics (NASDAQ: RGLS) in collaboration with Ionis Pharmaceuticals and GSK (Table 2). RG-101 is an N-acetyl-D-galactosamine- conjugated RNA antagomiR that also targets miR-122 in HCV infected hepatocytes (45). RG-101, like Miravirsen, shows considerable efficacy with patients displaying undetectable HCV RNA levels (45). However, some serious adverse events of severe jaundice were recently declared in a clinical trial and the FDA put the studies on hold until the situation is clarified. It is worth mentioning that Regulus was also working on RG-125 (also described as AZD4076), an antagomiR targeting miR-103/107, in phase 1 clinical trial for treatment of nonalcoholic steatohepatitis (NASH; Identifier NCT02612662 and NCT02826525) as well as RGLS5040, an anti-miR-27 aiming to reduce cholestatic diseases. However, development of these latter two were recently suspended.

Applications in fibrotic disease

Regulus has also worked with Genzyme (Sanofi) to test the efficacy of RG-012, an antagomiR against miR-21, which reduces the fibrogenesis of organs associated with Alport syndrome (46). This is an X-linked disease and is characterized by kidney disease, hearing loss and visual impairment caused by mutations of the genes encoding type-IV collagen (47). The use of a modified single-stranded antagomiR with phosphorothioate, 2'-O-methoxyethoxy and constrained ethyl modifications showed an important improvement in the survival of a Alport mouse model with a reduction of kidney disease progression (46). Despite interesting results, the phase 1 clinical trial of RG-012 has recently been discontinued mid-2018 because of the reorganization between Regulus and Sanofi (Clinical trial identifier NCT03373786).

Another promising company is MiRagen Therapeutics (NASDAQ: MGEN), based in Boulder, Colorado. First, the company developed MRG-201, also known as Remlarsen, a miRNA mimic

that aims to restore the levels of miR-29b, which is a negative regulator of the extracellular matrix deposition processes. The miR-29 family (miR-29a/b/c) is constantly downregulated in fibrotic diseases. MRG-201 is an LNA RNA mimic that is administered by intradermal injection and the phase 2 clinical trial is currently underway (Identifier: NCT03601052). Remlarsen could virtually be used for the treatment or prevention of pathological cutaneous fibrosis, as well as of other fibrotic diseases, including idiopathic pulmonary fibrosis (48).

Applications in cancer

While some companies are having great successes, others struggle to positively impact patient outcomes. This was the case of MiRNA Therapeutics (NASDAQ: MIRN) and a miRNA mimic, MRX34. MiR-34, is a well characterized tumor suppressor downregulated in a broad range of cancers (49-51). MRX34 was delivered as a double stranded RNA encapsulated into a liposome-formulated nanoparticle. Preclinical studies were promising when used in several cancer types such as renal cell carcinoma, acral melanoma and hepatocellular carcinoma (52). However, the FDA halted their phase 1 clinical trial when many immune-related serious adverse events leading to death were registered. It reached a point where MiRNA Therapeutics reduced its staff before Synlogic Inc (NASDAQ: SYBX) finally acquired it in 2017.

MiRagen Therapeutics is actively developing MRG-106, also known as Cobomarsen, an LNA antagomiR that targets miR-155. This miRNA is involved in the differentiation and proliferation of blood and lymphoid cells. Cobormarsen is actually involved in phase 1 trials (Identifier NCT02580552) and phase 2 clinical trials (Identifier NCT03713320), with the goal of treating certain types of lymphoma and leukemia (53). Similarly, MRG-107 also targets miR-155 to alleviate symptoms associated with amyotrophic lateral sclerosis (ALS) but has not yet entered clinical trials. In an ever-growing pipeline, they also work on MRG-110 in collaboration with Servier. This LNA antagomiR targets miR-92 in order to treat ischemic conditions such as heart failure (48). Its phase 1 clinical trial is currently recruiting (Identifier NCT03603431).

Pharmaceutical and biotech companies are heavily engaged in developing successful products and RNA biologics are closer than ever to entering the market. Another indicator of this effervescence is the acquisition of RNA-based companies by pharmaceuticals giants. Santaris Pharma was acquired by Roche in 2014, SiRNA Therapeutics by Merck in 2007, followed by the acquisition of this division by Alnylam Pharmaceuticals in 2014, and more recently MiRNA Therapeutics by Synlogic Inc. However, even more companies are currently testing new miRNA therapeutics. For example, ENGeneIC is currently designing and producing Mesomir, a miRNA mimic that aims to replace miR-16, a tumor suppressor that is reduced in cases of cancer, such as malignant pleural mesothelioma (54). It successfully completed phase 1 clinical trial and will soon start phase 2 (55). On another hand, Abivax produces ABX464, a small molecular compound that triggers the increase of miR-124 to reduce the symptoms of inflammatory bowel disease for patients refractory to anti-TNF biologics and corticosteroids. It is currently in preparation for a phase 2b clinical trial for ulcerative colitis and phase 2a for Crohn's disease.

Finally, a multitude of companies work in preclinical and large screening studies to identify potential biologic miRNA such as Opko with their CURNA program, Alnylam Pharmaceuticals, Interna Technologies and Mello Biotech. These companies could therefore increase, in the next several years, the number of miRNA therapeutics being tested or entering the market.

CONCLUSION

Enthusiasm, promise and hope are evident in the miRNA industry. As described, multiple companies are dedicating significant efforts and resources to develop miRNA-based products. The diagnostic field is definitely the most advanced with some miRNA panels already offered to clinicians and covered by major insurance companies. However, considering the thousands of publications in this area, miRNAs as diagnostic products can still be considered in their infancy. On the therapeutic side, despite the potentials, the miRNA-based therapeutic breakthroughs have not arrived yet. Recently, an analytical model based on technological growth metrics showed that miRNAs still require time to reach the maturity point needed to yield a significant number of products that could enter the market (56). For this reason, most of the technologies discussed are currently in clinical trials.

The development and commercialization of new diagnostic and therapeutic tools is definitely a long process. Considering the first evidence of the involvement of miRNA in human disease in 2002 and the first detection of miRNAs in blood in 2008, only a decade later, tremendous progress and effort has been made to bring these small RNAs from the bench to the bedside (57, 58).

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Role of sepsis modulated circulating microRNAs

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ABSTRACT

Sepsis is a life-threating condition with dysregulated systemic host response to microbial pathogens leading to disproportionate inflammatory response and multi-organ failure. Various biomarkers are available for the diagnosis and prognosis of sepsis; however, these laboratory parameters may show limitations in these severe clinical conditions. MicroRNAs (miRNA) are single-stranded non-coding RNAs with the function of post-transcriptional gene silencing. They normally control numerous intracellular events, such as signaling cascade downstream of Toll-like receptors (TLRs) to avoid excessive inflammation after infection. In contrast, abnormal miRNA expression contributes to the development of sepsis correlating with its clinical features and outcomes. Based on recent clinical studies altered levels of circulating miRNAs can act as potential diagnostic and prognostic biomarkers in sepsis. In this review, we summarized the available data about TLR-mediated inflammatory signaling with its intracellular response in immune cells and platelets upon sepsis, which are, at least in part, under the regulation of miRNAs. Furthermore, the role of circulating miRNAs is also described as potential laboratory biomarkers in sepsis.

INTRODUCTION

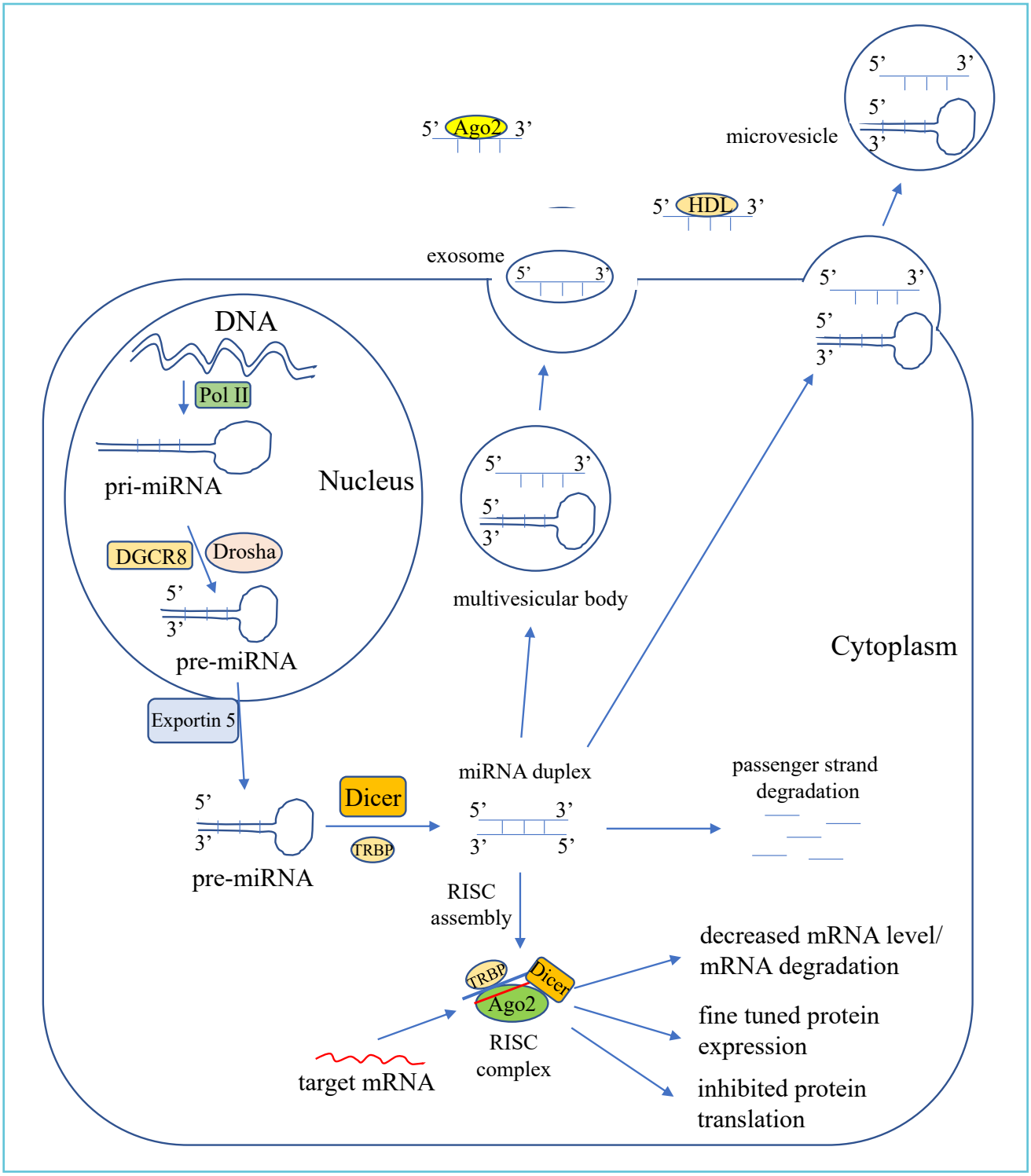
Sepsis is a life-threating condition with dysregulated systemic host response to microbial pathogens defined by the Third International Consensus Task Force (Sepsis-3), while septic shock is a subset of sepsis with circulatory, metabolic and cellular abnormalities (1). Based on the etiology of insults of sepsis, we distinguish pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) (2). Since similar mediators are released in both conditions that react with Toll-like receptors (TLRs), they inflict similar excessive inflammatory response (2). Sepsis still results in about 17% mortality due to multiorgan failure (3), which is caused by delayed diagnosis and treatment of patients as well as the inappropriate administration of broad spectrum antibiotics that contributes to the development of antibiotic resistance (4). Several clinical trials have aimed to test a large number of biomarkers for the early diagnosis of sepsis (5). Currently, FDA-(Food and Drug Administration, USA) approved procalcitonin (PCT) can effectively differentiate culture-negative and culture-positive sepsis from non-infectious systemic inflammatory response syndrome (SIRS) (6). In addition, several other diagnostic and prognostic biomarkers are available in sepsis, such as C-reactive protein (CRP) (7), serum lactate (8), and interleukin-6 (IL-6) (9). However, these parameters may be elevated in non-septic conditions as well (10).

MicroRNAs (miRNA) are evolutionarily conserved, single-stranded, non-coding RNAs of 20-25 nucleotides in length with the function of post-transcriptional gene silencing via decreasing messenger RNA (mRNA) levels to fine tune protein expression or via degradation of mRNA to inhibit translation (11). Each miRNA target hundreds of mRNAs, and each mRNA is under the control of several miRNAs. Among physiological conditions, miRNAs with a dynamic nature, tightly control intracellular processes to maintain homeostasis (11). For example, miRNAs are the fine-tuners of signaling downstream of TLRs to avoid excessive inflammation after infection (12). On the other hand, they have been implicated in the development of various human diseases, such as cardiovascular, autoimmune and malignant disorders (13-15). miRNAs secreted from the cells and their presence in plasma/serum denote the role of circulating (cell-free) miRNAs in pathogenesis (16). Although immune response is predominantly controlled at the transcriptional level, miRNA-mediated RNA interference operates at the translation level (17). Consequently, dysregulated intracellular and circulating miRNA expression has been correlated with the clinical features of SIRS (18), critically ill polytrauma (19), and sepsis (20-22). The function of miRNAs in the regulation of immune response and development of sepsis seems to be critical (23, 24), thus a better understanding of these mechanisms may result in improved diagnostic and therapeutic strategies in sepsis. In this review, we focus on TLR4-mediated inflammatory signaling with subsequent cellular events in immune cells and platelets upon sepsis, which are, at least in part, under the regulation of miRNAs. Furthermore, the role of circulating miRNAs is also summarized as potential diagnostic and prognostic biomarkers in sepsis.

BIOGENESIS AND RELEASE OF miRNAS

As the first step, primary miRNAs (pri-miRNAs) are usually transcribed by the function of RNA polymerase II in the cell nucleus (25). Pri-miRNAs are then processed into 70 bp “hairpin” miRNA precursors (pre-miRNAs) by to the endonuclease activity of Drosha (26). This reaction is catalyzed by the DiGeorge Syndrome Critical Region 8 (DGCR8) complex. Pre-miRNAs bind to the Exportin-5 transporter protein, which shifts them from the nucleus into the cytoplasm. Ribonuclease (RNAse) III called Dicer further

Figure 1 **Key steps in miRNA biogenesis***



**miRNAs originate from the nucleus as pri-miRNA precursor molecules. They are processed by the RNAse III-type enzyme, Drosha, in association with DGCR8, into smaller pre-miRNAs, then exported to cytoplasm, where they are cleaved by Dicer to their mature form of cc. 22 nucleotides double stranded miRNA. The guide strand of the mature miRNA is incorporated into RISC, where it binds to target mRNA by partial complementarity with its 3' UTR. This results in decreased mRNA*

levels to fine tune protein expression or mRNA degradation to inhibit translation. Mature miRNAs with pre-miRNAs can be released from the cells into microvesicles, exosomes, or circulate in a free form bound to Ago2 or HDL.

Abbreviations: Pol II (RNA polymerase II), DGCR8 (DiGeorge Syndrome Critical Region 8), RISC (RNA-induced silencing complex), TRBP (transactivation-responsive RNA-binding protein), Ago2 (Argonaute-2), UTR (untranslated region), pre-miRNA (precursor miRNA).

processes pre-miRNAs into mature miRNA duplexes (27). The RNA-induced silencing complex (RISC) is formed by TRBP (transactivation-responsive RNA-binding protein), Dicer and Ago2 (Argonaute-2) proteins, which guides miRNAs to post-transcriptionally regulate mRNAs by binding to their 3' untranslated region (UTR). In humans, they fine tune protein expression rather than inhibit it (11). The main steps of miRNA maturation are depicted in Figure 1.

miRNAs can be released in several ways from parent cells into the plasma and remain stable in the circulation (28). They are highly resistant to endo- and exogenous RNase activity, excessive pH and temperature conditions (29). These characteristics are partly achieved by transport within exosomes and microvesicles, or being carried by RNA-binding protein, such as Ago2 protein or high-density lipoprotein (28). Microvesicles are generated by the budding of the plasma membrane and transfer functional miRNAs and also pre-miRNAs to target cells (Figure 1). These "RNA vectors" can alter cellular functions and induce biological responses (30). Cell-free miRNAs have been detected in various body fluids, such as plasma, serum, urine or saliva (31).

SIGNALING PATHWAY INVOLVED IN TLR4 ACTIVATION IN IMMUNE CELLS

Stimulation of TLRs induces the activation of NF-κB (nuclear factor kappa B) and MAPK (mitogen activated protein kinase) pathway causing the production of proinflammatory cytokines in macrophages and monocytes during the development of sepsis (32). Ten human subtypes of TLRs (TLR1-TLR10) are known to exist (33). These receptors become functional by diverse stimuli (e.g. TLR2 is activated by peptidoglycan of Gram-positive bacteria), and are localized in various cellular compartments, such as TLR2 and TLR4 are present on the cell surface, while TLR3 and TLR7-TLR9 sensing nucleotide derivatives, are located in the membrane of intracellular vesicles (33). One of the most characterized receptor in sepsis is TLR4 with agonist of lipopolysaccharide (LPS) of Gram-negative bacteria. Signaling pathway downstream of TLR4 has been investigated in detail (34). Briefly, after recognition of LPS, TLR4 recruits the myeloid differentiation primary response protein 88 (MyD88). MyD88 then recruits IL-1R-associated kinases (IRAK4, IRAK1 and IRAK2) that activate and ubiquitinylate TNFR-associated factor 6 (TRAF6). Due to the subsequent ubiquitination of TAK1-binding protein 2 (TAB2), TAK1 becomes activated. These events lead to the activation of the inhibitor of NF-κB kinase (IKK) complex consisting of IKK-α, IKK-β and NF-κB essential modulator (NEMO, or IKK-γ), which phosphorylates IκBα and thereby releases NF-κB transcription factor containing p50 and p65 for translocation to the nucleus. This allows the transcription of proinflammatory genes, such as *IL6* and *TNF-α* (34). Figure 2 depicts the major signaling events of TLR4-induced MyD88-dependent signaling with the inhibitory effect of miRNAs against different components of this pathway (described below) (Figure 2).

In parallel, MyD88-independent TLR4 signaling is also induced upon infection causing production of type I interferons (35). This pathway is also under a broad regulation of miRNAs (36). Although it is evident that deregulated TLR4-induced NF-κB inflammatory response is predominantly involved in sepsis, administration of

Figure 2 TLR4-mediated signaling with the normal inhibitory function of miRNAs*

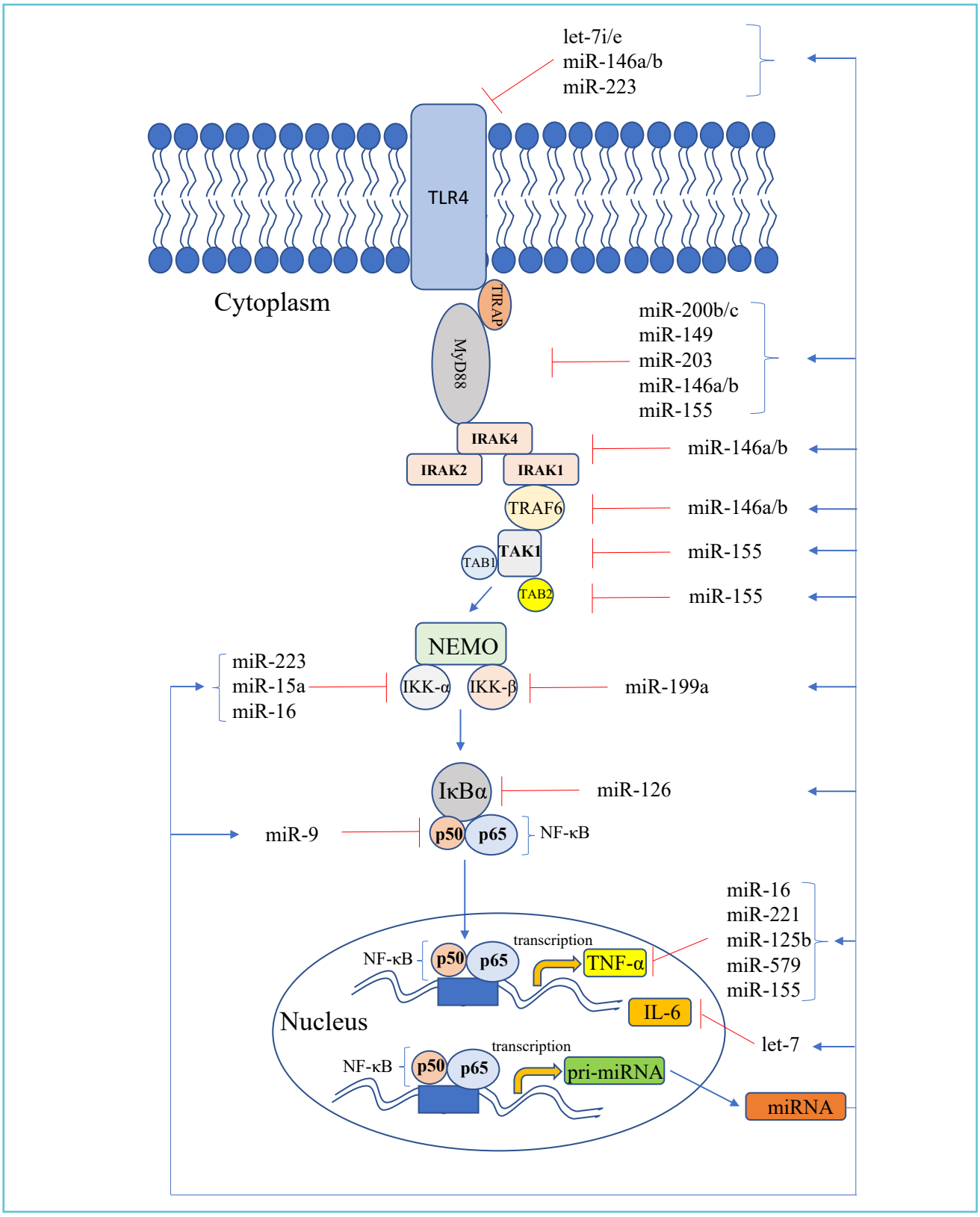


Figure 2 - Legend:
Intracellular miRNAs are expressed as a consequence of TLR4-induced signaling cascade to regulate the expression and function of signaling cascade components and that of induced proinflammatory proteins in the immune cells upon infection.
Abbreviations: TLR4 (Toll-like receptor 4), NF-κB (nuclear factor kappa B), MyD88 (myeloid differentiation primary response protein 88), IRAK (IL-1R-associated kinase), TRAF6 (TNFR-associated factor 6), TAB2 (TAK1-binding protein 2), IKK (inhibitor of NF-κB kinase), NEMO (NF-κB essential modulator).

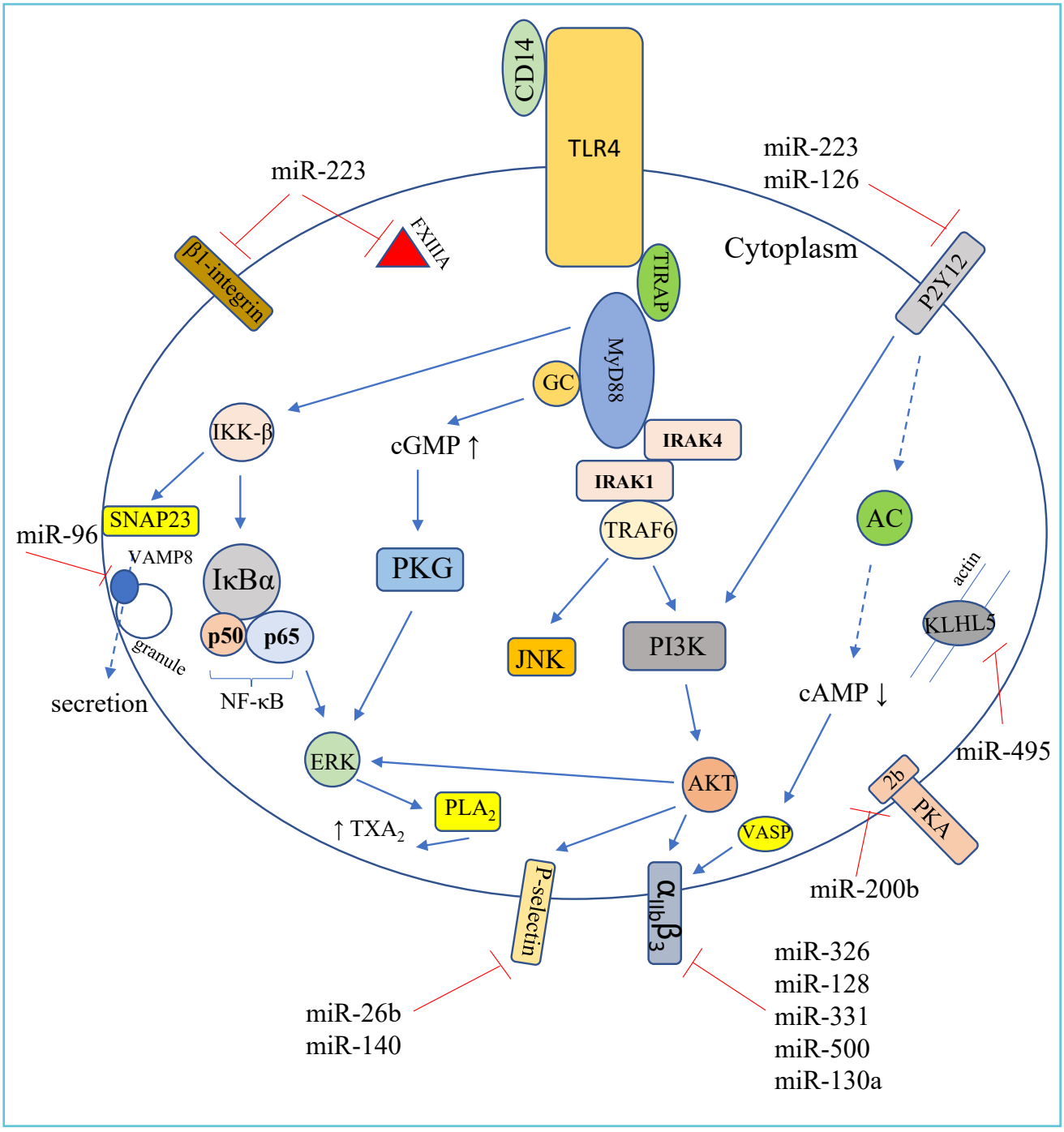
different anti-inflammatory drugs, such as TNF-α antagonist (37) or corticosteroids (38) resulted in only a moderate improvement in sepsis therapy suggesting that other regulatory factors may be also associated with sepsis.

TLR4-MEDIATED PLATELET ACTIVATION AND MEGAKARYOCYTE FUNCTION

Most TLR members are expressed on platelets and megakaryocytes (39). Hence, platelets participate in amplified inflammatory and immune response, and TLR2 and TLR4 can vastly contribute to sepsis related thrombotic complications. TLR-induced platelet activation causes platelet adhesion, aggregation, heterotypic aggregates formation, expression and release of proinflammatory cytokines and thrombin generation (39). Of note, platelets express TLR4, but not CD14, thus a low amount of soluble CD14 is required to initiate LPS-mediated platelet response (40). Upon infection, platelet activation is not directly induced by LPS via TLR4, but it is primed after stimulation elicited by other platelet agonists (41). As a result, release of soluble CD40L and platelet factor-4 is increased without higher P-selectin expression (42). Our group previously demonstrated that the rough form of LPS from *S. minnesota* induced platelet CD40L expression with elevated microparticle levels and potentiated platelet aggregation at low concentration of thrombin receptor activating peptide, however, P-selectin positivity was not enhanced (43). Septic patients frequently show increased platelet activation that may turn into severe thrombocytopenia because of neutrophil-dependent sequestration of activated platelets into lungs in a TLR4-dependent fashion (44). Moreover, platelet TLR4 is involved in TNF-α production after LPS administration (45), induces platelet binding to neutrophils causing formation of neutrophil extracellular nets (46), and propagates the splicing of unprocessed IL-1β and tissue factor to be translated in platelets (47, 48). Interestingly, low concentration of LPS (without induced systemic TNF-α production) caused platelet activation with enhanced platelet clearance and production increasing the thrombotic risk, while high LPS levels resulted in altered platelet reactivity not merely due to TLR4 function (49). Hence, platelets with TLR4 act at the crossroads of sepsis linking inflammation with coagulation abnormalities via propagation of thrombin generation (50) and expression and secretion of proinflammatory cytokine (47).

The molecular machinery of TLR4-mediated signaling in platelets is not fully elucidated as yet. TLR4 can trigger platelet activation via different signaling molecules. TLR4 interacts with MyD88 and TIR domain containing adaptor protein (TIRAP), and downstream proteins IRAK1, IRAK4 and TRAF6 are activated resulting in JUN N-terminal kinase (JNK) and PI3K/Akt pathway activation (51). MyD88 also forms complex with guanylyl cyclase (GC) causing cGMP protein kinase (PKG) activation and ERK phosphorylation (52). Additionally, platelets contain an intact, functional, and complete NF-κB pathway with non-genomic functions that becomes phosphorylated upon platelet stimulation (53, 54). Recently, the TLR4-PI3K-Akt-ERK-cPLA2-TXA2 pathway has been described during platelet activation (55). Figure 3 demonstrates the key intracellular elements of TLR4-induced signaling in platelets (Figure 3).

Figure 3 **Signaling pathway downstream of TLR4 receptor in platelets with related cellular events and their regulatory miRNAs***



**TLR4-induced platelet activation initiates different signaling cascades causing:*
i) guanylyl cyclase (GC) activation causing cGMP protein kinase (PKG) activation and ERK phosphorylation;
ii) NF-κB pathway activation with ERK phosphorylation; and
iii) PI3K-Akt-ERK-cPLA2-TXA2 pathway activation.
These signaling events lead to the enhanced expression/function of $\alpha_{IIb}\beta_3$ receptor, ADP receptor P2Y12, and P-selectin, as well as regulation of granule secretion.

TLR4 is also involved in the regulation of platelet production. Expression of TLR4 was observed in human megakaryoblastic leukemia cell line MEG-01 (56), and also in human megakaryocytes in patients with myelodysplasia (57). TLR4 was elevated during maturation of murine megakaryocytes showing higher CD41 expression (44). TLR4-deficient mice showed decreased platelet count, turnover with lower RNA content and less responsiveness to thrombin-activated expression of P-selectin compared to wild type littermates (58).

In low-grade endotoxemia, platelet turnover often increases, which causes a larger number of newly formed, more active platelets (59). These platelets carry higher and altered RNA levels being more prone to produce proteins and to participate in thrombus formation compared to older platelets (60). This phenomenon was supported by a former animal model when megakaryocytes produced platelets with altered mRNA profile within 24 hours in septic mice and these platelets mediated lymphotoxicity via granzyme B (61). Accordingly, TLR4-induced signaling can modulate thrombopoiesis as well.

ALTERED LEVEL OF miRNAs BY TLR4 SIGNALING IN IMMUNE CELLS

TLR4-mediated signaling enables to modulate miRNA expression. Several miRNAs are up-regulated following LPS stimulation, such as miR-155, miR-146a, miR-21, miR-223, miR-9 and let-7e, etc. in monocytes and macrophages. Among them, there are subtypes with early (e.g. miR-155) and late (e.g. miR-21) response in expression, but these all are NF-κB-dependent.

On the other hand, some miRNAs are down-regulated (e.g. miR-125b and let-7i) due to TLR signaling via transcriptional repression or destabilization of miRNA transcripts (12).

REGULATION OF TLR4-MEDIATED SIGNALING CASCADE BY miRNAs IN SEPSIS

miRNAs have been implicated as an important link between the innate and adaptive immune systems, and their dysregulation might have a role in the pathogenesis of inflammatory diseases (12). miRNAs directly target signaling proteins and control NF-κB activity in immune cells, thus have been identified as novel regulators of immune system (12, 62). Figure 2 depicts those key cellular miRNAs, which negatively regulate the components of TLR4-induced NF-κB signaling pathway (Figure 2). TLR4 expression is highly restricted to immune cell types, such as macrophages, dendritic cells, T- and B-cells. The expression of receptor is regulated by let-7 family. As such, overexpression of let-7i resulted in reduced level of TLR4 in human cholangiocytes (63), while transfection of antisense miRNA to let-7e in macrophages caused enhanced LPS-induced cytokine response via higher expression of TLR4 (64). MiR-146b also targets TLR4 based on luciferase reporter assays of HEK293 cells when there was decreased activity of reporter genes containing the 3'-UTR of TLR4 when miR-146b was transfected (65). In addition, myeloid-specific miR-223, which has an important role in granulopoiesis (66), is also a regulator of TLR4 (67) (Figure 2). Based on these results, miRNAs can tone down TLR4 expression.

Signaling molecules downstream of TLR4 function under the control of miRNAs. Expression of MyD88 protein is affected by miR-200b and miR-200c (68). Furthermore, miR-149 negatively regulated MyD88 protein levels in RAW264.7 cells when lentiviral vector expressing miR-149 was transfected (69). Overexpression of miR-203 resulted in significantly repressed translation of MyD88 in macrophages (70). Finally, miR-155 decreased MyD88 expression at protein but not mRNA level suggesting that the

miR-155-mediated inhibition is a post-transcriptional event in HEK293 cells (71). MiR-146 family negatively regulates the translation of IRAK1 and TRAF6. Taganov *et al.* reported for the first time that there are miRNAs which are up-regulated by TLR signaling, and in turn, miR-146a and miR-146b were found to suppress IRAK1 and TRAF6 (72). This role of miR-146b was later confirmed by others (65). The IKK complex is essential for NF-κB activation. Decreased levels of miR-15a, miR-16 and miR-223 were detected with elevated IKK-α levels when human monocytes were differentiated with granulocyte-monocyte colony stimulating factor (73). IKK-β has complementary sequences for miR-199a and the transfection of this miRNA caused reduced IKK-β level studied in ovarian cancer cells (74).

Similarly, a direct regulatory association between miR-126 and IκBα was described in HT29 cells (75) (Figure 2).

Via targeting transcription factors along TLR4 pathway, miRNAs can have a global impact on TLR4-induced gene expression (76). miRNAs induced by a particular signaling can inhibit the transcription factor, which is necessary for its expression. For example, elevated level of miR-155 feeds back and targets *FOXP3* to decrease its expression (77). *NFKB1* that is cleaved to form the NF-κB subunit p50, was shown to be targeted by miR-9 in human monocytes and neutrophils (78) (Figure 2).

TLR4-mediated NF-κB activation causes excessive production of proinflammatory cytokines, e.g. TNF-α and IL-6. Expression of these cytokines is directly targeted by miRNAs as well. The repressive effect of miR-579, miR-221, and miR-125 was studied on TNF-α during LPS tolerance in THP-1 cells (79). In addition, TNF-α translation could be also influenced by miR-16 (80) and miR-155 (81). Inflammatory response mediated by NF-κB rapidly reduced let-7 levels resulting in higher levels of IL-6 in cancer cells. As IL-6 activates NF-κB, thereby completes a positive feedback loop that maintains the epigenetic transformed state in the absence of the inducing signal (82) (Figure 2).

Among TLR4 signaling regulators, acetylcholinesterase that blocks NF-κB translocation, is regulated by miR-132 (83), translation inhibitor PDCD4 is targeted by miR-21 (84), while negative regulator *SHIP1* is a primary target of miR-155 (85) in macrophages. These two later miRNAs with these effects can indeed fine tune TLR4 signaling that can be important for LPS tolerance or in the resolution of TLR4-induced responses (12). Overall, accumulating data above clearly demonstrate that miRNAs highly control each level of this very complex machinery of TLR-mediated signaling in immune cells.

IMPACT OF miRNAs ON PLATELET FUNCTION

Platelets play an important role in vascular integrity. They circulate in a resting state and become activated after vessel injury by exposed collagen and von Willebrand factor to adhere and aggregate for prevention of bleeding (86). miRNAs are also carried by platelets due to the delivery of miRNAs with mRNAs from megakaryocyte (87). The fact about functional miRNAs in platelets without nucleus was questioned for a long time, thus it was also obscure whether platelets are able to produce proteins *de novo* when being exposed to different challenges. Platelet miRNAs have been studied in relation to the expression of platelet receptors or other activation-related intracellular proteins (88). As yet, only a few miRNAs have been proved as regulator of platelet proteins, such miR-223 regulates ADP receptor P2Y12, intracellular FXIII-A, and integrin β1 expression (89). P2Y12 receptor is targeted by miR-126 as well (90). VAMP8/endobrevin, a critical v-SNARE protein

involved in platelet granule secretion, is regulated by miR-96 (91), while the expression of αIIbβ3 receptor is controlled by miR-326, miR-128, miR-331, miR-500 (92) and miR-130a (93).

Our group has recently reported that miR-26b and miR-140 could affect *SELP* (P-selectin) mRNA level in MEG-01 cells (94). In addition, Nagalla *et al.* described miR-200b:*PRKAR2B* (encoding the β-regulatory chain of cAMP-dependent protein kinase type II, PKA) and miR-495:*KLHL5* (encoding a Kelch-like protein that binds actin) interactions in platelets (95) (Figure 3). Of note, these functions of platelet miRNAs above were not investigated specifically among septic conditions as yet.

CIRCULATING miRNAs AS LABORATORY BIOMARKERS IN SEPSIS

Besides their normal function in the regulation of gene expression, altered miRNA levels in plasma/serum have been intensively investigated with regards to their role as possible biomarkers in different human diseases, also in sepsis (20, 23, 24). Abnormal circulating miRNA levels reflected the pathophysiological processes during severe inflammation and bacterial infection, which was profiled in sepsis mice model using cecal ligation and puncture (CLP) (96). Cell-free miRNAs are stable in the circulation, and can be quantified more rapidly compared to time consuming microbial cultures. Hence, plasma or serum miRNAs may serve as potential biomarkers in the differential diagnosis of sepsis from SIRS, and may act as prognostic parameters under treatment (23). We have summarized the results of previous clinical studies profiling serum or plasma miRNAs (Table 1), and some key miRNAs are further described in detail below.

miR-150

This miRNA was formerly identified as a key regulator of immune cell differentiation and activation (17). During the maturation of B- and T-cells, miR-150 expression is down-regulated. When LPS was injected into humans, miR-150 levels went down in leukocytes (92). Since then, several trails reported that plasma/serum miR-150 was decreased in patients with sepsis at different degree (98, 99). Furthermore, the reduction of miR-150 showed a strong correlation with the severity of sepsis and the concentrations

Table 1 Circulating miRNA alterations in sepsis vs. SIRS subjects or healthy controls

miRNAs	Body fluid/ sample	Study populations	Methods	Results	Reference
miR-150	plasma/blood leukocytes	24 sepsis vs. 32 healthy controls	microarray	↓ (sepsis vs. controls)	98
	serum	138 sepsis, 85 ICU w/o sepsis vs. 76 healthy controls	qRT-PCR	↓ (non-survival vs. survival)	99
	plasma/blood leukocytes	23 sepsis, 22 SIRS, 21 healthy controls	HiSeq Sequencing	↓ (sepsis vs. SIRS) (sepsis vs. controls)	100
	plasma	120 sepsis, 50 healthy controls	qRT-PCR	↓ (sepsis vs. controls)	101

miR-146a	serum	50 sepsis, 30 SIRS, 20 healthy controls	qRT-PCR	↓ (sepsis vs. SIRS) (sepsis vs. controls)	105
	plasma	14 sepsis, 14 SIRS	qRT-PCR	↓ (sepsis vs. SIRS)	106
	blood leukocytes	226 sepsis, 206 healthy controls	qRT-PCR	↓ (sepsis vs. controls)	108
	blood leukocytes	32 sepsis, 38 healthy controls	microarray	↓ (sepsis vs. controls)	107
miR-223	serum	117 sepsis survivor, 97 sepsis non-survivor	Solexa Sequencing	↓ (non-survival vs. survival)	111
	serum	50 sepsis, 30 SIRS, 20 healthy controls	qRT-PCR	↓ (sepsis vs. SIRS) (sepsis vs. controls)	105
	serum	137 sepsis, 84 healthy controls	qRT-PCR	↔ (sepsis vs. controls)	110
	plasma	25 neonatal sepsis, 25 non-sepsis controls	qRT-PCR	↓ (sepsis vs. controls)	109
miR-15a/16	serum	166 sepsis, 32 SIRS, 24 healthy controls	qRT-PCR	↑ (sepsis vs. SIRS) (sepsis vs. controls)	113
	serum	46 neonatal sepsis, 41 non-sepsis controls	qRT-PCR	↑ (sepsis vs. controls)	114
	serum	117 sepsis survivor, 97 sepsis non-survivor	Solexa Sequencing	↑*/↓** (non-survival vs. survival)	111
	plasma	29 sepsis w/ shock, 33 sepsis w/o shock, 32 controls	qRT-PCR	↑ (septic shock vs. sepsis)	115
miR-122	serum	117 sepsis survivor, 97 sepsis non-survivor	Solexa Sequencing	↑ (non-survival vs. survival)	111
	serum	54 sepsis w/coagulation disorder (CD), 69 sepsis w/o CD	qRT-PCR	↑ (CD sepsis vs. non-CD sepsis)	117
	serum	108 sepsis, 20 healthy controls	qRT-PCR	↑ (sepsis vs. controls)	118

** denotes alteration of miR-15a, ** denotes change in miR-16 levels.*

of plasma IL-18 (98). On the other hand, higher miR-150 correlated with the survival of septic patients suggesting its reliable prognostic value (99). Based on a genome-wide sequencing of cellular miRNAs, miR-150 was able to discriminate between patients who had SIRS and those with sepsis (100). Recently, plasma miR-150 was found to be lower than normal that correlated with renal dysfunction and 28-day survival as well as plasma levels of IL-6 and TNF-α (101). MiR-150 expression was significantly decreased in human umbilical vein endothelial cells (HUVECs) *in vitro* after LPS treatment, and overexpression of miR-150 could protect HUVECs from LPS-induced inflammatory response and apoptosis targeting *NF-κB1* (101). Finally, reduced miR-150 level in peripheral leukocytes correlated with Gram-negative bacterial sepsis in the urogenital tract (102).

miR-146a and miR-223

MiR-146a has been widely studied in connection with immune response (72) and chronic inflammatory disorders (103). In parallel, miR-223 has been considered to play a key role in hematopoietic lineage differentiation (66), and was found to be up-regulated in the mucosa of colon in inflammatory bowel disease (104). Wang *et al.* described for the first time that there were significantly reduced serum levels of these miRNAs in septic patients compared to SIRS individuals and healthy controls (105). Additionally, the areas under the receiver operating characteristic curve (AUC-ROC) value was much higher in case of miR-223 (0.858) and miR-146a (0.804) vs. traditional biomarkers IL-6 (0.785) and CRP (0.589) (105). Similarly, septic subjects demonstrated lower plasma miR-146a levels than patients with SIRS (106). The expression of miR-146a was also down-regulated in blood leukocytes from sepsis (107, 108). Similarly, plasma miR-223 was decreased in neonatal sepsis vs. non-sepsis controls (109), while Benz *et al.* did not find significant difference in this miRNA level (110). However, when miR-223 was correlated with the outcome of sepsis, miR-223 was significantly lower in non-survivors in contrast to survivors of sepsis (111). Taken together, these studies suggested that decreased miR-223 and miR-146a levels were optimal for diagnosis of sepsis.

miR-15a and miR-16

These miRNAs were originally identified as tumor suppressors and dysregulated levels of miR-15a and miR-16 was found in certain solid tumors, such as in ovarian cancer (112). Since then, miR-15a and miR-16 were associated with innate immune system by targeting IκBα mRNA upstream of NF-κB pathway (73). Both miRNAs were found to be higher in both sepsis and SIRS subjects vs. normal controls (113). In addition, miR-15a had substantial AUC value (0.858) for the diagnosis of sepsis compared CRP (0.572) and PCT (0.605) and showed 94.4% specificity (113). Up-regulated miR-15a/16 was reported from the serum of neonatal sepsis patients, while transfection of miR15a/16 mimics into RAW264.7 macrophages down-regulated *TLR4* and *IRAK-1* in LPS-treated cells (114). Interestingly, diverse expression was seen in these miRNAs among survivor and non-survivors, i.e. serum miR-15a was significantly higher in non-survivors, while miR-16 was lower in this subgroup (111). Finally, higher level of miR-15a was associated with the development of septic shock in contrast to those with sepsis only (115).

miR-122

Although miR-122 is a liver-related miRNA, and thus showed elevated levels in liver injury earlier (116), its potential role in the diagnosis and prognosis of sepsis has been revealed as well. The level of miR-122 was elevated in sepsis, and even higher expression was found in those

who did not survive sepsis (111). Moreover, increased serum miR-122 was correlated to coagulation disorders in sepsis (117). Very recently, multivariate regression analysis showed that serum miR-122 was an independent prognostic factor for 30 day-mortality based on Sepsis-3 criteria (118).

Other miRNAs

In addition to the outlined miRNAs, a large number of other miRNAs have been investigated in relation to sepsis. MiR-486 and miR-182 were overexpressed, while miR-342 was down-regulated in peripheral blood leukocytes of septic individuals (98). Altered levels of miRNAs predicted the outcome of sepsis. Serum miR-483 (111) and miR-297 (119) were reduced, while miR-574 (119) was increased in survivors than non-survivors. Tacke *et al.* found that miR-133a levels were elevated in sepsis that correlated with sepsis severity, SOFA (Sequential Organ Failure Assessment) scores and CRP/PCT concentrations (120). The members of miR-4772 family were up-regulated in both *ex vivo* blood leukocytes and *in vitro* stimulated monocytes by LPS, however it was not able to differentiate sepsis from SIRS (100). A miRNA regulatory network with pathway analysis, disease ontology analysis and protein-protein interaction network analysis were applied to test miRNA reliability. Huang *et al.* identified 7 miRNAs, which have the potential to be diagnostic (miR-15a, miR-16, miR-122, miR-146a, miR-223, miR-499, miR-150) and 3 prognostic sepsis biomarkers (miR-483, miR-574, miR-193) (121).

CELL-FREE miRNAs AS THROMBOSIS SENTINELS

Although the role of miRNAs in the regulation of hemostasis and in the development of coagulation disorders has not been totally clarified as yet, panels of plasma miRNAs may aid to diagnose and monitor coagulation-related diseases (122). For example, cell-free miRNAs alterations were directly associated with coronary artery disease, acute ischemic stroke, antiphospholipid syndrome reviewed in Ref. 122. Since platelets are major source of circulating miRNAs, change in their plasma levels can effectively indicate platelet activation and related vascular disorders (123). Further clinical studies are also required to evaluate the potential of circulating miRNAs for time-course detection of sepsis-induced platelet activation with or without disseminated intravascular coagulopathy, as it was performed in rats after non-lethal endotoxin injection (124).

CONCLUSIONS

The properties of miRNAs hold potentials for analyzing them as novel diagnostic and prognostic biomarkers. Although there are several circumstances that may challenge the analysis of circulating miRNAs, they may become routinely accessible, non-invasive molecular biomarkers in the near future based on the results of recent clinical trials (14). In addition, circulating miRNAs seem to be critical components of the pathogenesis of diseases like other already established biomarkers, such as *BCR-ABL* or *HER2* in malignancy, thus they are not only the molecular remnants of different cell types, but rather of functional importance (125).

Consequently, miRNAs represent not only a new diagnostic repertoire, but targeted drugs can be developed to inhibit diseases with altered miRNA profile.

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In this issue: Non-coding RNAs as potential laboratory biomarkers

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MicroRNAs in endocrine tumors

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ABSTRACT

MicroRNAs (miRNAs) are small, protein noncoding RNAs that regulate gene expression post-transcriptionally. Their role is considered to set the gene expression to the optimal level, or in other words to provide "fine tuning" of gene expression. They regulate essential physiological processes such as differentiation, cell growth, apoptosis and their role is known in tumor development too.

At tissue level differential miRNA expression in endocrine disorders including endocrine malignancies has also been reported. A new era of miRNAs-related research started when miRNAs were successfully detected outside of cells, in biofluids, in cell-free environments. Their significant role has been demonstrated in cell-cell communication in tumor biology.

Due to their stability circulating miRNAs can serve as potential biomarkers. In common diseases circulating miRNAs can be potentially proposed as screening biomarkers and they are also useful to detect tumor recurrence hence they can be applied in post-surgery follow-up too. MiRNAs as diagnostic markers can also be helpful at tissue level when certain

histology diagnosis is challenging. Beside diagnosis, tissue miRNAs have the potential to predict prognosis.

Intensive research is carried out regarding endocrine tumors as well in terms of miRNAs. However, until now miRNAs as biomarkers do not applied in routine diagnostics, probably due to the challenging preanalytics. In this review we summarized tissue and circulating miRNAs found in thyroid, adrenal, pituitary and neuroendocrine tumors. We aimed to highlight the most important, selected miRNAs with potential diagnostic and prognostic value both in tissue and circulation. Common miRNAs across different endocrine neoplasms are summarized and miRNAs enriched at 14q31 locus are also highlighted suggesting their general role in tumorigenesis of endocrine glands.

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INTRODUCTION

MicroRNAs (miRNAs) are small, protein noncoding RNAs that regulate gene expression post-transcriptionally. Through RNA interference miRNAs target mRNAs mainly at 3' untranslated regions but even the coding sequence or 5'UTR were described to be miRNA target regions [1]. After biogenesis the mature miRNA incorporates into miRNA-induced silencing complex (miRISC) [1]. In the miRISC complex, based on sequence complementarity miRNAs cause translational repression, mRNA destabilization or mRNA cleavage. However in some particular cases miRNAs can enhance gene expression as well [1]. It is thought that approximately 30-50% of all protein-coding genes might be controlled by miRNAs [2]. One miRNA potentially targets several transcripts, and one gene's transcription is influenced by numerous miRNAs. Therefore, the net physiological outcome is the result of a miRNA target network. Their role is considered to set the gene expression to the optimal level, or in other words provide "fine tuning" and adaptive setting of gene expression [3]. Their roles have been described in the regulation of several physiological and pathophysiological cellular processes such as proliferation, differentiation, metabolism and apoptosis.

A new era of miRNA-related research started when miRNAs' presence was proved outside of cells, in biofluids. Henceforth extracellular miRNAs have been considered as a novel type of biomarkers that are secreted and can be taken up by various cells. Furthermore, as signal mediators they can function similarly to hormones or cytokines. Several studies showed correlations between circulating miRNA dysregulation and pathophysiological conditions. Regarding neoplastic diseases extracellular miRNAs recently have been investigated and linked to diagnosis, prognosis and recurrence [4]. Differential miRNA expression in endocrine disorders including malignancies has also been reported [5, 6].

Extracellular (EC) miRNAs are secreted by nearly all kinds of cells and therefore they are detectable practically in all body fluids [7]. As unprotected miRNAs are sensitive to degradation mainly through RNAses present in large amount in these fluids, miRNAs in circulation are bound to proteins (mainly Argonaute (AGO)) or they are packaged into vesicles that protect miRNAs against degradation or cleavage. Based on size, EC vesicles are mainly categorized as exosomes, microvesicles or apoptotic bodies. Exosomes are small (approximately 30-100 nm) membrane-limited secreted vesicles [8]. They are formed in the endosomal compartments of cell (multivesicular endosomes or MVEs) and can be released to extracellular space. Microvesicles are more variable in size, typically between 50-1000 nm. They are generated by directly budding or shedding off the plasma membrane. Both exosomes and microvesicles contain various molecules including mRNA, miRNA, proteins, cytokines and different surface receptors

specific for their cell origin. Interestingly, several miRNAs were found in high density lipoprotein (HDL) particles as well [8].

Since their discovery there is exponentially increasing information regarding EC vesicle function. Studies showed their significant role in cell-cell communication in immunology and tumor biology. For instance, exosomes secreted by dendritic cells carry antigens and are able to induce immune response [9]. They can mediate paracrine signals of cancer cells influencing tumor microenvironment by exosome secretion in promoting growth by inhibiting antitumor immune response and by facilitating angiogenesis, cell migration and metastasis [10]. Also, tumor cells were found to exhibit self-promoting effect by secreting microvesicles [11]. However, gastric cancer cells were detected to eliminate tumor-suppressor miRNAs by exosome secretion [12].

On the whole, in a malignant tumor overexpressed (oncogenic) and downregulated (tumor suppressor) miRNAs can be useful as potential biomarkers. MiRNAs are tissue specific, they may be unique identifiers of certain tumor types both in tissue and in circulation. The purpose of miRNA biomarkers can be various and they have a great potential in many ways. In frequent diseases, e.g., thyroid nodule (where prevalence is 2-6%, 19-35% and 8-65% by palpation, ultrasound and autopsy, respectively) circulating miRNAs can be potentially recommended as screening biomarkers [13]. miRNAs as diagnostic markers can also be helpful when cytology following fine-needle aspiration biopsy (FNAB) has to be performed, as from 3-6% to 10-25% of FNAB are interpreted as indeterminate without definitive diagnosis regarding thyroid tumors [13]. Additionally, in any tumor where histological diagnosis can be challenging (e.g. adrenal carcinoma or pheochromocytoma) miRNA biomarkers can be used to help diagnosis. Beside diagnosis, tissue miRNAs have the potential to predict prognosis and therapy response as well. Circulating miRNAs are also useful to detect tumor recurrence hence they can be applied in post-surgery follow-up.

In this review we focus on tissue and circulating miRNAs in thyroid, adrenal, pituitary and neuroendocrine tumors. Although the frame of this review cannot allow assessing all miRNAs in all the above mentioned neoplasms, for that many excellent reviews are available targeting a single tumor type, rather we would like to highlight the most important, selected miRNAs as potential diagnostic and prognostic tissue and circulating biomarkers.

THYROID TUMORS

Thyroid cancer is the most frequent endocrine malignancy. The majority of thyroid tumors (~95%) arise from follicular cells and they are categorized as papillary (PTC, 75-80%) and follicular (FTC, 10-15%) or anaplastic thyroid cancer (ATC, 0.2-2%). Tumors developing from parafollicular C cells are called medullary thyroid cancer (MTC) representing ~5-10% of all thyroid cancers. Although commonly they occur sporadically, some of them (25-30%) are hereditary and part of multiple endocrine neoplasia type 2 (MEN2), caused by germline mutations of the RET proto-oncogene [14]. Most of the well differentiated thyroid cancers (DTC, including PTC, FTC) have excellent prognosis, however patients with ATC have 6-12 months' median survival [13]. Thyroglobulin is widely applied as tumor marker for tumors arising from follicular cells. It is used for evaluation of tumor residuum or recurrence in patients treated by total thyroidectomy and/or radioidine ablation. On the other side calcitonin, a product of parafollicular C cells, is used for the diagnosis and follow-up of MTC. Both tumor markers have limitations therefore miRNAs can be practical in these diseases too [15].

Diagnostic miRNAs

Interestingly, global miRNA downregulation was detected in malignant thyroid cancer compared to normal tissue together with decreased DICER gene expression that was associated with aggressive features [13, 15].

Since the first publication on miRNAs in thyroid cancer in 2005, numerous studies have been

Table 1 miRNAs discriminating benign vs. malignant thyroid lesions from FNAB samples

Authors	Panel of markers	Groups	n (sample number)	Sensitivity (%)	Specificity (%)	Diagnostic accuracy (%)
Stokowy *et al*. 2016	miR-484, miR-184b-3p	mutation-negative follicular thyroid carcinomas and follicular thyroid adenomas	44	89	87	na
Paskas *et al*. 2015	BRAF V600E, miR-221, miR-222, galectin-3	benign vs. malignant thyroid nodule	120	73.5	89.8	75.7
Panebianco *et al*. 2015	miR-146b, miR-222, KIT, TC1	benign vs. malignant thyroid nodule	118	92.3	94.7	96
Shen *et al*. 2012	miR-146b, miR-221, miR-187, miR-30d	benign vs. malignant thyroid nodule	68	88.9	78.3	85.3
Keutgen *et al*. 2012	miR-328, miR-222, miR-21, miR-197	benign vs. malignant thyroid lesions	72	100	86	90
Mazeh *et al*. 2012	miR-21, miR-31, miR-146b, miR-187, miR-221, miR-222	benign vs. malignant thyroid nodule	11	88	100	90

published and extensively reviewed e.g., by Malek *et al.* and Celano *et al.* in 2017. Among several miRNA alterations described, recent meta-analyses emphasize the role of couple of commonly upregulated miRNAs: miR-146b, miR-221, miR-222, miR-181b in PTC compared to normal tissue [13, 15]. Regarding the role of some of these miRNAs, downregulation of KIT was identified as the common potential regulating mechanism [16]. In FTC, miR-637, miR-181c-3p, miR-206, and miR-7-5p were discovered as de novo potential FTC markers based on another meta-analysis comprising 3 independent datasets [17]. In anaplastic thyroid cancer, similarly to DTC cases, overexpression of miR-146b, miR-221, and miR-222 was described together with downregulation of miR-200 and miR-30 families leading to enhanced epithelial-to-mesenchymal transition (EMT) [18]. miRNAs have been dysregulated in MTC too, and this dysregulation was described as a probable early event in C-cell carcinogenesis [19]. Similar to ATC, the underexpressed miR-200 family through regulating E-cadherin by directly targeting ZEB1 (zinc finger E-box binding homeobox 1) and (ZEB2 zinc finger E-box binding homeobox 2) leads to enhanced expression of transforming growth factor β (TGFβ)-2 and TGFβ-1 [13, 15].

Apart from tissue samples fine needle aspiration biopsy (FNAB) specimens were also subjected to miRNA analysis and yielded highly comparable results [15]. Meta-analyses showed that multiple miRNA assays showed a higher diagnostic accuracy than single miRNA and the test results indicated 77% sensitivity, 75% specificity with 0.83 AUC in a receiver operating characteristic analysis [20]. However, several other studies reported promising sets of miRNAs in discriminating benign vs. malignant thyroid lesions from FNAB samples (Table 1).

Circulating miRNAs in both serum and plasma were tested and excellently summarized by Celano *et al*. Although the summary reported a highly variable miRNA expression [15], some miRNAs were identified by multiple groups. In PTC patients, the levels of miR-146b, miR-221 and miR-222 were detected to be higher compared to controls [13, 15, 21]. Post-surgically, significantly reduced miR-151 and miR-222 expression was reported compared to pre-operative samples in more than one study [21]. Also, miR-146b reportedly discriminated benign and malignant tumors with 61.4% sensitivity and 74.3% specificity, while miR-155 had 57.9% sensitivity and 63.2% specificity in serum/plasma [13, 15]. The levels of these two miRNAs were also correlated with lymph node metastases and tumor size [13, 15].

Plasma exosomal miRNAs were also assessed and miR-31 was found to be over-represented in the samples of patients with PTC compared to benign tumors, while miR-21 helped to distinguish between FTC and benign tumors. Using both miR-21 and miR-181a-5p helped to distinguish between PTC and FTC with 100 % sensitivity and 77 % specificity [23].

Prognostic miRNAs

Several studies investigated the potential prognostic value of miRNAs that was summarized in Celano *et al*. Higher expression of miR-146b, miR-221 and miR-222 showed association with prognostic parameters on tissue level [15]. The expression of these miRNAs showed association with tumor size, capsular and vascular invasion, extra-thyroidal extension, lymph node metastases and TNM stage [15]. Besides, the overexpression of miR-146b correlated with multifocality and miR-221 with distant metastases as well in PTC. Overall survival was significantly decreased in patients with higher miR-146b expression in tumor tissue [24]. In a recent study 7 miRNAs (miR-146b, miR-184, miR-767, miR-6730, miR-6860, miR-196a-2 and miR-509-3) were associated with the overall survival and miR-184, miR-146b, miR-509-3 and lysophosphatidic acid receptor 5

(LPAR5) were identified as independent risk factors for prognosis by multivariate analysis [25]. In FTC, the level of miR-221-3p, miR-222-3p, miR-222-5p, miR-10b and miR-92a was higher in metastatic cases vs. non-metastatic. Regarding MTC, Abraham *et al.* published that miR-183 and miR-375 were associated with lymph node metastases, distant metastases and mortality [26]. Also, upregulation of miR-224 was described as potential prognostic biomarker associated with a better outcome in MTC patients [19].

Among circulating miRNAs the level of miR-222, miR-221, miR-146b and miR-151-5p was described to be decreased after tumor removal [15]. Serum level of miR-146a-5p and miR-221-3p was found to be consistent with response to therapy, including patients with structural evidence of disease whose thyreoglobulin (Tg) test remained negative [13, 15]. Therefore it is suggested that these two miRNAs could be applied as post-treatment monitoring biomarker of PTC patients, especially when Tg assay results are uninformative [13, 15].

ADRENAL TUMORS

Adrenal tumors can develop from the adrenal cortex or the medulla. Adrenocortical tumors (ACT) are common and their prevalence reaches 6% after the age of 60 years [27]. Most of them are benign (adrenocortical adenomas, ACA) in nature. Although the most common tumors, so called incidentalomas are non-functioning, some are associated with hormone overproduction syndromes (such as Cushing's, Conn's syndrome, hyperandrogenism). Adrenocortical carcinomas (ACC) however, are rare and aggressive tumors (0.5–2/million per year) [27] with a dismal prognosis where surgical resection is the only curative treatment. The differential diagnosis between ACA and localized ACC can be challenging as radiological and pathological features can be similar but the distinction between them is essential due to the completely different therapy.

HORMONE PRODUCING ADRENAL ADENOMAS

Regarding functional adenomas, peripheral blood hormone testing can help in diagnosis. In aldosterone producing adenomas (APAs) high expression of miR-23 and miR-34a was described [27]. He *et al*. reported 31 miRNAs which were significantly differentially expressed in APAs when compared to normal adrenal cortex. Of these, 23 were downregulated with miR-375 being the most underexpressed [28]. Between expression of miR-375 and miR-7 a strong positive correlation was found indicating a potential synergistic function [28]. MiR-375 substitution in NCI-H295R ACC cell line resulted in decreased cell growth and it inhibited its target gene metadherin (MTDH). Also, miR-375 expression was negatively correlated with APA tumor size reflecting its potential role as a prognostic marker. In cortisol producing adenoma and carcinoma, circulating, extracellular, vesicle-associated miR-22-3p, miR-27a-3p and miR-320b were significantly overrepresented compared to non-functional adenomas [29].

ADRENOCORTICAL CARCINOMAS (ACC)

Diagnostic miRNAs in ACC

Similarly to thyroid tumors, numerous publications reported differentially expressed miRNA profile in ACA and ACC which are exceedingly summarized by Igaz *et al*., Cherradi *et al*., and Hassan *et al*. [30–32]. The most frequently reported overexpressed miRNAs were miR-483-5p and -3p, miR-503, miR-210 and miR-184 in ACC vs ACA. Certainly, miR-483-5p and miR-483-3p have a significant role in adrenal tumorigenesis, its gene is located in the intron of the insulin like growth factor 2 (IGF2) gene, a well-known overexpressed

gene in ACC. Expectedly, a positive correlation was described between miR-483-5p and IGF2 expression levels [33]. Experiments, however, demonstrated that miR-483-5p has an independent role in ACC's pathogenesis as IGF2 transgenic animals did not develop tumors [31]. Indeed, downregulation of miR-483-5p (and miR-483-3p) led to decreased proliferation of ACC cell line [34], and it protected cells from apoptosis by targeting proapoptotic PUMA (p53-upregulated modulator of apoptosis or BBC3: BCL2 binding component 3) [30]. Wang *et al*, suggested the use of the combination of SMAD4 (SMAD family member 4) negative/low expression with elevated miR-483-3p expression that provided a diagnostic specificity of 92.8% for distinguishing ACA vs. ACC (while SMAD4 expression itself demonstrated high sensitivity of 92%) [35]. The overexpression of miR-210 has been also documented by several publications [31, 32]. This miRNA is widely overexpressed in different tumors and it is also called as a "hypoxamir" due to its upregulation by both hypoxia inducible factor 1 subunit alpha and beta (HIF1α, HIF1β). It has a role in tumorigenesis through regulating arrest of cell proliferation, repression of mitochondrial respiration, arrest of DNA repair, vascular biology, and angiogenesis [36]. Among downregulated miRNAs in ACC miR-195 has been frequently reported [31, 32]. As a member of miR-15/16/195/424/497/6838 family it promotes apoptosis together with inhibiting cell proliferation in ACC and other cells targeting cell cycle regulators such as cyclin D1 (CCND1) or cyclin dependent kinase 6 (CDK6) [31].

Beside adrenocortical tumor tissues, miR-483-5p was found to be increased in serum, plasma and circulating exosomes derived from patients with ACC compared to patients with ACA [31, 32]. Circulating miR-100, miR-181b, miR-184, miR-210 and miR-34a were found to be upregulated and miR-195, miR-335, miR-376a downregulated in ACC samples compared to ACA [31, 32]. After tumor removal, the initially highly expressed miR-483-5p decreased and low level of miR-195-5p increased in blood stream of patients with ACC. Therefore, it is suggested that these miRNAs directly derived from ACC tissues [32]. Interestingly, opposite expression of miRNAs in tissue vs. serum was also described in independent publications too. While miR-34a was detected increased in serum and decreased in ACC tissues, miR-376a showed the opposite pattern suggesting a potential selecting mechanism of extracellular secretion [31, 32]. Assessing diagnostic potential of miRNAs Chabre *et al.* also investigated miR-195, miR-335 and miR-376a and found that miR-195 showed the highest sensitivity (90,9%) and specificity (100%) in discriminating ACC patients [37].

Prognostic miRNAs in ACC

The decreased expression of miR-195 and miR-497 in ACC was reported to directly regulate TARBP2 (TARBP2 subunit of RISC loading complex) and DICER1 (dicer 1, ribonuclease III) expression in ACC cells therefore contributing to a global downregulation of miRNA expression [32, 34]. Also, a significant overexpression of TARBP2, DICER, and DROSHA (drosha ribonuclease III) in ACC compared with ACA or normal adrenal cortices were found and inhibition of TARBP2 in human ACC cell line resulted in a decreased cell proliferation and induction of apoptosis [38]. Interestingly, low DICER1 expression was also associated with poor clinical outcome in adrenocortical carcinoma [30–32].

Lower expression of miR-483-5p in combination with increased miR-195 level was reported as predictor of poor prognosis in ACC [30–32]. High expression of miR-503 was associated with shorter survival [34], and increased expression of miR-210 with ACC clinicopathologic parameters of aggressiveness and a poor prognosis [39,

40]. In a recent study, three miRNA clusters were identified related to prognosis [40]. "Mi1" and "Mi2" miRNA clusters including 11 upregulated miRNAs located at Xq27.3 and 38 downregulated miRNAs derived from 14q32 locus were associated with better prognosis (C1B molecular group) while "Mi3" miRNA cluster associated with poor prognosis (C1A group).

Using next-generation sequencing expression levels of 6 microRNAs (miR-503-5p, miR-483-3p, miR-450a-5p, miR-210, miR-483-5p and miR-421) predicted malignant/non-malignant status with over 95% accuracy [41]. In this study the best single miRNA for malignancy was miR-483-3p [41]. MiR-139-5p and miR-376a levels significantly increased in aggressive ACC compared with non-aggressive ACC patients in tumor samples but not in circulation [37]. Additionally, high circulating levels of miR-483-5p or low circulating levels of miR-195 were associated with both shorter recurrence-free survival and shorter overall survival in the study of Chabre *et al*. 2013 [37].

PHEOCHROMOCYTOMA-PARAGANGLIOMA

Although the localization is different pheochromocytomas (PCC) and paragangliomas (PGL) arise from the same type of neural crest tissue of the sympathetic and parasympathetic paraganglia [42]. Tumors of the adrenal medulla are called PCCs and neoplasms developing from thoracic, abdominal or pelvic region paraganglia are named as PGLs. It represents a rare disease as its estimated incidence is 1-8 cases per million worldwide annually [42]. PCCs and PGLs are usually benign (10-year overall survival is around ~96%), however 10% of PCC and even 40% of PGL become metastatic resulting in a poorer prognosis (5-year survival below 50%) [42]. Unfortunately, there are neither clear histopathological signs of malignant behavior nor efficient therapy for malignant PCC/PGL. Therefore, investigating miRNAs as potential biomarkers can be useful in this regard. Nonetheless, compared to thyroid and adrenocortical neoplasms miRNAs are not so extensively investigated in PCC/PGL.

More than 30% of the cases are attributed to germline mutation leading to autosomal dominant genetic syndromes such as multiple endocrine neoplasia type 2A and 2B caused by *RET* mutations, von Hippel Lindau syndrome due to *VHL* mutations, neurofibromatosis type 1 with *NF1* mutations or hereditary PG syndromes caused by mutations of succinate dehydrogenase (SDH) genes. Lately, as a result of next-generation sequencing, novel genes, including *KIF1b, PHD2, TMEM127, MAX, FH, MDH2, GOT2* and *SLC25A11* were identified in the pathogenesis of PCC/PGL [43].

Diagnostic miRNAs

miRNA profile in different genetic subtypes is also distinct. Hypoxia induced miR-210 was found overexpressed commonly in pseudohypoxia-associated PCC/PGLs harboring *SDHB* and *VHL* mutations. Upregulation of miR-139-3p, miR-541, miR-765 and miR-133b was described in *VHL* associated tumors, while miR-96 and miR-183 were found to be overexpressed in neoplasms with *SDHB* mutations [30, 44, 45]. NGF (nerve growth factor) treatment *in vitro* in PCC cell line significantly decreased the level of miR-139-3p and miR-210 and led to differentiation raising the role of these two miRNAs in tumor development [46]. Similarly, through targeting ezrin, miR-96 and miR-183 also suppressed cell adhesion and differentiation [30]. In MEN2B associated PCs miR-885-5p was repeatedly found to be overexpressed [30]. miR-885-5p regulates molecules involved in apoptosis (Casp3) and cell cycle (CDK2) [30], and by targeting IGF-binding proteins its role was supposed in *RET* mutation associated PCC/PGL pathogenesis

[30]. Interestingly, miR-137 and miR-382 can be considered as general PCC/PGL markers as they were found overexpressed in most cases except for *MAX* mutation associated tumors [30]. Interestingly, similarly to ACCs, miRNAs coded at 14q32 genomic region (DLK-MEG3 region) was described to be downregulated in *MAX* mutated and a subset of sporadic PC samples as well [42]. The DLK-MEG3 locus was reported to be hypermethylated in approximately 10% of PC samples and the role of downregulated miRNAs located here was also proposed [30].

Prognostic miRNAs

Similarly to ACC, miR-483-5p was found to be upregulated in malignant PCC/PGL in more than one study [30]. Also, the well-known tumor suppressor miR-15a, miR-16 were underexpressed in malignant PCC vs. benign tumors [47]. These miRNAs were downregulated in several neoplasms and they are considered tumor suppressors by targeting BCL2 and CCND1 [30, 47]. Igaz *et al*. described miR-1225-3p overexpressed compared to sporadic non-recurring, MEN2-, VHL-, and NF1-associated PCs [30]. By targeting Notch signaling, the role of miR-1225-3p is considered in PC tumorigenesis as in *in vitro* experiments Notch-1 inhibited proliferation of PC cell [30].

Compared with benign PCCs, miR-101 level was higher in patients with malignant PCCs and the level of miR-101 was higher in *SDHD* mutation associated tumors [48]. In discriminating malignant from benign PCs AUCs for miR-101 in all investigated PCCs samples were 0.79 and 0.77 for non-SDHD mutant samples [48]. Another study by Patterson *et al.* also proposed that miR-483-5p, miR-101, and miR-183 could serve as useful diagnostic markers (AUC: 0.7; 0.78 and 0.82, respectively) for distinguishing malignant from benign PCCs [44].

PITUITARY TUMORS

Pituitary adenomas are among the most frequent intracranial tumors with a high incidence rate, approx. 10–15 % [49]. Although the great majority of pituitary tumors are benign they can lead to significant morbidity through compressing adjacent structures (visual impairment, headache) or by hormonal disturbance (either hypo- or hyperfunction). Of these 95% are sporadic, only the remaining 5% are associated with genetic syndromes such as MEN1, MEN4, Carney complex or McCune-Albright syndrome. Interestingly, miRNAs are an extensively investigated field in pituitary tumors. Since the first study published in 2005 by Bottoni *et al*.[50], more than a hundred hits have appeared for a search with keywords “microrna” AND “pituitary” AND “adenoma” in Pubmed in February, 2019. These studies broadly evaluated not only miRNA signature characteristics of different types of pituitary adenomas but also target genes and miRNA function as well. Accordingly, several reviews summarizing the expression profile and role of tissue miRNAs widely investigated in pituitary adenoma have been published, one of the most recent by Feng *et al*. in 2018 [51] who extensively summarized miRNAs reported in pituitary adenomas dissected by adenoma subtypes. Here, we aim to highlight the roles of miRNAs by their targets’ function. Although the expression profile and role of tissue miRNAs are widely investigated in pituitary adenoma, we lack information about the diagnostic potential of miRNAs present in circulation. Pituitary adenomas are hormone secreting which give an excellent opportunity to monitor tumor growth and function by hormone tests, therefore the role of miRNAs might be less important. However, regarding non-functional pituitary adenomas a blood-based miRNA biomarker could help the diagnosis and patient follow-up after surgery.

Diagnostic miRNAs

Pituitary subtype-specific miRNA profile was described by several studies [51].

In ***GH producing adenomas*** several miRNAs target insulin growth factor binding proteins (IGFBP-3, -6, -7) that regulates organ development and growth [51]. The overexpressed miR-26a has a key role in the pathogenesis of GH-secreting adenoma by directly regulating PI3K/Akt signaling pathway and LEF-1 that is involved in anterior pituitary development [51]. The overexpression of Pituitary Tumor-Transforming Gene 1 (PTTG1) was demonstrated in ~90% of pituitary adenoma independently of adenoma type. A set of downregulated miRNAs (miR-126, miR-381, miR-665, miR-300, miR-381, miR-329) is suggested to contribute to *PTTG1* overexpression as restoring their level *in vitro* led to suppressed viability and proliferation of pituitary cells [52]. High Mobility Group AT-Hook Protein 2 encodes a non-histone chromosomal high mobility group (HMG) protein, as a transcriptional regulator it controls cell cycle and pituitary cell proliferation. Numerous miRNAs were identified targeting HMGA1 and 2 in GH, PRL and non-functioning pituitary adenomas [51]. In sporadic GH-producing adenomas miR-34a and miR-107 were found to regulate *AIP* a well-known tumor suppressor frequently mutated in familial pituitary adenomas.

In ***corticotroph adenomas*** miR-26a was massively overexpressed compared to normal pituitary. MiR-26a downregulated Cyclin A and Cyclin E and it targeted PRKCD (protein kinase C delta) that was reported to suppress ACTH secreting pituitary cells [51].

In ***prolactinomas*** HMGA1 and 2 as a target of several miRNAs were also validated. Interestingly the expression of miR-432 were found to be positively correlated with serum prolactin [51]. The role of downregulated miR-410 was also proved in pathogenesis of prolactinoma by targeting Cyclin B1 [51].

In ***non-functioning pituitary adenomas*** (NFPAs) the role of HMGA1 and 2 proteins also has to be mentioned regulated by several miRNAs [51]. Cell cycle in NFPAs is regulated in G2M transition by miRNAs through targeting Wee1 and CDC25A [53]. The TGFβ signaling is also controlled by miRNAs through SMAD3 [54]. Additionally, miR-106b influenced migration and invasion of pituitary adenoma cells via regulating PTEN and further activity of the PI3K/AKT signaling pathway and MMP-9 expression [51].

In pituitary carcinoma metastasis expression of miR-20a, miR-106b and miR-17-5p were increased compared to the primary neoplasm and these miRNAs were proved to be involved in pituitary carcinoma metastasis by attenuating PTEN and TIMP2 (TIMP metallopeptidase inhibitor 2) [55]. In ACTH producing carcinomas miR-493 was significantly upregulated compared to ACTH adenomas while miR-122 overexpressed in both corticotroph adenomas and carcinomas compared to normal pituitary [56]. LGALS3 and RUNX2 are both predicted targets of miR-493 and these genes have been shown to have roles in pituitary tumor cell growth [56].

Prognostic miRNAs

Level of several miRNAs (miR-24, miR-34a, miR-93, miR-148-3p, miR-152, miR-132, miR-15a, and miR-16) are significantly lower in invasive pituitary adenomas compared with non-invasive ones [57]. In prolactinomas, miR-183 was found downregulated in aggressive tumors and it inhibited tumor cell proliferation by inhibiting KIAA0101 that is involved in cell cycle activation and inhibition of p53-p21 mediated cell cycle arrest [58]. In corticotroph adenomas miR-93-3p, miR-93-5p, miR-25-3p and miR-106b-5p were detected to be overexpressed in invasive tumors compared to non-invasive ones through

targeting *MCM7* the overexpression of these miRNAs led to increased invasiveness and unfavorable outcomes after resection [51, 59]. Patients with corticotroph adenomas with decreased level of miR-141 had higher chance of remission [60].

In NFPAs several miRNAs showed correlation with tumor size some of the (miR-450b-5p, miR-424, miR-503, miR-542-3p, miR-629, and miR-214) were underexpressed and target *SMAD3* [54]. In invasive adenomas expression levels of miR-181b-5p, miR-181d, miR-191-3p, and miR-598 were upregulated, and the expression levels of miR-3676-5p and miR-383 were downregulated [61]. In GH3 cells Caveolin-1 (CAV1) was reported to promote invasion while silencing *CAV1* indirectly induced miR-145, miR-124, and miR-183 that suppressed the migration and invasion of pituitary adenoma cells through targeting *FSCN1, PTTG1IP* and *EZR,* respectively [57].

NEUROENDOCRINE TUMORS (NETs)

Neuroendocrine tumors (NETs) consist of heterogeneous neoplasms of different origin arising from neuroendocrine cells throughout the body (most commonly from the lungs, pancreas, small intestine, and rectum). Gastro-entero-pancreatic NETs (GEP-NETs) represent less than 1% of digestive cancers and 7-21% of all neuroendocrine neoplasms [62]. Lung NETs originate from pulmonary neuroendocrine cells accounting for approximately 25% of primary lung neoplasms. Lung NETs classified into the following subtypes: typical carcinoids (TCs, well differentiated, low-grade); atypical carcinoids (ACs, well-differentiated, intermediate-grade); large cell neuroendocrine carcinomas (LCNECs, poorly differentiated, high-grade); and small cell lung cancer (SCLCs, poorly differentiated, high-grade) [63]. NET as a heterogeneous group have different behavior and prognosis but they express neuroendocrine markers such as chromogranin A (CgA) and synaptophysin. Based on the World Health Organization (WHO) classification the prognosis is characterized by the grade of neuroendocrine differentiation and the proliferative index (Ki-67) from G1-G3. However, guidelines indicate that prognosis is also influenced by several other factors, such as patient age, tumor site, metastatic spread and hormonal production [64]. Unfortunately as more than 80% of patients usually present with metastatic disease, NET prognosis is poor, due to the relative lack of effective therapy [63, 64]. Among circulating biomarkers, CgA has been measured in several NETs, but its value as a prognostic biomarker in NETs is limited [64], therefore identification of prognostic factors to predict outcome would be fundamental.

Diagnostic miRNAs

Interestingly, little information is available regarding miRNA profile in ***gastric NETs (gNETs)***, however it was described that gastrin-induced miR-222 overexpression resulted in reduced p27, which in turn caused actin remodeling and increased migration in human stably CCK2 receptor expressing gastric adenocarcinoma cell line [65].

In circulation, serum miR-222 expression was increased in hypergastrinemic patients with autoimmune atrophic gastritis and type 1 gastric NET. Because its level decreased in patients after CCKR2 agonist treatment miR-222 was proposed to be a promising biomarker for gastrin induced premalignant changes in the stomach [65].

However, different miRNA sets with altered expression were described regarding ***pancreatic NET (pNET)***, comparing functional, nonfunctional NET, normal pancreas, normal pancreatic islets in any combinations that is reviewed in detail by Malczewska *et al*. and Zatelli *et al*.

[64, 65]. The overexpressed miR-103, miR-107 and the underexpressed miR-155 discriminated sporadic pancreatic NET (insulinoma and non-functioning) from acinar cell carcinomas. The role of downregulated miR-155 was suggested in pathogenesis by targeting proapoptotic tumor protein p53 inducible nuclear protein 1 (TP53INP). Also, miR-144/451 cluster and miR-21 was found overexpressed compared to normal pancreatic islets [65]. MiR-204 was found primarily expressed in insulinomas and correlated with immunohistochemical expression of insulin [66]. Interestingly, several miRNAs located at 14q32 region showed dysregulated expression, including miR-144/451 cluster [65]. Interestingly, these miRNAs showed overexpression in insulinomas compared to other endocrine tumors where miRNAs of this region are frequently downregulated. In *in vitro* experiments miR-144 induced cell proliferation in murine pancreatic β cells and regulated Akt signaling by targeting *PTEN* [65]. Additionally, miR-451 also promoted cell proliferation by regulating cell cycle through targeting p19 [67]. Overexpression of miR-21 were reported overexpressed in pNET [65, 66]. Expression of miR-642 correlated with Ki67 (MiB1) score and miR-210 correlated with metastatic disease [68].

13 miRNAs were identified by comparing serum from pNET patients and healthy volunteers and miR-193b was up-regulated in both pNET tissue and serum when compared to controls described by Thorns *et al*. [68]. In addition, miR-1290 showed overexpression in pNETs and the latter could accurately distinguish patients with low-stage pancreatic cancer from healthy controls and subjects with chronic pancreatitis [69].

MiRNA profile of ***small bowel NET (sbNET)*** was also investigated, however fewer experimental information are available regarding miRNA's function studies and target validation. MiR-7-5p, miR-182, miR-183 and miR-96-5p were found to be upregulated in sbNET compared to normal small bowel consequently in different studies [65]. Furthermore, miR-182, miR-183 and miR-96 overexpressed in NET metastases compared to primary tumors [65]. Similarly, upregulation of miR-196a was described in numerous studies however its role in cell proliferation could not be confirmed [65, 69]. In addition, the downregulation of miR-129-5p and miR-133a was also established in sbNET metastases vs. primary tumors [65].

Similarly to tissue, overexpression of miR-182, miR-196a and miR-200a and downregulation of miR-31, miR-129-5p and miR-133a were detected in blood of sbNET patients and the level of some of them changed upon somatostatin treatment [65].

Similarly to sbNET, in ***appendiceal carcinoids*** without metastases low levels of miR-96 and high levels of miR-133a were detected [65]. In ***colorectal NET (cNET)*** patients underexpression of miR-186 was found in tumor tissue, blood and stool samples compared to controls. In parallel, PTTG1 upregulation was detected in the same samples together with decreased miR-186 expression therefore the authors suggested that upregulation of PTTG1 was induced by the loss of miR-186 [70].

Interestingly, based on miRNA expression profile analysis common origin for pulmonary carcinoids and GI-NETs was suggested by Yoshimoto *et al.* [71].

Lung NETs. The expression profiles of pulmonary carcinoids and SCLCs were quite different, indicating the distinct genesis of these neuroendocrine neoplasms [71].

Prognostic miRNAs

A study by Sadanandam *et al*. identified molecular subtypes of ***pNET***s (islet-like, intermediate and metastasis-like primary types) [72]. As these subtypes exhibit distinct metabolic profiles marked by differential pyruvate

metabolism, substantiating the significance of their separate identities they may have role to predict different behavior. Expectedly, the three subtypes have distinct mRNA and miRNA signatures as well [72]. Roldo *et al*. reported the overexpression of miR-21 as positively correlated with Ki-67 proliferation index and presence of liver metastases [66]. Besides, expression of miR-642 was also described to correlate with Ki67 index score while miR-210 correlated with metastatic disease [68]. Additionally miR-196a was identified as prognostic factor in pNET as its expression significantly associated with stage, and mitotic count [64]. Also, high miR-196a level was associated with decreased overall survival and disease-free survival. The hazard ratio for recurrence of patients with high miR-196a expression was 16.267 [73].

In ***sbNET*** increased plasma miR-21 and decreased miR-150-5p were characteristic to metastatic tumors [74]. In line with this, low plasma miR-21 and high miR-150-5p levels were associated with significantly prolonged overall survival [74]

In ***rectal carcinoids*** miR-885-5p was identified as upregulated in tumors with lymphovascular invasion. Also, high miR-885-5p expression was independently associated with lymphovascular invasion. Therefore miR-885-5p is suggested as a potential biomarker for predicting malignancy [75].

Regarding NET in the lung Zatelli *et al*. thoroughly summarized miRNAs with prognostic role [64]. miR-150 and miR-886-3p were found downregulated while miR-92a2* and miR-7 upregulated in SCLC that showed correlation with overall or disease-free survival. The latter miRNAs were found to be associated with chemoresistance too [64]. In other publications including typical, atypical carcinoids and large cell neuroendocrine carcinomas the upregulated miR-21 and the downregulated miR-409-3p, miR-409-5p, and miR-431-5p correlated with the presence of lymph node metastases and set of other 5 upregulated miRNAs with overall survival [64].

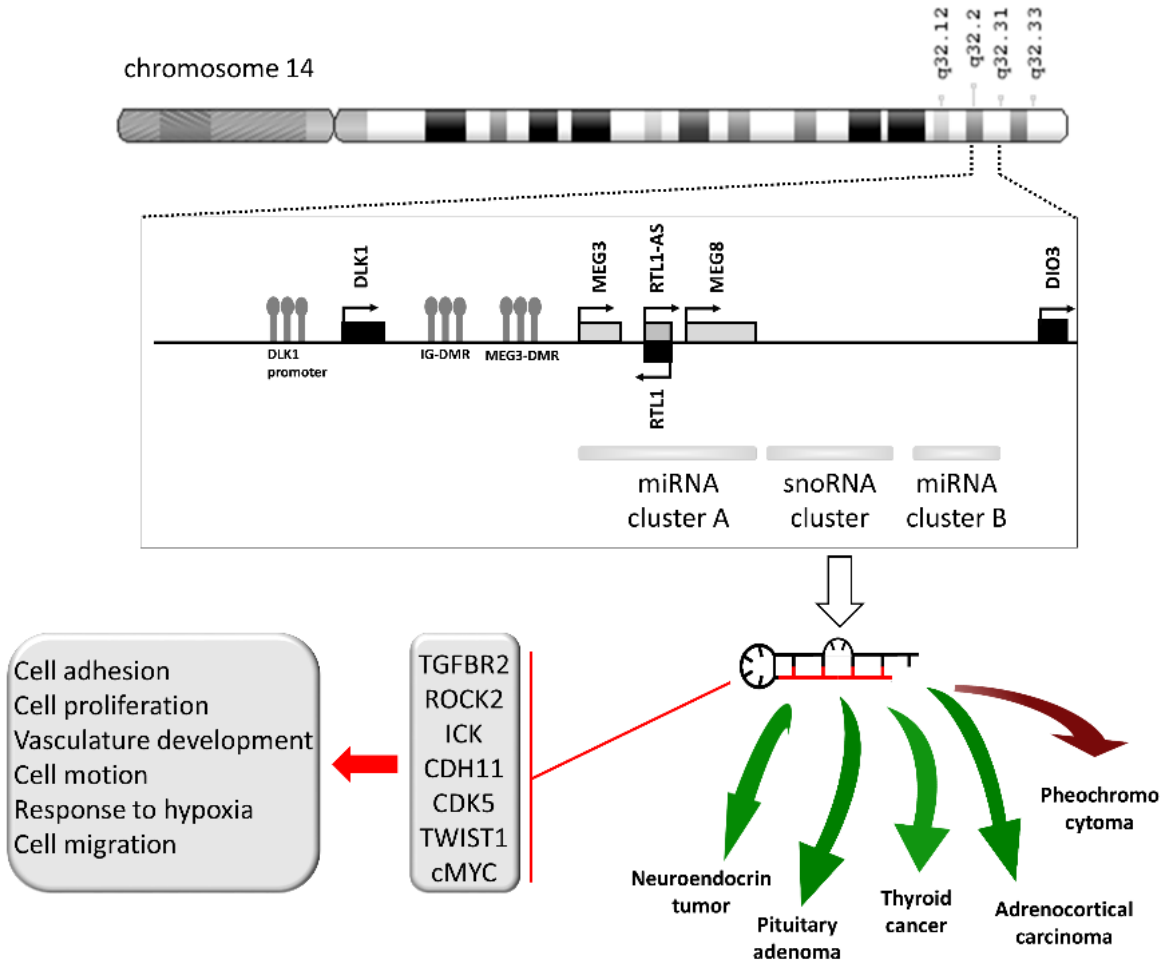
COMMON miRNAs DETECTED IN VARIOUS ENDOCRINE TUMORS

Although miRNAs' expression and their functions are tissue specific, discussing common miRNAs among different endocrine tumors can still be an interesting aspect reflecting potential common pathomechanism. For instance, miR-210 was overexpressed in several endocrine tumors, such as ACC and PCC/PGL [30, 31]. Also, its increased expression seems to be an unfavorable prognostic factor correlating clinicopathologic parameters of aggressiveness and a poor prognosis in ACC and in pNET [39, 40, 68]. Usually, the increased expression of miR-210 might be a consequence of hypoxic environment within tumor tissues or activation of pseudohypoxia signaling pathway in PPC/PGL. As a general phenomenon increase of miR-210 was also described in other, non-endocrine solid tumors (i.e. breast, lung, head and neck, pancreatic and glioblastoma).

Overexpression of miR-483-5p and miR-483-3p in tissues and in circulation (serum, plasma and exosomes) is almost a unique characteristic of ACC and is considered as a prognostic marker [30, 31, 32, 33]. It predicted malignant/non-malignant status and it associated with both shorter recurrence-free survival and shorter overall survival. Interestingly, not only for tumors of adrenal cortex but for tumors originated from adrenal medulla (PCC/PGL) miR-483-5p is also proposed as useful diagnostic markers for malignancy [30].

Two well-known tumor suppressor miRNAs: miR-15a and miR-16 are downregulated in pituitary adenomas and in PCC where they were described to be able to discriminate benign

Figure 1 **miRNAs coded at 14q32 is commonly dysregulated in endocrine neoplasms: downregulated in neuroendocrine tumors, pituitary adenomas, thyroid cancer and adrenocortical carcinoma (indicated by *green arrows*) and overexpressed in pheochromocytomas (showed by *red arrow*)***



**At 14q32 region paternally imprinted genes (DLK1, RTL1, and DIO3) are illustrated by black, maternally imprinted noncoding RNAs (MEG3, anti-RTL1, and MEG8) are represented by grey boxes.*
Lollipops indicate methylation sites at DLK1 promoter region, intergenic differentially methylated region (IG-DMR), and MEG3 differentially methylated region (MEG3-DMR) region. MiRNAs at 14q32 region are located in 2 clusters separated by a small nucleolar RNA (snoRNA) cluster. miRNA cluster 1 contains 10 miRNAs and cluster 2 encodes more than 40 miRNAs. Therefore, alteration in methylation, chromatin remodeling, genomic imprinting imbalance or structural loss can lead to dysregulated miRNA expression. 14q32 miRNAs influence cell proliferation, cell adhesion, and migration through regulating targets involved in transforming growth factor beta signaling or epithelial–mesenchymal transition.

TGFBR2: Transforming Growth Factor Beta Receptor 2; ROCK2: Rho Associated Coiled-Coil Containing Protein Kinase 2; ICK: Intestinal Cell Kinase; CDH11: Cadherin 11; CDK5: Cyclin Dependent Kinase 5; TWIST1: Twist Family BHLH Transcription Factor 1; cMYC: MYC Proto-Oncogene; RTL1: retrotransposon-like gene RTL1; RTL1-AS: retrotransposon-like gene RTL1 antisense; DLK1: Delta Like Non-Canonical Notch Ligand 1; MEG3: Maternally Expressed 3; MEG8: Maternally Expressed 8, Small Nucleolar RNA Host Gene; DIO3: Iodothyronine Deiodinase 3.

and malignant forms of the disease [47, 51]. In addition, the lost expression of general tumor suppressor miRNAs targeting BCL2 and CCND1 have been described in other, non-endocrine tumors as well [30, 47].

Additionally, Pituitary Tumor-Transforming Gene 1 (PTTG1) is another target gene that is regulated by miRNAs in endocrine tumors. Several miRNAs (miR-126, miR-381, miR-665, miR-300, miR-381, miR-329) are suggested to contribute to PTTG1 overexpression in pituitary adenomas [51], that is also upregulated in colorectal NET by the loss of miR-186 [70]. However, PTTG1 overexpression is not unique for endocrine tumors as it was described in several other malignancies with prognostic potential.

MiR-21, a widely investigated oncomiR, has been found up-regulated in many types of human tumors. Indeed, in lung NET and pNET it was described overexpressed and in pNET its expression positively correlated with proliferation index and with metastases [65, 66]. Additionally, its level was reported elevated in circulation in sbNET where it was described to be characteristic to metastatic tumors and associated with significantly prolonged overall survival [74]. In follicular thyroid cancer plasma level of exosomal miR-21 helped to distinguish between FTC and benign tumors [23].

Similarly to miR-21, miR-222 is differentially expressed in several endocrine tumors, such as in gastric NET (where it is induced by gastrin) and in thyroid cancer (both papillary and anaplastic) [13, 15, 18, 65], whereas in follicular thyroid cancer the tissue level of miR-222 was prognostic (higher in metastatic cases vs. non-metastatic). In the blood stream of PTC patients, miR-222 expression significantly decreased after surgery [21].

CONCLUSIONS AND FUTURE PERSPECTIVES

It is obvious that miRNAs are potentially useful and promising tissue and circulating biomarkers. They are extensively investigated in different neoplasms including tumors of the neuroendocrine system. Interestingly, miRNAs coded at 14q32 region seems to be dysregulated in almost all endocrine neoplasms (Figure 1) and several other tumors. This region is frequently found hypermethylated in cancer compared to normal tissues. The downregulation of miRNAs located at 14q32 region shows important correlations with poor prognosis and aggressiveness in different cancer types. Studies also suggest chromatin remodeling by lncRNA-mediated mechanisms in this region beside DNA methylation that may also influence miRNA expression. Additionally, as 14q32 is an imprinted region and imprinting imbalance could also result in alteration of paternally and maternally expressed genes.

Regarding their application to routine diagnostics thyroid tumors and FNAB samples seem to be investigated the most intensively. Although high sensitivity and diagnostic accuracy of miRNAs have been reported in thyroid cancer which may be suitable for differentiation of benign vs malignant thyroid nodules, routine use of miRNAs as biomarkers has not been widespread yet either in thyroid or other endocrine and non-endocrine tumors. In literature it is suggested that this may be due to challenging preanalytics, especially in case of bio-fluids. Additionally, there are no IVD-CE (IVD: applicable for In Vitro Diagnostic, CE: complies with the essential requirements of the relevant European health, safety and environmental protection legislations) marked assays to measure miRNAs in clinical care. That also may delay miRNAs' application as potential diagnostic biomarkers.

However, regarding thyroid nodules there are common miRNAs among different studies discriminating benign and malignant nodules (e.g. miR-221, miR-222, and miR-146b). These miRNAs should be further investigated as potential candidates for clinical use.

In pituitary neoplasms, however there is the most information gathered regarding the pathogenesis and miRNA-regulated networks, there is basically no information on miRNAs in biofluids. It can be a consequence of negative results or the difficulty of sample group homogenization due to the different adenoma groups especially in the light of the 2017 WHO classification. In adrenocortical carcinoma the role of one miRNA (miR-483-5p) has been repeatedly proved by several studies in both tissue level and in circulation. And finally, the prognostic role of miRNAs is also intensively investigated in NETs however, again, due to the heterogeneous tumor group there is still a need for data regarding the application of any miRNA biomarker in clinical use. Behind the discrepant results found in literature not only the tumor heterogeneity but also the differences in study design and technical implementation could be suspected. Regarding circulating biomarkers, currently there are many biological (age, gender, BMI, smoking status, disease stage, other accompanying illnesses, medications, etc.) and technical (serum, plasma, extracellular vesicle as source, different nucleic acid extraction methods, miRNA detection methods, etc.) factors known and unknown which need standardization and harmonization in order to establish evidences before application of miRNAs as biomarkers.

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In this issue: Non-coding RNAs as potential laboratory biomarkers

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The role of microRNAs in congenital heart disease

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ABSTRACT

Congenital heart diseases (CHDs) are the leading inherited cause of perinatal and infant mortality. CHD refers to structural anomalies of the heart and blood vessels that arise during cardiac development and represents a broad spectrum of malformations, including septal and valve defects, lesions affecting the outflow tract and ventricules. Advanced treatment strategies have greatly improved life expectancy and led to expanded population of adult patients with CHD. Thus, a better understanding of the pathogenesis and molecular mechanisms underlying CHDs is essential to improve the diagnosis and prognosis of patients. The etiology of CHD is largely unknown, genetic and environmental factors may contribute to the disease. In addition to the mutations affecting genomic DNA, epigenetic changes are being increasingly acknowledged as key factors in the development and progression of CHDs. The posttranscriptional regulation of gene expression by microRNAs (miRs) controls the highly complex multi-cell lineage process of cardiac tissue formation. In recent years, multiplex experimental models have provided evidence

that changes in expression levels of miRs are associated with human cardiovascular disease, including CHD. The newly described correlations between miRs and heart development suggest the potential importance of miRs as diagnostic markers in human cardiovascular diseases. In the future, more intensive research is likely to be carried out to clarify their contribution to personalized management and treatment of CHD patients. In this paper, we discuss the current knowledge on the causative role of miRs in cardiac development and CHDs.

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INTRODUCTION

CHD defines functional and structural disorders of the heart and blood vessels that arise during embryogenesis. CHD is the most common cause of perinatal and infant mortality. The reported birth prevalence varies widely across countries and continents, the best estimation generally accepted is 8 per 1,000 live births (1). CHDs are the leading cause of infant morbidity and mortality and account for approximately 40% of prenatal deaths and 20% of mortality in the first year of life (2, 3). CHDs encompass a wide spectrum of phenotypes including atrial septal defect (ASD), ventricular septal defect (VSD), patent ductus arteriosus (PDA), transposition of the great arteries (TGA), pulmonary valve atresia (PVA), coarctation of the aorta (CoA), tricuspid atresia (TA), tetralogy of Fallot (TOF), hypoplastic left heart syndrome (HLHS) and univentricular heart (UH). The severity of the different forms of CHD varies extensively from simple mild lesions with follow-up for decades without any treatment to complex cyanotic malformations requiring urgent surgical intervention (4). Recent advances in pre- and postnatal diagnosis as well as surgical treatment approaches have increased life expectancy of patients with CHD. Nowadays more than 75% of CHD patients live to adulthood, increasing the pediatric CHD cohort and requiring lifelong specialized cardiac care. As a result, an increased number of patients will reach the reproductive age and can transmit the disease with a high risk (5, 6). Therefore, a better understanding of the etiology is essential in order to give early diagnosis, allow timely interventions, effective patient management and proper genetic counseling. To date, approximately 20% of CHD cases have known causes, such as teratogen effects or genetic alteration. Half of the genetic abnormalities are chromosomal aneuploidy, submicroscopic copy number variation or nucleotide sequence mutations. These abnormalities may cause syndromes (with extracardiac symptoms) or isolated heart defects. Clarification of genetic background is complicated due to low penetrance, high phenotypic variability and variable expression of symptoms (7, 8). Very little is known about the etiology of the remaining 80% of cases, most of them follow multifactorial inheritance including genetic and environmental factors in disease development (7). Recently, epigenetics is being increasingly acknowledged as a key mechanism in the pathophysiology of cardiovascular diseases, including CHDs. Epigenetics refers to a set of mechanisms that regulate gene expression without changing the underlying nucleotide sequence. Beside DNA methylation and histone modification as two basic elements of epigenetic machinery, noncoding RNA (ncRNA) molecules can also induce epigenetic modifications by post-transcriptional regulation of gene expression. The best studied group of noncoding RNAs are miRs, which are small, evolutionarily conserved, single-stranded RNA molecules, approximately 22 nucleotides in length at mature stage. They suppress expression of target genes by inhibiting translation or encouraging degradation of their mRNA by complementary base pairing at 3'UTR. The degree and nature of complementary sites

between the miR and target mRNA appear to determine the gene silencing mechanism. Since the first description in the early 1990s, more than 1000 miRs have been identified in mammals regulating more than 30% of genes. A single miR can regulate multiple target mRNAs, and individual mRNA can be targeted by many miRs (2, 9, 10).

The aim of this paper is to review the current knowledge of the potential role of miRs in abnormal cardiac development and consequently in CHD.

THE ROLE OF MICRORNAS IN HEART DEVELOPMENT

Various roles of miRs were identified in the pathogenesis, progression, and remodeling of cardiovascular diseases. The process of cardiac tissue formation and cardiac gene expression is so highly complex that it requires formation of diverse cell types, including cardiomyocytes, valvular and endothelial cells, conduction system, smooth muscle cells, in a tightly regulated, spatiotemporal manner. MiRs increase this complexity even further by adding another layer of regulation at the post-transcriptional level (11, 12). The significance of miRs in cardiogenesis was first revealed in gene deletion experiments in animal models, using mice and zebrafish.

Dicer is an essential endonuclease in the miR biogenesis pathway, disruption of its function removes all mature miRs. Dicer deletion in mice and mutation in zebrafish caused lethality by arresting gastrulation, providing convincing evidence on the essential role of miRs in early animal development. To better understand the function of miRs in cardiac tissues, studies on tissue specific Dicer deletion were carried out on mouse heart at different developmental stages. Deletion of cardiac specific Dicer prenatally resulted in defective heart morphogenesis and embryonic lethality. However, heart specific Dicer deletion after birth led to abnormal expression of contractile proteins, and remarkable sarcomere disarray coupled with reduced cardiac function and rapid progression to dilated cardiomyopathy and heart failure. This cardiac phenotype closely resembled human dilated cardiomyopathy and heart failure (13-15). To understand the contribution of individual miRs to cardiac development, much research has been undertaken with gain- and loss-of-function studies of specific miRs (16-18). The outcomes of these studies clearly indicated that single miRs have crucial roles in cardiac development and function.

MiR-1 (miR-1-1, miR-1-2, miR-206) and miR-133 (miR-133a-1, miR-133a-2, miR-133b) families are highly conservative. They are abundantly, but not exclusively expressed in the heart. These miRs are produced from the same polycistronic transcript, encoded by two separate genes in the mouse and human genomes, therefore they have identical sequences and consequently target the same mRNAs.

Last decades of intensive research using developmental models of the heart have revealed that these miRs control fundamental cardiac transcription factors, such as *GATA4*, *TBX1*, *MEF2C*, *SRF* and *MHC* genes. MiR-1/-133 have a crucial role in regulation of cardiomyocyte proliferation. They are also known to induce mesoderm formation and differentiation of embryonic stem cells. In the developing mouse heart, excessive expression of miR-1 inhibited ventricular myocyte proliferation (17).

In contrast, targeted deletion of miR-1-2 was found to be lethal at nearly 50% of mouse embryos at weaning age, and some animals showed ventricular septal or conduction system defects in later stage (18). Deletion of either miR-133a-1 or miR-133a-2 did not affect seriously the cardiogenesis, but resulted in VSD and chamber dilatation leading to late embryonic or

early postnatal death (19). Both miR-1 and miR-133 seemed to regulate growth-related cardiac genes as growth suppressors and had an opposite role in cardiomyocyte apoptosis (20, 21). Furthermore, miR-1 was linked to NOTCH1 receptor, whose ligand is essential in normal asymmetric division (22). To understand the exact molecular mechanism underlying the function of miRs, their target genes are required to be identified. In case of miR-1, one important validated target is *Hand2* cardiac transcription factor. Deletion of *Hand2* gene in mouse models leads to similar failure in ventricular myocytes as miR-1 overexpression, which also reduces expression of *Hand2* (17).

The miR-15 family includes six miRNAs: miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195, miR-497. By inhibiting these miRs, mitosis of myocardial cells increases and the suppression of the most important target gene, *CHEK1* is eliminated. Of particular importance is the miR-195, whose overexpression was detected during cardiac hypertrophy in human and mouse hearts, and it was sufficient alone to induce hypertrophic growth of cultured rat cardiomyocytes (23).

MiRs belonging to the miR-208 family (miR-208a, miR-208b, miR-499) are commonly referred to as MyomiRs, expressed specifically in the heart. MiR-208-null animals do not show gross developmental defects although their overexpression induces cardiac hypertrophy and conduction defects (24). Among their target genes, the *THRAP1* is involved in the negative regulation of myocardial growth and hypertrophy. MiR-499 is responsible for the expression of myosin genes in the heart muscle of mice (24-26).

The 17-92 miRs are clustered, and initially they were known as oncogenes (oncomiR). A few of these miRs (miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, miR-92a-1) play an important role in cardiogenesis, participating in the differentiation of progenitor cells in the heart muscle. Their elevated level causes CHDs due to inhibition of the expression of the main progenitor genes (*ISL1*, *TBX1*) (12).

The miR-143 family expressed in endocardial, myocardial and cardiac progenitor cells is involved in the regulation of the *ADD3* target gene, which encodes the F-actin capping protein. This pathway is involved in the proper design and function of the heart chambers. By knocking out these miRs, outflow tract or ventricular disorders can develop in the heart (27, 28).

Evolutionarily conserved miR-218a-1, miR-218a-2 and miR-218b together with *SLIT2* and *ROBO* genes provide the adequate development of the heart tube. Together with the transcription factor *TBX5*, they participate in the morphogenesis of the heart. Downregulation of miR-218 causes overexpression of *TBX5*, resulting in heart looping and chamber disorders (29).

ALTERED MICRORNA EXPRESSIONS IN CONGENITAL HEART DISEASES AND THEIR USE AS BIOMARKERS

During the past decade, findings of miR expression profiling experiments have demonstrated that significant changes of specific miR signatures occur in various forms of cardiovascular diseases, including CHDs (22). The first studies documented deregulated miR expression patterns in humans mostly in the myocardial tissues, such as ventricular myocardium or outflow tract tissues (2, 10, 30). Subsequently, it became clear that some miRs released from their cells of origin can also be detected in different bodily fluids (31-33). Numerous studies started to investigate tissue- and disease-specific miR profiles in bodily fluids in different human pathological conditions (34-36). Extracellular miRs became attractive candidates as noninvasive

biomarkers for early diagnosis or prognosis of CHDs (34, 37, 38) (Table 1).

However, extracellular miR profiling as a valid diagnostic or prognostic tool is still in the descriptive phase. The highly variable miR assessments from blood are derived from the different extraction methods, analysis platforms applied and the fact that the extracellular miRs are either associated with proteins or contained in cellular fragments (e.g. microparticles, exosomes). An excellent review summarizes the currently used methodological approaches in utilizing miRs as circulating biomarkers.

The authors highlighted optimal miR isolation protocols, the advantages and disadvantages of various expression methods and provided a workflow in designing miR profiling from plasma or serum samples (39).

Table 1 MicroRNA profiling methods and expression changes in CHD*

Sample	CHD	Control	Methods		MicroRNAs			Reference
			Screening	Confirmatory	Candidate	Confirmed	Expression	
Cardiac tissue	TOF (n=16)	n=8 normally developing hearts	Microarray	RT-qPCR	miRs (n=61)	miR-27b miR-421 miR-1275	Upregulated	(10)
						miR-122 miR-1201	Downregulated	
Cardiac tissue	TOF (n=21)	n=6 normal controls	Microarray	RT-qPCR	miRs (n=41)	miR-19a miR-130b miR-146b-5p miR-154 miR-155 miR-181c miR-181d miR-192 miR-222 miR-337-5p miR-363 miR-424 miR-424* miR-660 miR-708	Upregulated	(30)
						miR-29c miR-181a* miR-720	Downregulated	

Cardiac tissue	VSD (n=28)	n=9 normal heart tissues	Microarray	RT-qPCR	miRs (n=25)	miR-181c	Upregulated	(41)
						miR-1-1	Downregulated	
Cardiac tissue	TOF (n=16)	n=8 normally developing hearts	Microarray	RT-qPCR	miRs (n=61)	miR-421	Upregulated	(44)
Cardiac tissue	TOF (n=26)	n=15 healthy individuals	Microarray	RT-qPCR	miRs (n=75)	miR-940	Downregulated	(46)
Cardiac tissue	HLHS (n=15)	n=6 non-failing control hearts	Microarray	RT-qPCR	miRs (n=93)	miR-204	Upregulated	(49)
						miR-99a miR-100 miR-137-3p miR-145a	Downregulated	
Cardiac tissue	CHD (n=30)	n=30 normal samples	Gene expression microarray	RT-qPCR	miR-145 miR-182	miR-145	Downregulated	(52)
Cardiac tissue	CHD (TOF, VSD, PA) (n=10)	n=11 acyanotic cardiac defects	-	RT-qPCR	-	miR-138	Upregulated	(45)
Cardiac tissue	TOF (n=30)	n=10 normal samples	Gene expression microarray	RT-qPCR	miR-1 miR-19a miR-30a miR-30c miR-30d miR-30e miR-130a miR-130b miR-144 miR-206	miR-1 miR-206	Downregulated	(36)
Cardiac tissue	Cyanotic CHD (n=10)	n=10 acyanotic cardiac defects	-	RT-qPCR	-	miR-184	Downregulated	(53)

Serum	TGA (n=26)	n=20 healthy subjects	Microarray	RT-qPCR	miRs (n=23)	hsa-let-7e miR-16 miR-18a miR-25 miR-93 miR-106a miR-144* miR-451 miR-486-3p miR-486-5p miR-505*	Upregu-lated	(47)
Serum	CHD (n=61)	n=20 normal individuals	-	RT-qPCR	-	miR-873	Upregu-lated	(54)
Serum	Cyanotic CHD (n=32)	n=20 acyanotic CHD individuals	-	RT-qPCR	-	miR-182	Downregu-lated	(55)
Plasma	VSD (n=20)	n=15 VSD-free partici-pants	Microarray	RT-qPCR	miRs (n=36)	miR-498	Upregu-lated	(40)
						hsa-let-7e-5p miR-155-5p miR-222-3p miR-379-5p miR-409-3p miR-433 miR-487b	Downregu-lated	
Plasma	CHD (ASD, VSD, AVSD) (n=26)	n=27 healthy children	miRNA PCR Array	RT-qPCR	miRs (n=84)	hsa-let-7a hsa-let-7b miR-486	Upregu-lated	(37)
Maternal serum	CHD (ASD, VSD, TOF) (n=30)	n=30 normal fetuses	SOLiD sequencing	RT-qPCR	miRs (n=11)	miR-19b miR-22 miR-29c miR-375	Upregu-lated	(42)

Venous blood	TOF (n=37)	n=15 healthy controls	Microarray	RT-qPCR	miRs (n=49)	miR-183-5p miR-421 miR-625-5p miR-1233-3p	Upregulated	(11)
						miR-140-3p miR-142-5p miR-181d-5p miR-206 miR-339-5p	Downregulated	

**ASD: atrial septal defect, AVSD: atrioventricular septal defect, CHD: congenital heart disease, HLHS: hypoplastic left heart syndrome, miRs: microRNAs, PA: pulmonary atresia, RT-qPCR: reverse transcription-quantitative polymerase chain reaction, TGA: transposition of the great arteries, TOF: tetralogy of Fallot, VSD: ventricular septal defect.*

To date, especially the common and/or severe CHDs have been studied extensively by miR expression profiling. VSD, as a discontinuation in the septal wall between left and right ventricles, accounts for approximately 20-40% of CHDs (Fig. 1b) (40). Large defects lead to severe heart failure requiring urgent surgical intervention, while smaller ones may be asymptomatic and close spontaneously. The more severe forms can result in left-to-right shunt with consecutive left ventricle overload and pulmonary hypertension. Although its embryology and pathology have been clarified, its etiology remains unknown. After initial microarray screening, Li *et al* (41) compared the expression of a set of 25 candidate miRs in heart tissues of patients with VSD and healthy controls by qRT-PCR. They found that miR-1-1 expression was decreased in patient samples and associated with the increased level of its target genes, *GJA1* and *SOX9*. The miR-181c overexpression, linked with the downregulation of *BMPR2* gene, was identified as well. Later on, another research group reported for the first time circulating miR profiles for patients with VSD with upregulation of miR-498 and downregulation of hsa-let-7e-5p, miR-155-5p, miR-222-3p, miR-379-5p, miR-409-3p, miR-433 and miR-487b in plasma samples of patients (40). Zhu *et al* hypothetize that miRs in maternal serum can be used as biomarkers for the prenatal detection of fetal CHD in early pregnancy. They analyzed miR profiles in serum samples from pregnant women, who had fetuses with ASD, VSD or TOF and from women with normal pregnancies. They identified significantly higher expression of miR-19b and miR-29c in case of VSD and upregulation of miR-22 and miR-375 in TOF. Results of this study raised the possibility of using miRs in the maternal serum for early diagnosis of fetal cardiac disorders as noninvasive biomarkers (42). However, because of the limitations of the study, further research is required to confirm the usefulness of miRs in the clinical practice for prenatal diagnosis of CHD.

There are much less data on the expression profile of miRs in congenital ASD (Fig. 1c). In a more recent study Song and colleagues (37) identified significant upregulation of hsa-let-7a, hsa-let-7b and miR-486 in children with ASD, VSD and AVSD. The hsa-let-7a and hsa-let-7b were specifically overexpressed in ASD cases supporting their hypothesis that specific miRs are associated with specific types of CHD. Similar expression profiles of hsa-let-7a and hsa-let-7b were detected in mothers of ASD children. Based on their results, not only do these miRs have diagnostic but also predicting roles for CHD risk in offspring.

Figure 1 Altered expression of miRs in CHDs*

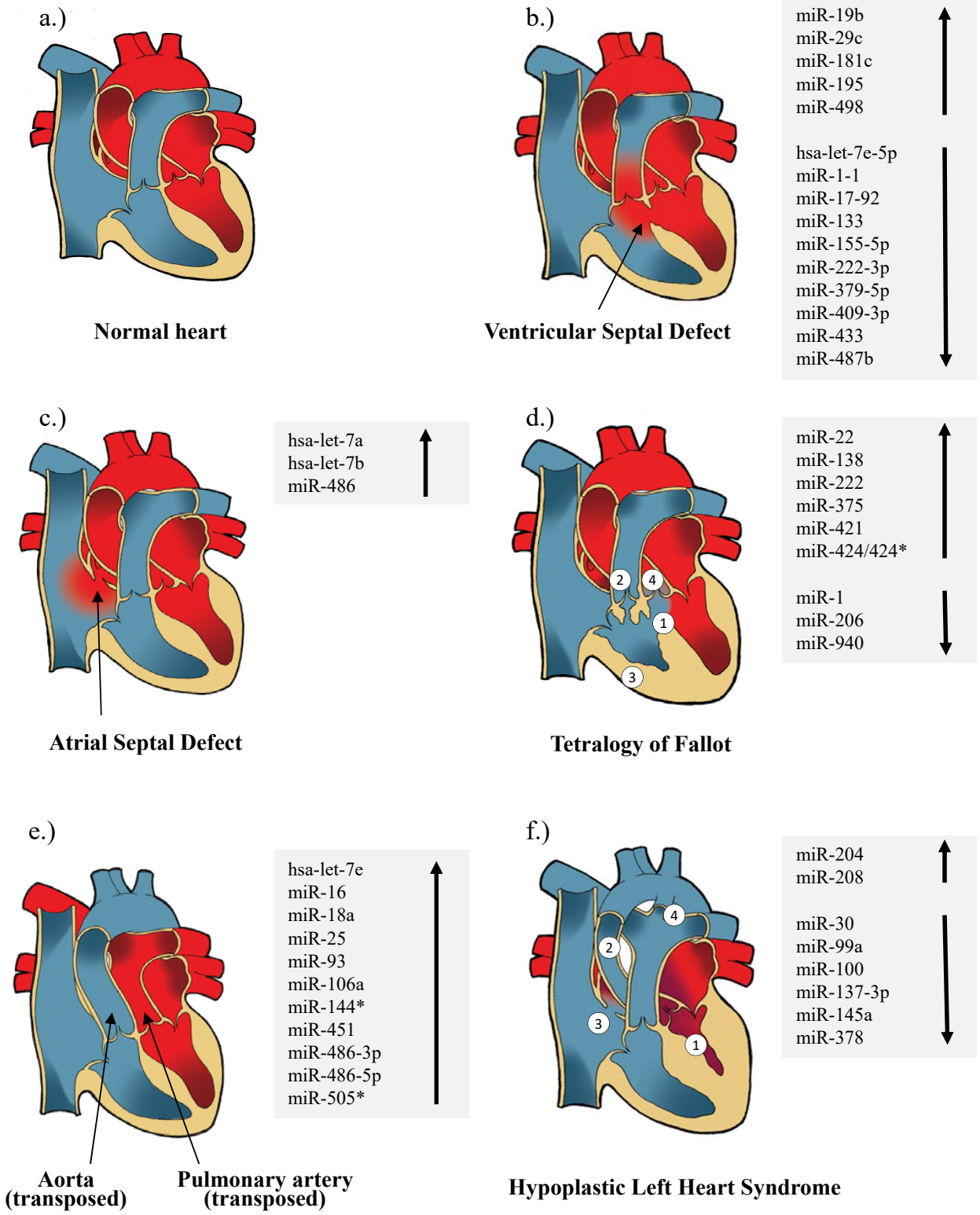

Figure 1 - Legend
a) normal heart structure
b) Ventricular Septal Defect (VSD): a hole in the wall between the lower chambers of the heart (the ventricles)
c) Atrial Septal Defect (ASD): a hole in the wall between the upper chambers of the heart (the atria)
d) Tetralogy of Fallot (TOF): a combination of four heart defects that are: 1. ventricular septal defect, 2. pulmonary stenosis, 3. right ventricular hypertrophy, 4. overriding aorta
e) Transposition of the Great Arteries (TGA): the two major arteries (aorta and pulmonary artery) that carry blood away from the heart are switched (transposed)
f) Hypoplastic Left Heart Syndrome (HLHS): a combination of: 1. underdeveloped left ventricle, 2. hypoplastic aorta, 3. atrial septal defect, 4. patent ductus arteriosus.

↑ *Upregulation* ↓ *Downregulation*

References: (10-12, 30, 36, 37, 40-42, 44-47, 49).

TOF (Fig. 1d) is the most common form of cyanotic CHD that represents 5-8% of all CHDs (43). It is characterized by: 1) pulmonary outflow tract obstruction; 2) VSD; 3) overriding aortic root; and 4) right ventricular hypertrophy. Without surgery, patients have poor prognosis and follow-up studies show that even after repair of TOF they have a higher risk for heart failure. Despite its prevalence and clinical significance, the underlying mechanism is poorly understood.

More recently, miRs have been investigated as etiological factors in the pathogenesis of TOF. O'Brien and colleagues (10) examined expression patterns of miRs in right ventricular myocardium from infants with nonsyndromic TOF compared to infants with normally developing heart. Microarray analysis revealed that the levels of 61 miRs significantly changed, especially miR-1275, miR-27b, miR-421, miR-1201 and miR-122, moreover the levels of expressions were similar to those in the fetal myocardium. Potential targets of miRs with altered expression were concentrated in gene networks critical to cardiac development. Subsequently, this group performed a follow-up study focusing on miR-421 by under- and overexpressing of miR-421 on primary cells from ventricular myocardium of TOF heart.

The authors found an inverse correlation between expression of miR-421 and *SOX4* that is a key regulator of the Notch signaling pathway, which suggests the association of miR-421 with TOF (44). He *et al* (45) studied miR-138 expression in myocardial samples from patients with cyanotic TOF and identified a two-fold increase compared to patients with acyanotic CHD. Based on studies from Liang and colleagues (46) miR-940 is the most downregulated miR in myocardium from patients with TOF among the identified 75 dysregulated miRs. Zhang *et al* (30) applied microarray analysis to identify deregulation of miRs in right ventricular outflow tract tissue in infants with TOF. They found 18 miRs with significantly altered expression, among which miR-424/424* and miR-222 were shown to affect cardiomyocyte proliferation and differentiation. MiR target gene network analysis showed that 16 of the 18 miRs targeted 97 genes involved in heart development.

Abnormal expression of connexin 43 (*Cx43*) has been documented in conotruncal anomalies although the underlying mechanism is unknown. Wu and colleagues (36) investigated the potential role of miRs in altered expression of *Cx43* in myocardium samples of patients with TOF. They showed that miR-1 and miR-206 were significantly decreased in the TOF patients as compared with controls, which may cause upregulation of *Cx43* protein synthesis and suggest the role of these miRs in the pathogenesis of TOF.

Little is known about the expression profile of miRs in CHD with systemic right ventricle. In patients with TGA (Fig. 1e) after atrial switch

operation, late systemic right ventricular dysfunction develops. Lai *et al* (47) tested the circulating miR expression profile in these patients. They found that 23 miRs were upregulated, 11 were validated to be increased in patients compared with controls. MiR-18a and miR-486-5p expression signature correlated negatively with systemic ventricular contractility.

HLHS (Fig. 1f) represents 2% to 9% of CHDs and accounts for 23% of neonatal deaths. Characteristics of HLHS include varying degrees of hypoplasia of the left ventricle, mitral valve and aortic valve atresia/stenosis, and hypoplasia of the ascending aorta. This is one of the most serious cardiac malformation and leads to death in the newborn period without treatment. Nowadays as a result of surgical palliative techniques a five-year survival can be reached in more than half the cases (48). Sucharov *et al* (49) investigated the miR expression in the right ventricle myocardium of HLHS patients. They found that pediatric HLHS population has a unique miR profile, and some miRs (miR-100, miR-145a, miR-99a, miR-137-3p, miR-204) are modulated by changes in volume loading of the right ventricle. Biostatistical analysis revealed that target genes of these miRs are important for cardiac development and disease.

The most common genetic defect leading to CHD is the trisomy 21 in Down syndrome. Five miRs are overexpressed in Down syndrome, miR-99a, hsa-let-7c, miR-125-b2, miR-155 and miR-802, all of them are located on chromosome 21 (50).

Patients with DiGeorge syndrome, which is the most common microdeletion syndrome, have heterogeneous phenotype including CHD, immunodeficiency and hypocalcemia. The genetic cause is the deletion of 22q11.2 region including *TBX1*, which is essential in normal cardiac development. The *DGCR8* gene encodes a crucial component of the RNA induced silencing complex (RISC), necessary for miR biogenesis. Haploinsufficiency of this complex leads to impaired miR expression and development of CHD (51).

CONCLUSION

CHDs account for a significant part of cardiovascular diseases, perinatal morbidity and mortality. Improved survival of children with CHD has led to high number of adult patients facing multiple challenges throughout their lifetime. Despite our increasing knowledge on the genetic basis and signaling pathways involved in cardiac development, there are still huge gaps that require further examination.

In the cardiovascular research field, numerous studies demonstrated that miRs are required for proper heart development and function. Characteristic expression profiles of miRs have specific and generalized effects on cell signaling pathways associated with CHD.

MiRs are attractive diagnostic and prognostic biomarkers as they remain stable in bodily fluids and avoid RNA degrading enzymes and hereby may provide an additional diagnostic tool to assess heart disease.

According to recent reports miRs can serve as noninvasive biomarkers for the extent of myocardial damage and the postoperative clinical course of pediatric patients with CHDs following surgery.

Despite the results having been reported in the past decade, the exact role of miRs in CHDs is still unclear. Large-scale studies are needed to provide a better understanding of the molecular interactions causing CHD.

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In this issue: Non-coding RNAs as potential laboratory biomarkers

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Circulating microRNAs as possible biomarkers for coronary artery disease: a narrative review

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ABSTRACT

Coronary artery disease is one of the most common cardiovascular diseases in the world. Involvement of microRNAs on the pathogenesis of this disease was reported either in beneficial or detrimental way. Different studies have also speculated that circulating microRNAs can be applied as promising biomarkers for the diagnosis of coronary artery disease. Particularly, microRNA-133a seems to fulfill the criteria of ideal biomarkers due to its role in the diagnosis, severity assessment and in prognosis. The panel of circulating microRNAs has also improved the predictive power of coronary artery disease compared to single microRNAs. In this review, the role of circulating microRNAs for early detection, severity assessment and prognosis of coronary artery disease were reviewed.

INTRODUCTION

Coronary arteries supply blood to the heart muscle and consist of two main arteries: the right and left coronary arteries, and their two branches, the circumflex artery and the left anterior descending artery (1). Analogous to other arteries, normal coronary artery consists of three well-defined layers: the intima, media, and adventitia. These three layers are separated by layers of elastin. Internal elastic lamina separates intima from media and external elastic lamina separates media from the adventitia (2).

Coronary artery disease (CAD) is the leading cause of cardiovascular deaths (CVD) globally (3). In 2020, it is estimated that this disease will account for death of 11.1 million patients globally (4). Someone suffers from coronary disease every 26 seconds, and someone dies from every minute in the USA (5). In Europe, between 1 in 5 and 1 in 7 women die from CAD, and the disease accounts for between 16% and 25% of all deaths in European men (6). Studies suggest that the average age-adjusted incidence rates of CAD per 1,000 person-years are 12.5 for white men, 10.6 for black men and 4.0 for white women (7). The clinical spectrum of CAD ranges from stable angina pectoris (stable CAD) to acute coronary syndromes (ACS) which includes unstable angina (unstable CAD) and myocardial infarction (8). The myocardial infarction (MI) is further classified into ST segment elevated MI (STEMI) and non-ST segment elevated MI (NSTEMI). In terms of arterial occultation STEMI is characterized by a complete occultation of epicardial coronary blood vessel and elevated ST in electrocardiogram whereas NSTEMI is characterized by a sever coronary artery narrowing. However, both of them are accompanied by necrosis of myocardial cell and elevated cardiac biomarkers (9). Only a few previous articles have reviewed the potential of circulating microRNAs (miRNAs) as biomarker on different phases of CAD. Consequently, this review narrates the role of circulating miRNAs in the early detection, diagnosis, severity assessment of CAD as well as restenosis, and their role as a prognosis marker. Furthermore, it describes the pathogenesis, current diagnosis modalities, and limitation of miRNAs as a biomarker of CAD.

PATHOGENESIS OF CORONARY ARTERY DISEASE

The primary pathologic process causing CAD is atherosclerosis of the large and medium sized coronary artery. The increment of cholesterol level which binds with low density lipoprotein and very low-density lipoprotein increases the chance of infiltration of these molecules into the artery wall and leads to oxidation (10). This can initiate migration of smooth muscle cells from the tunica media to intima of the artery (11). Activated smooth muscle cells produce fibrotic extra cellular matrix (ECM), which changes the lipid rich fatty streak into more advanced lesion (12). The ECM forms the fibrous cap that has an important role in maintaining the mechanical stability of the plaque. In addition to calcification, neo-vascularization affects the structure of the plaque. As the plaque size increases, the oxygen from the bloodstream does not reach all areas of the lesion, and the inner section gets hypoxic and these neo-vessels also cause small hemorrhages inside the plaque which subsequently increases its size rapidly (13).

In most cases, ischemia and infarctions are caused by physical disruption of the fibrous cap of the lesion, which allows thrombogenic material to interact with blood cells (14). This contact leads to formation of a thrombosis, which can block the blood flow in the artery. Inflammatory cells also destabilize the plaque by secreting pro-inflammatory cytokines, proteases, coagulation factors and vaso-active molecules. These molecules inhibit the formation of stable fibrous

cap, degrade the collagen in the cap and initiate the formation of the clot (15).

Recently, the discovery of miRNAs involvement on the pathogenesis of CAD reignited the lesson for using them as diagnosis and prognostic marker for cardiovascular disease (CVD). Now, it is accepted that miRNAs are involved either in a beneficial or detrimental way in almost all steps of atherogenesis, including endothelial damage and dysfunction, monocyte-wall invasion and activation, lipoprotein formation, plaque stability, remodeling of the CV system, and platelet and vascular smooth muscle cell function (16).

microRNAs regulate gene expression post transcriptionally by degrading messenger RNA targets and/or by blocking their translation (17, 18). Each miRNA can target multiple mRNAs and regulate ~60% of mammalian protein-coding genes (19). They have diverse functions in the regulation of several key biological and cellular processes including differentiation, proliferation, and apoptosis in cardiovascular system (20).

In recent years, circulating miRNAs have created great interest and have been investigated as a source of novel biomarkers for several human diseases (21-23). They are reported from whole blood, peripheral blood mononuclear cells, platelets, serum, plasma, and other body fluids (24). Regarding, using miRNAs as a biomarker in CAD abundant researches have been undertaken. They revealed that determining the expression level of miRNAs in body fluids have a potential role intended for early detection, diagnosis, severity assessment markers and prognostic indicators.

EXISTING DIAGNOSIS MODALITIES FOR CORONARY ARTERY DISEASE

Currently, common diagnosis of CAD relies on visualization of the anatomic structure of coronary artery and functional assessment of the heart. Coronary angiography is considered as a gold standard method for diagnosis of CAD (25). However, coronary angiography may overestimate or underestimate disease due to the fact that it is influenced by technical factors and complexity of coronary anatomy and plaque configuration (26, 27).

Furthermore, complications from the technique including those related to local anesthesia and use of contrast material, as well as contrast induced nephropathy, infection, local vascular injury, myocardial infarction, stroke, and death are also common (28, 29). Additionally, prevailing of non-flow limiting CAD in women which is undetectable through this technique also compromises its value (30). As a result, the emerging of noninvasive techniques, whether imaging or non-imaging, hold great prospects (31). MicroRNAs in this regard might have potential to skip these bottlenecks.

DETECTION METHODS OF miRNAs

High throughput sequencing, quantitative real time polymerase chain reaction (RT-qPCR) and microarrays are the major quantification methods that are currently being used (32). Sequencing is the best technique for discovering new miRNAs whereas qPCR is the gold standard technique for quantification of miRNAs (33). On the other hand, microarray technique is the best alternative method for genome-wide assays on a larger scale (34). However, quantification of miRNAs, compared to protein, still lacks standardized methods and clear recommendation about which body fluid is appropriate?

SAMPLE PREPARATION AND NORMALIZATION TECHNIQUE OF miRNAs

Selecting the appropriate sample is the basic issue for analyzing miRNAs. MicroRNAs are found intracellularly or can be actively secreted by

cells (35). Even though there is high extracellular RNase activity, miRNAs are stable in extracellular area, due to their packaging in apoptotic bodies, microvesicles (MV), exosomes, lipoproteins (Lp), and special proteins. Previous studies showed that miRNAs are found in blood, urine, breast milk (36), saliva, tears, and other body fluids (37). In this review, blood and its components like plasma, serum, a peripheral blood mononuclear cell (PBMC) were the major sample for miRNAs determination (Table 1).

MicroRNAs can be extracted by different techniques. The most common technique is selected based on the desired purity and amount of miRNA. Some of the extraction methods are TRIzol based, miRNeasy and mirVANA (38). TRIzol based method was the technique used by various studies (Table 1).

Quantification of miRNA expression needs data normalization. The normalizer might be either endogenous or exogenous reference genes. However, there is no consensus on optimal normalization strategy, particularly the choice of reference genes. In terms of the source of the reference, it might be endogenous or exogenous whereas in terms of their nature, it might be miRNAs, synthetic RNA or other genes (39). In this review, the most common exogenous reference gene found in various studies is a miRNA obtained from *C. elegans* which is the cel-miRNA-39. The small non-coding RNA (RNU6) was also the most frequently used non miRNA endogenous reference genes (Table 1). Furthermore, miRNA-156a and miRNA-16 were used as endogenous miRNA normalizer as well.

ROLE OF miRNAs AS POSSIBLE BIOMARKERS FOR CORONARY ARTERY DISEASE

MicroRNAs are small non-coding endogenous RNAs and can regulate different developmental and physiological processes of cardio-vascular system (34). These molecules are also highly valuable biomarkers due to their cell-type specificity, abundance, and stability in most solid and liquid clinical specimens (40). Gustafson *et al.* stated the beneficial aspect of miRNA-guided diagnostics as an increasingly and powerful molecular approach for deriving clinically significant information from patient samples. Li *et al*. also proved that these miRNA molecules can be used as diagnosis, management, and monitoring of numerous diseases. They are also helpful for stratifying the type of CAD patients and even the type of ACS in different groups. For instance, a study done by Ward *et al*. showed that miRNA-25-3p, miRNA-221-3p, and miRNA-374b-5p were highly associated with STEMI, and miRNAs 221-3p and 483-5p were highly correlated to NSTEMI (41).

Early detection of coronary artery disease

Various guidelines (42-44) support to screen individuals having family history of premature CAD and diabetes mellitus (DM). Screening of CAD includes remarkable investigation starting from the easy Framingham risk score screening tool to more complicated and relatively accurate coronary angiography (45). As a result, highly sensitive and specific screening tests with low cost and invasiveness are essential for a better monitoring program of CAD. microRNAs expressed and released from platelet, monocyte, endothelial cells at the initiation stage of CAD may take their share in this regard. Wang *et al.* recommended that circulating levels of miRNA-31 and miRNA-720 have a potential role for early detection of CAD. They proved that these miRNAs can regulate endothelial progenitor cell (EPC) function via the suppression of FAT4 and thromboxane A2 receptor which are expressed in EPC's of CAD patients early (46). Their expressions were remarkably low in CAD patients compared to non-CAD patients.

Table 1 Summary of miRNAs from selected studies in coronary artery disease*

miRNAs	Altera-tion	Study population	Method	Role	Sample type	Reference gene/ miRNA	Extrac-tion	Refer-ence
miR-31 miR-720	Down Down	CAD (n=20) vs. HC (n=15)	qRT-PCR	prognosis, diagnosis	Plasma EPCs	miRNA-16a	TRIzol based	(46)
miR-181a	Down	Obese (n= 21) vs. non-obese (n=125)	Microarray qRT-PCR	diagnosis	monocytes	RNU5G	-	(47)
miR-149 miR-424 miR-765	Down Down Up	CAD (n= 95) vs. HC (n=32)	qRT-PCR	diagnosis	plasma	miRNA-156a	TRIzol based	(49)
miR-765 miR-149	Up Down	SCAD (n= 37) UCAD (n=32) vs. HC (n=20)	Microarray, qRT-PCR	diagnosis	Plasma	miRNA-156a	TRIzol based	(50)
miR-133	Up	AMI (n=13), AP (n=176), vs. HC (n=127)	qRT-PCR	diagnosis	Plasma	RNU6	TRIzol based	(51)
miR-135a miR-147	Up Down	SAP (n= 25) UAP (n= 25) vs controls (n= 20)	qRT-PCR	diagnosis	PBMC	let-7a and miRNA-16	-	(56)
miR-1 miR-126 miR-483 miR-133a	Up Up Up Up	SAP (n= 34) UAP (n=19) vs. non-CAD (n= 20)	qRT-PCR	severity evaluation	Plasma	miRNA-16	TRIzol based	(52)

miR-126 miR-17 miR-92a miR-155 miR-145 miR-133a miR-208a	Down Down Down Down Down Up Up	CAD (n=36) vs. non-CAD (n=17)	qRT-PCR	diagnosis	Serum/ plasma	cel-miRNA-39	TRIzol based	(97)
miR-206 miR-574	Up Up	CAD (n=67) vs. non-CAD (n=67)	Microarray qRT-PCR	diagnosis	Plasma	RNU6	MirVANA	(54)
miR-34a miR-21 miR-23a	Up Up Up	CAD (n=32) non-CAD (n=20)	Microarray qRT-PCR	diagnosis	Plasma	RNU6	TRIzol based	(55)
miR-2861 miR-3135b miR-191	Up Up Up	CAD (n=90), vs. non-CAD (n=70)	Microarray qRT-PCR	Severity assessment	Plasma	cel-miRNA-39	mirVanaTM	(74)
miR-126 miR-199a	Up Up	CAD (n=176)	qRT-PCR	prognostic	Plasma, MVs Exosomes	cel-miR-39	TRIzol based	(75)
miR-197 miR-223	Up Up	(ACS, SAP) (n=873)	qRT-PCR	prognostic	Serum	cel-miRNA-39	TRIzol based	(76)
miR-133a miR-208b	Up Up	ACS (n=444)	qRT-PCR	Prognostic, diagnostic	Plasma	-	-	(77)
miR-208a	Up	CHD (n=290) vs. HC (n=110)	qRT-PCR	Severity	Plasma	RNU6B	TRIzol based	(60)
miR-155	Up	CHD (n=300) vs. HC (n=100)	qRT-PCR	Severity	Serum	RNU6B	TRIzol based	(60)

miR-483 miR-451a miR-155	Up Down Up	SCAD (n=59)	qRT-PCR	Severity	Plasma	cel-miRNA-39	miRNeasy	(68)
miR-486a miR-92a	Up Up	CAD (n=95) SAP (n=30) UAP (n=39) MI (n=26) vs. HC (n=16)	qRT PCR	Severity	Lipo-protein fractions	cel-miRNA-39	miRNeasy	(70)
miR-100 miR-143 miR-145 miR-21	Down Down Down Up	ISR (n=51) non-ISR (n=130) vs HC (n=52)	qRT PCR	Severity	Plasma	RNU6	TRIzol based	(80)
miR-425 miR-93	Up Up	ISR (n=39) vs. non-ISR (n=39)	miRNA PCR array	Severity	Plasma	-	-	(81)
miR-181b miR-155 miR-185	Down Up Up	ISR (n=6) vs non-ISR (N=43)	qRT-PCR	Restenosis	Plasma/ cell culture	miR-24	TRIzol based	(82)

**Abbreviations - ACS: Acute Coronary Syndromes, AP: Angina Pectoris, CAD: Coronary Artery Disease, CHD: Coronary Heart Disease, ISR: In-Stent Restenosis, HC: Health Control, MI: Myocardial Infarction, miR: microRNA, MV: Microvesicles, n: number of participants, qRT-PCR: quantitative Real-Time Polymerase Chain Reaction, SAP: Stable Angina Pectoris, UAP: Unstable Angina Pectoris, RNU: small non-coding RNAs.*

On the other hand, Hulsmans *et al.* showed the down regulation of the monocyte derived three isoforms of miRNA-181 in coronary artery disease. Particularly, miRNA-181-a was associated with CAD even after adjustment for traditional risk factors: obesity and metabolic syndrome (47). They also described that miRNA-181 related with inflammatory toll-like receptor and nuclear factor κB signaling and it may be potential biomarker for early detection of obesity related coronary artery disease. However, expression of miRNA-181 has been observed to be regulated by other toll-like receptor signaling factors that may potentially reduce its specificity of the prediction.

Bialek *et al.* also showed that plasma miRNA-208a is an interesting and promising candidate for a new biomarker released early after onset of myocardial infarction. The peak of miRNA-208a was observed earlier than the traditional biomarkers (cTnI and CK-MB mass). This implies that miRNAs will have an importance as early biomarker role in emergency department than the traditional markers (48).

Differentiate patients with CAD from non-CAD

Diagnostic values, which is commonly expressed in area under the curve (AUC) of the receiver operating characteristics (ROC) in this review, ranges from "bad" classification power (AUC, 0.5-0.6) for some miRNAs to "excellent" for others (AUC, 0.9-1.0).

Various miRNAs have a potential to classify CAD patients from non-CAD. Sayed *et al.* have assayed three plasma miRNAs: miRNA-765, miRNA-149, and miRNA-424 in CAD patients with non-CAD controls. All of them showed promising results to discriminate stable and unstable CAD from controls. ROC-AUC value of down-regulated plasma miRNA-149 classified stable and unstable CAD patients from non-CAD (0.938 and 0.951), respectively. Up-regulated miRNA-765 also distinguished CAD from non-CAD patients (49).

Discriminatory powers of miRNA-149 and miRNA-765 plasma levels were also repeated in other study (50). They classified unstable CAD from the controls with AUC values of 0.972 and 0.977, respectively. Whereas, stable CAD were differentiated from controls with 0.959 and 0.938 AUC values for miRNA-765 and miRNA-149, respectively. With this significant classification power, however, plasma levels of miRNA-765 were significantly correlated with age in all groups. This ultimately affects the characteristics of ideal biomarker. In contrast, plasma levels of miRNA-149 was not statistically significant in this aspect.

Furthermore, Wang *et al.* have revealed that miRNA-133a classified CAD from non-CAD individuals and exceeded the prediction potentials of the demographical data (age, sex, smoke, hypertension, diabetes, hyperlipidemia, etc.) and cardiac troponin I (cTnI) (51). The cTnI, clinical data, and miRNA-133a individually showed AUC value of 0.741, 0.785 and 0.918, respectively. Interestingly, the addition of miRNA-133a to the clinical data and cTnI remarkably increased the AUC values that were 0.942 and 0.925, respectively. Another study also showed that plasma miRNA-133a level was useful for diagnosis of unstable CAD (AUC = 0.906) (52). The combination of other two miRNAs (miRNA-1 and miRNA-126) increased the efficiency of detecting unstable CAD from controls. Moreover, miRNA-1 and miRNA-126 could differentiate both stable and unstable CAD from the controls independently with a potential of ≥ 0.85 value of AUC in the above study. Both of them were up-regulated in CAD patients (53).

Classification of CAD from non-CAD with "satisfactory" power was also reported by Zhou *et al.* through plasma expression of miRNA-206 and miRNA-574-5p (AUC value of 0.607 and 0.699, respectively) (54). Bioinformatics analysis revealed that their potential target gene might be involved in the onset and development of CAD that extend our understanding to validate them for early diagnosis of CAD.

Other studies without ROC curve analysis showed that different miRNAs have statistically significant difference between CAD and non-CAD. Han *et al.* (55) showed that from miRNA-34a, miRNA-21, miRNA-23a, miRNA-30a and miRNA-106b; miRNA-34a and miRNA-21 were significantly higher in the plasma of CAD patients compared to controls, whereas miRNA-23a had reduced expression among CAD patients (all $P<0.01$). The ratio of miRNA-135a to miRNA-147 concentration PBMC had showed 19 fold increment in CAD patients compared with controls. MiRNA/target gene/biological function linkage analysis suggested that the change in PBMC miRNA signature in CAD patients is probably associated with a change in intracellular cadherin/Wnt signaling (56). Dong *et al.* identified a panel of PBMC miRNA (miRNA-24, miRNA-33, miRNA-103a, and miRNA-122) that provided a high diagnostic accuracy of CAD (AUC=0.911, 95% CI 0.880-0.942) (57). Faccin

et al. also showed that a combination of three miRNAs (miRNA-155,-145 and let-7c) revealed a better classification power than the single miRNA alone (58).

Severity assessment of coronary artery disease

The Synergy between percutaneous coronary intervention with Taxus and cardiac surgery (SYNTAX) and gensini score are the two anatomical tools used to assess severity of CAD (59). Various circulating miRNAs have also correlated with the severity of CAD. They are correlated with the level of stenosis, complexity of stenosis and stability of the plaque in CAD. Circulating miRNA-133a expression is one of the miRNA that correlates with the severity of coronary artery stenosis in terms of complexity and level of stenosis.

Quantitative analysis revealed that circulating miRNA-133a level was significantly elevated in CAD patients having stenosis of coronary artery compared to non-coronary heart disease (CHD) patients. It was also moderately correlated with gensini scores and it was a better indicator of severity assessment relative to cTnI (51). However, miRNA-133a couldn't significantly differentiated low level stenosis from non-CHD individuals. Furthermore, in the other studies, miRNA-208a (60), miRNA-155(61) and miRNA-223 (62) were strongly correlated with gensini scores.

Guo *et al.* also tried to correlate plasma level of miRNA-145 with number of diseased vessel, SYNTAX score and stability of the plaques. They found that significantly lower levels of miRNA-145 in patients with three-vessel disease and high SYNTAX score compared with those with one or two-vessel disease and low or intermediate SYNTAX score, respectively. However, the result revealed that the level of miRNAs-145 between patients with one-vessel and two vessel disease, and between low score and intermediate score groups were not significantly different (63). Furthermore, miRNAs-214 tends to correlate with the SYNTAX score (64).

Every year, a large portion of CAD patients experience a sudden cardiac arrest due to unstable plaques rupturing (65). This produce subtotal or total occlusion and leading to ACS. Consequently, noninvasive biomarkers which can identify one of the severe form of CAD is clinically demanding.

In this regard, a study done by *Li X et al.* revealed the expression of miRNAs-122, -140-3p, -720, -2861, and -3149 have been highly elevated in the ACS group compared with the non-ACS groups and have good potential to identify patients. The discriminatory powers of these miRNAs were greater than AUC of 0.8 except for miRNAs-3149, i.e., 0.670. Using panel of miRNAs-122, -2861, and -3149 had a better classification power compared to using it alone (66). Other miRNAs such as: miRNA-106b, miRNA-25, miRNA-92a, miRNA-21, miRNA-590-5p, miRNA-126 and miRNA-451 also classified ACS from non ACS (67). In line with this, a study done by *Li S et al.* showed that combinations of miRNA-483-5p and miRNA-451a can discriminate plaque rapture with an excellent classification power, AUC (0.982; CI: 0.907-0.999). A panel of miRNA-483-5p and miRNA-155-5p had also showed the highest AUC (0.898; CI: 0.790-0.962) (68). In a study done by *Luque et al.* also showed that miRNA-638 was an independent predictor of plaque instability for carotid artery (69).

On the contrary, serum levels of 6 miRNAs including miRNA-92a and miRNA-122 could not differentiate ACS from non-ACS in other study. However, analysis of lipoprotein sub fraction level of miRNA-486 and -92a revealed good distinguishing power of ACS from non ACS (70). Level of high density lipoprotein-2 (HDL-2) miRNA-92a and HDL-3 miRNA-486 could classify

the ACS and non ACS up to an accuracy of 84% with adjustment for age, gender and serum lipids. Coronary bifurcation lesion is also one of the severe forms of CAD. Hence, it is the most challenging lesion in percutaneous coronary intervention (PCI) medicine due to rate of re-stenosis and major adverse cardiac event (71). As a result, determining whether the lesion is bifurcated or not is crucial for effective management of CAD. *Liu et al.* showed that miRNA-30-d was up-regulated and miRNA-1246 down-regulated in bifurcated compared to patients with non-bifurcated lesion (72).

Furthermore, miRNAs have been correlated with the characteristic of different plaques. For instance, more calcified plaque and less calcified plaque have diverse array of clinical outcome and miRNAs which are correlated to the level of calcification may have a potential to assess the severity of CAD. miRNA-21 expression in macrophages of non-calcified coronary artery lesions was significantly higher with an AUC value of 0.655 (73). *Liu et al.* also obtained biomarkers that can classify calcified from non-calcified lesion. Out of 8 miRNAs, further validation of miRNA-2861, miRNA-3135b and miRNA-191-3p showed better classification power (74).

Prognostic markers of coronary artery disease

Though limited information has been reported so far regarding correlation of miRNAs with CAD prognosis, reports indicated that some miRNAs might have a potential. Their ability of involvement in all aspects of CAD progression like vascular performance and cardiac remodeling either in beneficial or detrimental way might make them capable of predicting future consequence of the diseases (16).

To appreciate this, *Jansen et al.* determined plasma and microvesicles (MV) level of 10 miRNAs: miRNA-126, miRNA-222, miRNA-let7d, miRNA-21, miRNA-20a, miRNA-27a, miRNA-92a, miRNA-17, miRNA-130, and miRNA-199a, which are involved in vascular activities. There were no significant association between cardiovascular events and plasma level of the above miRNAs. In contrast, increased expression of miRNA-126 and miRNA-199a in circulating MVs was significantly associated with a lower major adverse CV event rate (75).

Likewise, *Schulte et al.* confirmed in a large cohort that baseline serum levels of miRNA-126 was not a helpful prognostic marker of CAD, even with the adjustment of cases into ACS and stable CAD groups (76). However, Elevated levels of miRNA-197 and miRNA-223 reliably predicted future cardiovascular death. *Widera et al.* also investigated the prognostic value of plasma levels of cardiomyocyte-enriched miRNAs (miRNA-1, miRNA-133a, miRNA-133b, miRNA-208a, miRNA-208b, and miRNA-499) among ACS patients. Out of them, only miRNA-133a and miRNA-208b levels were significantly associated with the risk of death (77).

Association of restenosis with miRNA expression

Restenosis is a common adverse event of endovascular procedure that is characterized by recurrence of narrowing of a blood vessel. If restenosis occurs after stenting, this is called in-stent restenosis (ISR) (78). In general, the threshold value for restenosis is a ≥50% narrowing (79). microRNAs are also associated with the occurrence of restenosis in CAD patients. He *et al*. showed that the reduction of circulating miRNA-143 and miRNA-145 levels were associated with the occurrence of ISR and could serve as novel non-invasive biomarkers for ISR (80). Furthermore, a study done by O'Sullivan *et al.* indicated that miRNA-93-5p independently predicted ISR after adjustment for traditional CAD risk factors (81). Fejes *et al.* examined the role of miRNA-181b, miRNA-185 and miRNA-155 to distinguish ISR patients from non-ISR.

miRNA-181b were downregulated, while both miRNA-185 and miRNA-155 were upregulated in ISR patients compared to non ISR (82). The purpose and implications of various selected miRNAs which have diagnostic and prognostic role for CAD are listed in (Table 1).

PARAMETERS INFLUENCING miRNAs LEVELS

Former studies have proven that heparin administration to the patients prior to blood sampling interferes with result of miRNAs (83,84). Collecting blood samples with heparinized test tube had also mislead miRNA determination (85). *Boileau A et al.* also revealed that endogenous heparin has a great effect on miRNA quantification (86).

Furthermore, anti-platelet therapy has also an effect on miRNAs expression. *Russo et al.* reviewed that platelet-derived miRNAs, like miRNA-92a and miRNA-19b respond to aspirin therapy (87). *Willeitnet al.* also revealed that plasma levels of platelet miRNAs, such as miRNA-223, miRNA-191, and others, that is, miRNA-126 and miRNA-150, were reduced under anti-platelet treatment (88). Therefore, high caution is needed when selecting patients for *in vivo* studies of miRNA quantification with respect to heparin and anti-platelet administration prior to blood sampling. In fact, the addition of heparinase enzyme in the sample reversed the effect of heparin (89, 90).

Quantification of miRNA levels altered in CAD might also be influenced by the intake of medication, such as statins and angiotensin converting enzyme (ACE) inhibitors (91). These findings emphasize the importance of quantifying the drug- and metabolite-based influence on miRNAs in the clinical setting. At the same time the inconsistency of the data reflects the necessity of further studies evaluating pathways of how miRNA levels are influenced in circulating blood. Additionally, it needs to be considered, that levels of biomarkers can also be influenced by the speed of their elimination. *Gidlöf et al*. found cardiac miRNA levels strongly correlating with renal function indicating that the renal function might also influence the plasma levels of miRNAs (92).

The influence of high-altitude hypoxic environments on plasma miRNA profiles has also been observed. *Yan et al.* recently reported that 175 miRNAs differently expressed relative to altitude and their expression level were also correlated with red blood cell counts and hemoglobin values (93). Co-variability of miRNA level with demographic factors was also reported. *Neha Singh et al.* found that miRNA-126-5p and miRNA-92a-3p were co-variables with age and serum creatinine level (94).

LIMITATIONS OF UTILIZING miRNAs AS BIOMARKERS

The major drawback of using miRNAs as biomarkers for clinical diagnosis is their laborious isolation and detection procedures. In addition, the current technology employed to isolate and estimate levels of miRNA requires optimization (95).

Other most significant challenge is their lower tissue and disease specificity because of an apparent expression of miRNAs in different diseased state and tissues. For instance, *Witwer et al.* reviewed that the scenario of miRNA-141 which was increased in pregnant women, prostate cancer and other cancers originated from epithelial, breast, colon and lung (96). Such kind of scenario also exists in CAD.

CONCLUSION AND FUTURE PERSPECTIVE

Circulating miRNAs as blood-based biomarker in CAD is highly promising: for early detection, assessing severity and prognostic indicators. They have potentials of “excellent” to “satisfactory”

power of classifying patients with or without CAD as well as patients with stable CAD or unstable CAD. Furthermore, miRNAs are not specific, a single miRNA can be elevated or reduced in different disease conditions. As a result, developing an algorithm or a panel of tests might have a contribution to increase the specificity of miRNAs. In this review, modeling of panel tests revealed remarkable results for identifying CAD patients and grading of severity of the disease (53, 57, 58, 67, 68). As a result, extensive validation of panels of miRNAs in large cohorts with their physiological role might be an extraordinary finding.

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Acronyms

ACE: *angiotensin converting enzyme*

ACS*: Acute Coronary Syndrome*

AUC*: Area Under the Curve*

CAD*: Coronary Artery Disease*

CVD*: Cardiovascular Disease*

CHD*: Coronary Heart Disease*

cTnI*: Cardiac Troponin I*

CV*: Cardiovascular*

ECM*: Extra Cellular Matrix*

EPC: *Endothelial Progenitor Cell*

ISR*: In-Stent Restenosis*

Lp*: lipoproteins*

miRNA*: microRNA*

MI*: Myocardial Infarction*

MV*: Microvesicle*

PARS*: Post-Angioplasty Restenosis*

PBMC*: Peripheral Blood Mononuclear Cell*

PCI*: Percutaneous Coronary Intervention*

qRTPCR*: quantitative Real Time Polymerase Chain Reaction*

RNU*: small non-coding RNA*

ROC*: Receiver Operating Characteristics*

STEMI*: ST segment elevated MI*

SYNTAX: *Synergy between percutaneous coronary intervention with Taxus and cardiac surgery*

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Authors' contributions

TM conceived the idea and wrote the first draft of the review, HWB had contribution on revising the first draft of the review and guiding. All of the authors have amended the final version of the manuscript.

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In this issue: Non-coding RNAs as potential laboratory biomarkers

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MicroRNA profiles in B-cell non-Hodgkin lymphoma

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ABSTRACT

B-cell non-Hodgkin's lymphomas are tumors of B-cells that arise following clonal expansion and consequent invasion of immune organs by B-cells blocked at a certain step of the differentiation process. Genetic abnormalities with altered gene expression are common in the transformed state of B-cells at any stage of B-cell development. These stages are regulated by a combination of transcription factors, epigenetic modifications, microRNAs, and extrinsic signals. MicroRNAs are a class of short non-coding single-stranded RNAs implicated in the regulation of mRNA function and translation. Each microRNA can regulate multiple transcripts; and a transcript is under potential control by multiple microRNAs. Their dysregulation can contribute to the pathogenesis of B-cell non-Hodgkin lymphomas, and they could be used as a potential target for diagnosis, evaluation of prognosis and therapy monitoring. The mechanisms of microRNA dysregulation range from dysregulation of the DNA sequences encoding the microRNAs to transcriptional regulation of microRNA loci. In this review, we summarized the microRNA profiles of the

most common B-cell Non-Hodgkin Lymphomas for the pathogenesis, diagnosis and their potential therapeutic implications.

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INTRODUCTION

Lymphomas represent a heterogeneous group of cancers that vary in presentation, prognosis, and pathogenesis. According to the World Health Organization (WHO) classification report, more than 100 different lymphoma types have been identified. Non-Hodgkin's lymphoma (NHL) is a heterogeneous group of disorders that arises in lymphoid hematopoietic tissue and that can be grouped into B- and T-cell lymphomas which account for about 90% and 10% NHLs, respectively (1, 2). B-cells Non-Hodgkin Lymphomas (BCNHL) are tumors of B-cells that arise following clonal expansion and consequent invasion of immune organs by B-cells blocked at a certain step of the differentiation process (3, 4). B-cell lymphomas (BCLs) are a complex and heterogeneous group of tumors with different cellular origin, genetics and pathology. Patients diagnosed with these diseases show extremely variable clinical courses, ranging from very indolent to highly aggressive types, receive various types of treatment modalities, respond differently to therapy, and have extremely variable clinical outcomes (5).

This heterogeneity is partly reflective of the fact that these tumors are derived from different stages of mature B-cell differentiation. It encompasses a wide variety of disease subtypes in which the incidence pattern greatly varied. Diffuse large B cell lymphoma (DLBCL), Mantle cell Lymphoma (MCL), Follicular Lymphoma (FL), Burkitt's lymphoma (BL), Chronic Lymphocytic Leukaemia (CLL), Mucosa-Associated Lymphoid Tissue (MALT) and Marginal zone lymphoma (MZL) are the main subtypes (4). Majority of NHL B-cells have passed the germinal centre (GC) reaction and thus their immunoglobulin (IG) genes have undergone somatic hypermutation (SHM) and heavy chain class-switching. FL and DLBL are good examples of this lymphoma. Other subtypes like MCL and CLL are derived from GC-inexperienced B cells in at least a proportion of cases, or other cell types are marginal zone B cells e.g. MZL (6). The pathophysiology of these lymphomas come from intrinsic cellular aberrations like B-cell receptor (BCR) and NF-kB signaling defect affecting pathways of particular importance and the fact that the tumor cells are reliant on the microenvironment through cell-extrinsic communication and activation via different cell surface receptors (7-9).

Chromosomal translocations involving an oncogene and one of the immunoglobulin genes are a common phenomenon in BCNHLs (10). Three BCNHLs have characteristic genetic abnormalities that are important in determining their biologic features and are useful for differential diagnosis. These include: t(14;18) in FL, t(11;14) in MCL and t(8;14) in BL. Characteristic for these translocations is that a cellular proto-oncogene is placed under the control of the Ig promoter on chromosome 14q, resulting in constitutive activation of the gene which in turn gives to the cell survival or proliferative advantage. In FL, t(14;18) translocation results in overexpression of an anti-apoptosis gene called B cell lymphoma-2 (BCL-2). In MCL and BL, the translocations result in over-expression of cell cycle genes associated with proliferation of Cyclin D1 (CCND1) or Myc, respectively (11, 12). These lymphomas are influenced by aberrant genetic alterations, epigenetic dysregulation, aberrant pathway activation, and complex tumor–microenvironment interactions (13). Diagnosis and classification modalities of these multi-subclass of BCNHLs are challenging. Moreover, the molecular heterogeneity of these disease makes the treatment modalities difficult, accordingly patients who are treated similarly have variable

outcomes (14). However, the advancement of recent technologies in the use of effective and practical detection techniques, and identifications of novel biomarkers at the genetic, epigenetic, and protein level as well as at the tumor microenvironment enables and improves the diagnostic process, sub-typing, outcome stratification, and personalized therapy for lymphoma patients (15).

Among these biomarkers micro ribonucleic acids (miRNAs) are the most important one. Individual miRNA expression and miRNA signatures analysis allow specific cell differentiation stages to be easily identified in the pathogenesis of BCNHL (16). As a result, it can be taken as a powerful player in the pathogenesis, diagnosis and prognosis these diseases (17). In this review, the authors summarize the miRNA profiles and the implications of miRNA dysregulation in the pathogenesis, diagnosis and prognosis of BCNHL, as these molecules appear to be cell type and disease specific, unlike most other biomarkers currently available.

DEFINITION OF miRNAs, BIOGENESIS, MECHANISM OF GENE REGULATION AND THEIR ROLES IN BCNHL PATHOGENESIS

Definition and biogenesis of miRNA

MiRNAs are small, evolutionary highly conserved, 20-24 nucleotides long, single stranded non-coding RNA molecules. They are involved in the regulation of gene expression by binding to target mRNA in plants, animals and viral genome via post-transcriptional degradation or translational repression. Their mechanism of gene regulation is by translational silencing or by impairing the stability of their target messenger RNAs (mRNAs) (18, 19).

It is predicted that miRNA account for 1-5% of the human genome and regulate at least 30% of protein-coding genes (20). Until 2000's, 940 distinct miRNAs molecules have been identified within the human genome (21). Later on, the recent advancement in small RNA deep sequencing technology has now enabled the identification of over 2500 mature human miRNAs (22).

MiRNA precursors are commonly found in clusters through many different regions of the genome, most frequently within intergenic regions and introns of protein coding genes (23). Human miRNA biogenesis is a multistep process that begins in the nucleus where miRNA genes are transcribed by RNA polymerase II into long primary miRNAs (pri-miRNAs) molecules, and subsequently trimmed into smaller, stem-looped, hairpin-like miRNA precursor (pre-miRNA) by RNase III-type enzyme Drosha that form a microprocessor complex with its binding partners DiGeorge Syndrome critical region gene 8 (DGCR8). Then the pre-miRNAs are exported from the nucleus into the cytoplasm via Exportin-5 to be sliced by another RNase III-type enzyme called Dicer and its binding partners called the transactivator RNA-binding protein (TRBP), to generate a 19- to 23-nucleotides RNA duplex that contains both the mature miRNA strand and its complementary strand. The mature miRNA strand is preferentially incorporated into a miRNA-induced silencing complex (miRISC), while the other strand of miRNA is degraded by the RNA-induced silencing complex (RISC). The miRNA strand guides the RISC to its mRNA target, containing complementary sequence to the mature miRNA and subsequently induces the cleavage or silencing of the target mRNA. The complementarity between miRNA and target mRNA is a crucial factor for the post-transcriptional regulatory mechanism (24, 25).

Mechanisms of miRNAs in gene regulation

Normally, miRNAs play a role in heterochromatin formation, histone modification, DNA methylation, and gene silencing which affects the level of target gene expression (26). They generally bind to a specific target mRNA with a complementary

sequence to induce cleavage, or degradation or block translation by a feedback mechanism. MiRNAs also inhibit protein translation from the target mRNA (27). They can also speed up deadenylation, causing mRNAs to be degraded rapidly (28). For example, miRNA-430 in zebrafish, bantam-miRNA and miRNA-9 in Drosophila cultured cells causes translational repression by the disruption of translation initiation (29).

For the ease of understanding, some scholars categorized and summarized the mechanisms of miRNAs actions as follows: Cap-40S initiation inhibition; 60S Ribosomal unit joining inhibition; elongation inhibition; ribosome drop-off (premature termination); co-translational nascent protein degradation; sequestration in P-bodies; mRNA decay (destabilization); mRNA cleavage; and transcriptional inhibition through microRNA mediated chromatin reorganization followed by gene silencing (30).

MiRNAs may influence histone marks by regulating the expression of histone modifiers. For example, in MCL, downregulated expression of miRNA-15a, miRNA-16-1, and miRNA-29 are due to histone hyperacetylation at the promoter sites of their genes. The hyperacetylation is brought about by the overexpression of Myc: Myc binds to and represses Histone Deacetylase 3 (HDAC3), an enzyme that is responsible for removing acetyl groups from histone residues. This in turn results in the downregulation of miRNA-15a/16-1 (31).

MiRNAs virtually regulate all cellular processes including cell cycle, developmental, cell proliferation, apoptosis, differentiation, metabolism, organ development and morphogenesis, hematopoiesis and disease process including cancers (32, 33). MiRNAs regulate gene expression at the post-transcriptional level by binding to short motifs of complementary sites of transcribed target mRNA at the 3'-untranslated regions (3'-UTRs). When it binds to its specific target, it can cause either protein translational repression or transcript degradation of the mRNA molecule (34).

Deregulation of miRNA expression is a type of genetic alterations relevant for lymphomagenesis. The loss of miRNA-15 and miRNA-16 in CLL with deletion of the 13q14 region (35) and upregulation of miRNA-155 and its precursor mRNA BIC in DLBCL and are good examples (36).

MiRNAs usually play a critical role in tumor development since they are often located at fragile sites and genomic regions on chromosomes that are associated with cancer (37). They are important in cancer biology by regulating the expression levels of target mRNAs to assist tumor growth, metastasis, angiogenesis and immune evasion (38). They play an important role in determining cancer behavior since the non-coding regions of the genome are frequently deleted in cancer including BCNHL often contain miRNA genes (18).

Dysregulation of miRNA may result in the aberrant expression of miRNA target genes, and results in the acceleration of lymphomagenesis (39). Dysregulations of miRNAs can be associated with different diseases (40), chronic lymphocytic leukemia (CLL) being the first human disease known to be associated with miRNA dysregulation. In the pathogenesis of B cell malignancies, miRNAs participate in pathways fundamental to cell development like B-cell receptor (BCR) signalling, B-cell migration/adhesion, cell-cell interactions in immune niches and the production and immunoglobulins class-switching. MiRNAs influence B cell maturation, generation of pre-, marginal zone, follicular, B1, plasma and memory B cells. They also regulate B-cell proliferation through inhibiting proteins like E2F1 involved in the process of cell proliferation (41).

Each NHL subtype has their distinct miRNA signatures resulting from coordinately dysregulated expressions of miRNAs. This miRNA dysregulation

may be due to dysregulation of the DNA sequences encoding the miRNA to transcriptional regulation of miRNA loci, miRNA biogenesis pathway or messenger RNA (mRNA) targets. The activation of oncogenic pathways, and the reprogramming of BCNHL transcriptomes may be due to this abnormal miRNA expression. This widespread dysregulation suggests that miRNAs can be used as a diagnostic and prognostic tool. Profiling of different cell types and tissues indicated that the pattern of expression of miRNAs is cell type and tissue specific, suggesting that the program of expression of miRNAs is neatly cell type dependent and tightly associated with cell differentiation and development (42, 43).

miRNA PROFILE IN BCNHL PATHOGENESIS, DIAGNOSIS AND SUBTYPING

miRNA profile in BCNHL pathogenesis

MiRNAs are very crucial for the regulation of translation in physiological and pathological states, including the sequential differentiation of B-cells and lymphomagenesis (44).

MiRNAs control hematopoiesis through modulating different signaling pathways that are cell type and context specific (45). MiRNAs play an important role in hematopoiesis as it had been confirmed by the deletion of components of these miRNAs biosynthetic pathway in vivo demonstrates the critical role in hematopoiesis. For instance, miRNA miRNA-17-92 cluster, miRNA-34a, miRNA-125b, miRNA-150, miRNA-181a, and miRNA-212/132 are important for a correct early B-cell development process (46). They mainly involved in the regulations of germinal center (GC) B cell differentiation by targeting of activation induced cytidine deaminase (AID) (47, 48). However, miRNA-17-79 cluster, miRNA-24, miRNA-146, miRNA-155, miRNA-128, and miRNA-181b prevent the differentiation of early stage B cell progenitor cells. Other few miRNAs such as miRNA-16, miRNA-103, and miRNA-107 act later on, and miRNA-221, miRNA-222, and miRNA-223 regulate the terminal or end stages of hematopoietic development (49).

Potential regulatory role for miRNAs in discrete stages of mature B-cell differentiation have a direct role for the miRNA-mediated regulation of oncogenes and key transcription factors in B-cell differentiation (50). MiRNAs that play a determinant task in the lineage differentiation decision of B-cells includes the high expression levels of the cluster of miRNA-23a (miR-23a, miRNA-27a, and miRNA-24) and miRNA-125b reduce the differentiation to B-cell lymphocyte lineage in favor of myeloid differentiation (51, 52). Moreover, some miRNAs have an important role in early B-cell development like miRNA-150 up-regulation in hematopoietic progenitors reduces the normal quantity of mature B cells by blocking the maturation process at the pro-B cell stage (53). There is a strong association between changed miRNA expression and oncogenesis. MiRNAs that enhance cellular processes are associated with oncogenesis and tumor progression, with uncontrolled clonal expansion, increased invasiveness, and resistance to apoptosis. Those miRNAs involved in these processes are called 'oncomiRs', and are frequently upregulated. On the other hand, miRNAs that counteract these oncogenic characteristics are called 'tumor-suppressor miRNAs', and are often down regulated in cancer including BCNHL (54).

The importance of miRNAs in cancer has been underlined by the identification of changes in their target binding sites and the miRNA processing machinery of tumor cells (54, 55).

miRNAs influence B-cell maturation, generation of pre-B, marginal zone, follicular, B1, plasma and memory B cells. MiRNA-150, miRNA-155, miRNA-21, miRNA-34a, miRNA-17-92 and miRNA-15-16 are the major miRNAs having essential functions in malignant B-cell development

(56). The levels of miRNA155, miRNA200c, miRNA130a, miRNA125b and miRNA21 were found significantly upregulated whereas miRNA29c, miRNA451 and miRNA145 were found downregulated in BCNHL patients when compared with healthy controls (57).

The miRNA-17-92 cluster is a polycistronic miRNA encoded by chromosome 13 amplified in BCNHLs (58). This cluster of miRNA is the most complex and highly conserved sequence in humans that produces six mature miRNAs, (miRNA-17, miRNA-18a, miRNA-19a, miRNA-20a, miRNA-19b1, and miRNA-92-1) generated from the third exon of the open reading frame C13orf25 at loci 13q31.3. The 13q31.3 gene locus is a frequent site for gene amplification, which explains highly elevated levels of miRNA-17-92 observed within a variety of lymphomas. MiRNA-17-92 can be directly regulated by c-MYC and E2F transcription factors (E2Fs). However, E2F3 is thought to be the predominant regulator in BCNHL pathogenesis (59).

MiRNA-17-92 cluster is recurrently amplified in human B cell malignancies, causing the overexpression of these miRNAs in several lymphoma types, like MCL, FL, BL, GCB-DLBCL, but is never overexpressed in ABC-DLBCL (60). Forced overexpression of miRNA-155 results in the development of DLBCL (61) by suppressing the growth-inhibition of BMP2/4 and TGF-β1 via SMAD5 inhibition (62).

MiRNA-19a and miRNA-19b are two miRNAs in this cluster play key roles in the induction of BCNHL progression. Whereas miRNA-17-5p and miRNA-20a are the other two miRNAs which play a key role in controlling cell proliferation by regulating the transcription factor E2F1. As a result, miRNA-17-5p and miRNA-20a are considered as tumor suppressors, highlighting the complexity and versatility of miRNA-mediated regulation in cancer (54, 63).

miRNA profiles in the subtypes of BCNHL

miRNA profiles in Diffused Large B-Cell Lymphoma (DLBCL)

DLBCL is one of the most common, frequent and aggressive kind of BCNHL, accounting for nearly 30–40% of newly diagnosed lymphomas (1). It is a heterogeneous group of diseases with an aggressive clinical course (64) that accounts for approximately one third of patients with NHL. Due to its heterogeneity in genetic abnormalities, clinical features, response to treatment and prognosis (65, 66) and outcome prediction based on clinical and molecular features is difficult. Thus, an assessment of miRNA expression profiling can be used to get important information regarding diagnostic, subtyping and outcome prediction for DLBCL (67).

In light of miRNAs' potential as diagnostic markers for cancer prognostication there is an increasing interest in the possible role for miRNAs as markers for both B-cell differentiation stage and malignant transformation. It has been shown that miRNA expression patterns can characterize the stages of human B-cell differentiation (50, 68, 69). To date, a large number of microRNA signatures in lymphomas were identified, and the role of miRNAs in the development, classification and in the regulation of target genes is under intensive investigation (68).

miRNAs, such as miRNA-17-92, miR-15a/16-1 clusters, miRNA-222 and let-7f are highly expressed in DLBCL pathogenesis and common targets for copy number changes. Among the multiple miRNA loci on 12q that were frequently targeted by copy number gains, miRNA 26a-2 and let-7i were found also highly expressed in the prime tumors. These data suggest that the chromosome 12q miRNAs are more likely to contribute to the pathogenesis of GCB-type DLBCL (70).

The mechanisms of the above miRNAs in the pathogenesis of DLBCL are described below.

The role of miRNA-15a/miRNA-16-1 cluster in the pathogenesis of DLBCL via targeting BCL2 is to reduce apoptosis of lymphoma cells (71). Similarly, the increased expression of miRNA-17-92 cluster leading to increased expression of *MYC* and increases the development and the aggressiveness of lymphomas (72) and by reducing the degree of apoptosis of lymphoma cells (73). The function of miRNA-17–92 is associated with c-MYC, and a negative feedback loop may exist between miRNA-17–92 and c-MYC. This is important in the regulation of cell proliferation and apoptosis as it induces the growth of B-cell lymphoma by reducing apoptosis and promoting the proliferation of lymphoma cells. There are several other potential targets for miR-17–92, including proapoptotic BCL-2 interacting mediator of cell death, PTEN and E2F transcription factor 1, which is a direct target of MYC and promotes cell cycle progression (74).

On the other hand, the let-7f miRNA regulates the expression of the RAS proteins that regulate cell growth and differentiation through MAP kinase signaling. Hence, let-7f indirectly alters the cell proliferation rate through its downstream MAP signaling cascade and regulates the expression of oncogenes (75).

Additionally, miRNA-330, miRNA-17-5p, miRNA-106a, and miRNA-210 were found increasingly expressed DLBCL. The mechanism in the pathogenesis of the disease is that an alteration in miRNA expression levels in DLBCL causes an aberrant expression of miRNA target genes and consequent disruption of the gene expression profile, which can result in cancer development. Multiple mechanisms has been identified like genomic mutation of miRNA loci, epigenetic changes and deregulation of transcription factors contribute to the modulations of miRNA expression levels (76, 77). In the contrary of the above-mentioned miRNAs, miRNA-150, miRNA-145, miRNA-328, miRNA-139, miRNA-99a, miRNA-10a, miRNA-95, miRNA-149, miRNA-320, miRNA-151 and let7e had considerably decreased expression in DLBCL (78). As it had been reported by Fassina *et al.*, miRNA-17-92 cluster, miRNA-150 and miRNA-210 were found to be significantly overexpressed in GCB-DLBCL and allowed correct identification of 97% GCB-DLBCL cases (79).

As it had been reported by Thapa *et al.*, miRNA-17, miRNA-106a and miRNA-106b regulate the proliferation, apoptosis and invasion of DLBCL cells by repressing the expression of cyclin-dependent kinase inhibitor 1. In addition, higher expression level of miRNA-15a, miRNA-16, miRNA-17, miRNA-106, miRNA-21, miRNA-155 and miRNA-34a-5p are specific to DLBCL than in other malignancies. This suggesting that these miRNAs may be used as potential candidate biomarkers for DLBCL diagnosis (80). DLBCL tumors are also characterized by upregulated expression of miRNA-150, miRNA-17-5p, miRNA-145, and miRNA-328 when compared with samples taken from normal lymph nodes and follicular lymphoma (FL) (78).

MiRNA-155 is one of the best recognized miRNAs in lymphomas, particularly in DLBCL usually upregulated in several lymphoma subtypes (54, 61, 74) such as in primary mediastinal BCL (PMBCL) and DLBCL, especially of the ABC type (81, 82). It acts as an onco-miRNA in the pathogenesis and aggressiveness of these lymphoma subtypes. Levels of miRNA-155 expression in ABC- DLBCL subtype were found to be significantly higher than in GC-DLBCL, suggesting that miRNA-155 is diagnostically useful to distinguish ABC-DLBCLs from GC-DLBCL and may explain the poor prognosis of ABC-DLBCL patients (15). For example, forced over-expression of miRNA-155 in mice results in the development of a high grade BCL similar to DLBCL (61, 83) confirmed that the association between this miRNA expression and BCNHL development.

Down-regulation of the target mRNA is considered the main mechanism by which miRNAs modulate protein expression. MiRNA expression level can aid to distinguish between subtypes of DLBCL (GC-DLBCL and ABC-DLBCL) even though the subtype differentiation is based on validated FFPE technique by using Nanostring testing. For example, the miRNA-21, miRNA-144, miRNA-155, miRNA-221, miRNA-222 and miRNA-451 were found upregulated and more highly expressed in the ABC subtype than in the GCB subtype (69). On the other hand, miRNA-28, miRNA-151, miRNA-331, and miRNA-454-3p were found to be upregulated in the GC-type DLBCL (84). For example, the mechanism by which miRNA-21 upregulated expression influences the pathogenesis of ABC-DLBCL is associated with tumor growth, invasion and metastasis through targeting multiple tumor and metastasis suppressor genes, including programmed cell death 4 (neoplastic transformation inhibitor), tropomyosin 1-α and phosphatase and tensinhomolog (PTEN) (85, 86).

miRNA profiles in Burkitt Lymphoma (BL) diagnosis

Burkitt lymphoma (BL) is another aggressive type of BCL. BL is a highly aggressive type of BCNHL and is the fastest growing human tumor. It has two major subtypes, the endemic one that is predominantly affecting young children and common in equatorial Africa, and the systemic/sporadic type which is affecting adults as well and occurs in worldwide (87). Recent studies using NGS on BL have improved the understanding of the pathogenesis of these tumors. Mutations in the transcription factor 3 (TCF3) or its negative regulator ID3 occur in about 70% of sporadic and immunodeficiency-related BL and 40% of endemic cases. TCF3 promotes survival and proliferation in lymphoid cells by activating the B-cell receptor/phosphatidylinositol 3-kinase signaling pathways and modulating the expression of cyclin D3, which is also mutated in 30% of BL (1). The most frequently mutated genes in Burkitt lymphoma were *MYC* (40%) and Inhibitor of DNA binding 3 (*ID3*) (34%) (88).

It is characterized by a high degree of proliferation of the malignant cells and deregulation of the *c-Myc* gene caused by t(8;14)(q24;q32) leading to the constitutive expression of the Myc oncogene (89). BL is also characterized by the dysregulated expression of *Myc* as a consequence of translocations of immunoglobulin genes. It was found that miRNA-155 expression is highly reduced in BL because miRNA-155 suppresses activation induced cytidine deaminase (AID) mediated Myc-IGH translocation (90). Therefore, BL can be characterized by the unstable interaction between c-Myc and miRNAs like let-7a, miRNA-34b, miRNA-98, miRNA-331 and miRNA-363 (91). Upregulated expressions of miRNA-155 mediated by c-MYC play a role in the lymphomagenesis of pediatric BL (36).

In addition to histological, immunohistochemistry testing in conjunction with BCL2 and c-Myc testing, miRNA profiling can improve the differentiation of BL from DLBCL (1). Moreover, miRNA may have a clear role in pathogenesis, differentiating BL from other, but it is only investigational. For example, the loss of miRNA-155 expression in BL is useful distinctive marker in the differential diagnosis from DLBCL (92).

In BL patient's miRNA expression profiling, miRNA-150 having c-Myb and survivin protein targets had extremely decreased expression levels. Thus, deregulation of miRNA-150 is an important diagnostic biomarker for BL screening and diagnosis (93). In majority of the cases of BL, there is a *c-Myc* translocation, members of the miRNA-17-92 cluster (miRNA-17-3p, miRNA-18a, miRNA-19a, miRNA-19b and miRNA-92) are up regulated and let-7 family miRNAs are down regulated (94). Expressions of miRNA-21 and miRNA-23a are useful molecular

biomarkers in the diagnosis and prognosis for BL in children (95). MiRNA-221/222 is also critical mediator for BL pathogenesis (96).

miRNAs profiles in Follicular Lymphoma (FL)

Follicular lymphoma (FL) is another of the most common forms of B-cell lymphoma derived from germinal center B-cells. It comprises approximately 15–20% of newly diagnosed lymphomas (97).

A specific chromosomal translocations t(14;18)(q32;q21) involving the B-cell lymphoma-2 gene (*BCL2*) and immunoglobulin (Ig) loci is essential for FL development (11). In addition to t(14;18)(q32;q21) as the molecular hallmark of FL, chromosomal rearrangements affecting the *BCL6* locus constitute one of the most common cytogenetic finding (98, 99). FL is the slow growing BCNHLs accounting for about 20–30% of all NHL. It has the tendency to transform into DLBCL, with translocation t(14;18)(q23;q21) in 90% of cases and is associated with BCL2 activation which may lead to accumulation of GCB cells with prolonged lifespan (100).

The comparison study performed on miRNA expression profiles of FL and DLBCL shown that miRNA-155, miRNA-210, miRNA-106a, miRNA-149, and miRNA-139 were found overexpressed in both of these cancers when compared with normal lymph nodes. These overexpressed miRNAs are suggestive of lymphomagenesis (78). Other overexpressed miRNAs distinct to FL are miRNA-20a/b and miRNA-194 and they target cell proliferation inhibitors like *CDKN1A* and *SOCS2*, respectively (101).

The miRNAs that showed significantly decreased expressions in FL patients are miRNA-202, and miRNA-139-5p. However, miRNA-338-5p, miRNA-9, and miRNA-330-3p are significantly up regulated (94, 102).

miRNAs differentially expressed in BL, DLBCL and FL

Rapid and accurate differential diagnosis of BL versus DLBCL is very important for therapeutic decisions and patient prognosis. MiRNA-155 is the most significantly lost miRNA in BL, followed by miRNA-29b and miRNA-146a, whereas the most significantly gone miRNAs in DLBCL are miRNA-17-3p, miRNA-595 and miRNA-663. MiRNA-29b is downregulated in BL cases (103-105). In addition, miRNA-34b is also downregulated in BL (94, 106).

MiRNA-155, miRNA-21 and miRNA-26a are potential diagnostic biomarkers to differentiate BL from DLBCL and DLBCL/BL. In both BL and DLBCL/BL cases of lymphoma, miRNA-155, miRNA-21, and miRNA-26a showed considerably reduced level of expression than primary DLBCL (107). In addition to miRNA-155, miRNA-17-5P, miRNA-106A, and miRNA-210 are found significantly expressed at a higher concentration in DLBCL than in normal tissue. In opposite to this, miRNA-10a, miRNA-95, miRNA-99a, miRNA-139, miRNA-145, miRNA-149, miRNA-150, miRNA-151, miRNA-320 and miRNA-328 were found to be expressed at a significantly reduced level. MiRNA-330, miRNA-17-5P, miRNA-106A and miRNA-210 are the most discriminatory miRNAs - for DLBCL and FL diagnosis. Comparing DLBCL and FL with other subtypes, miRNA-17-5P and miRNA-92 were found overexpressed whereas eight miRNAs consisting of miRNA-330, miRNA-338, miRNA-135A, miRNA-150, miRNA-125B, miRNA-301, miRNA-126, and miRNA-213 were found down regulated in DLBCL (78).

miRNA profiles in Mantle Cell Lymphoma (MCL)

MCL constitutes approximately 5% to 10% of all newly diagnosed cases of NHLs (108) having the genetic hallmark of t(11;14)(q13;q32)

translocation that results in overexpression and displaces the cyclin D1(CCND1) gene on chromosome 11 downstream to the enhancer region of the IgH gene on chromosome 14 (109).

MiRNA expression profiling of B cells from MCL patients led to the identification of miRNA expression signature and frequent deregulation of a set of miRNAs. For example, miRNAs such as miRNA-124a, miRNA-155, miRNA-328, miRNA-326, miRNA 302c, miRNA-345, miRNA-373, and miRNA-210 were identified as upregulated in patients with MCL (110). The mechanism of this upregulated miRNA in the pathogenesis of MCL is inhibiting *CDK6* expression as well as phosphorylation of RB1. The good example of this mechanism is that miRNA-124a inhibit *CDK6* expression as well as phosphorylation of RB1, targeting of *CDK6* and *CCND1* prevents the downstream pro-survival signaling of the cyclin/CDK pathway (111).

Several miRNAs have been implicated in MCL pathogenesis. The major downregulated miRNAs include miRNA-29 family (miRNA-29a, -29b, and -29c), miRNA-142-3p/5p, miRNA-150, and miRNA-15a/b were found associated with short overall survival of patients with MCL (110). The pathophysiologic roles of miRNAs such as miRNA-15a, miRNA-16-1, and miRNA-29 are by transcriptional repression and its epigenetic regulation by c-Myc in MCL. They are downregulated due to histone hyperacetylation at the promoters of their genes. In this instance, the hyperacetylation is brought about by the overexpression of *Myc* gene in which the binding of *Myc* on target gene represses HDAC3, an enzyme that is responsible for removing acetyl groups from histone residues. This in turn results in the downregulation of miRNA-15a/16-1 (31). MiRNA-29 indirectly targets the de novo DNA methyltransferases thereby controlling gene expression. Therefore, loss of miRNA-29 may result in elevated MCL1 levels (25). MiRNA-29b is down regulated in malignant cells, consistent with MCL-1 protein up regulation. Enforced miRNA-29b expression reduced MCL-1 cellular protein levels and thus miRNA-29 is an endogenous regulator of MCL-1 protein expression and apoptosis. The miRNA-29 also targets CDK6, expression which is a known prognostic and pathogenetic factor in MCL. Furthermore, downregulation of miRNA-29 is in line with the CCND1 overexpression and the consequent CDK4/CDK6 activation, which is the primary event in MCL pathogenesis (112).

Another study showed that miRNA-31, miRNA-148a and miRNA-27b are also among the down regulated miRNAs in MCL, on the other hand miRNA-617, miRNA-370 and miRNA-654 are among the up regulated miRNAs. Of these, miRNA-31 is the most down regulated miRNA targets MAP3K14 (NIK) gene, which is essential for activation of the alternative NF-κB pathway (113). The miRNA clusters at locus 7q22 including miRNA-106b, miRNA-93 and miRNA-25 are also highly up regulated in MCL. MiRNA-106b specifically promotes cell-cycle progression by targeting cyclin-dependent kinase inhibitors p21/CDKN1a. In addition to this function, miRNA-106b overrides doxorubicin-induced DNA damage checkpoint (114). MiRNR-181a and miRNR-181b are down regulated in lymphoma and acts as a tumor suppressor by targeting the T cell lymphoma 1(TCL1) oncogene and indirectly regulate the levels of the oncogene mantle cell lymphoma1 (MCL1) (112). miRNA-21 overexpression also leads to pre-BCL, which is completely dependent on the continued expression of miRNA-21 (54, 115).

miRNAs profiles in Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma

MALT lymphoma usually presents as localized disease and typically arises from sites such as the stomach but characteristically disseminates, either within the same organ or to other extranodal sites where MALT lymphomas are

known to arise (116). Chromosomal alterations, especially trisomy 3, 12, and 18, are common in MALT lymphomas. Chromosomal translocations associated with MALT lymphomas include t(11:18)(q21: q21), resulting in the production of a chimeric protein (API2- MALT1) (117); and t(1;14)(p22;q32), t(14;18)(q32;q21), and t(3;14)(p14.1;q32), resulting respectively in transcriptional deregulation of BCL10, MALT1, and FOXP1 (118, 119).

Different studies conducted indicated that, there are miRNAs significantly up regulated in MALT lymphoma cases. The miRNA-200 family (miRNA-200a, b and c) is the most common one. The other miRNAs located in these clusters, miRNA-429 and miRNA-141, were also up regulated. The miRNA-200 family inhibits the initiating step of metastasis, the epithelial-mesenchymal transition, by maintaining the epithelial phenotype through directly targeting the transcriptional repressors (120). But miRNA-126 was found down regulated in the case of MALT (94, 120). Additionally, up regulation of miRNA-181c, miRNA-182, miRNA-183, miRNA-200c, miRNA-363, miRNA-654 and miRNA-768-5p were found in this subtype of BCNHL (94).

miRNAs profiles in Nodal Marginal Zone Lymphoma (MZL)

Nodal marginal zone lymphoma (NMZL) is a small B-cell neoplasm whose molecular pathogenesis is still essentially unknown and whose differentiation from other small B-cell lymphomas is hampered by the lack of specific markers (121).

Nodal MZL shows greater expression of miRNA-221, miRNA-223, and let-7f. Expression of these miRNAs is enhanced in nodal MZL, whereas FL strongly expresses miRNA-494. Upregulation of miRNA-223 and miRNA-221, which targets the germinal center-related genes LMO2 and CD10, could be partially responsible for expression of a marginal zone signature. In splenic MZL, the miRNA-29 cluster is commonly lost and its expression silenced (121). (Table 1)

miRNA PROFILES IN BCNHL PROGNOSIS

Best treatment for cancer requires accurately recognizing patients for risk-stratified therapy. Those individuals having a rapid response to initial treatment may benefit from shortened treatment regimens. The role of miRNA in cancers is also implicated in the prognosis

Table 1 A summary of dysregulated miRNAs and their potential implications for diagnosis and subtyping of BCNHL

MiRNA	Expression	B-cell NHL subtype	Potential Role	Reference
miRNA-155, -21, -221	Increased	ABC-DLBCL vs. GCB-DLBCL	Subtyping	(69)
		DLBCL, FL	Diagnosis	
miRNA-155, -21, -210	Increased	DLBCL	Diagnosis	(69, 76, 122)
miRNA-21	Increased	ABC-DLBCL vs. GCB-DLBCL	Subtyping	(122)
miRNA-330, -17-5p, -106a, -210	Dysregulated	DLBCL vs. FL	Subtyping	(78)

miRNA-125b, -143, -451, -145	Increased	DLBCL vs. FL	Subtyping	(123)
miRNA-223, -217, -222, -221,	Dysregulated	DLBCL vs. transformed FL	Subtyping	
miRNA-17-92 cluster, -29a, -106a, -720 , -1260, -1280	Increased	ABC-DLBCL vs. GCB-DLBCL	Subtyping	(124)
miRNA-20b, -26a, -92b, -487b	Increased	DLBCL vs. FL	Subtyping	
miRNA-17-92 cluster, -150, -210	Increased	GCB-DLBCL vs. high grade FL	Subtyping	(79)
miRNA-15a, -16-1, -29c, -155, -34a	Increased	DLBCL	Diagnosis	(85)
miRNA-451	Decreased	FL	Diagnosis	(102)
miRNA-338-5p	Increased	FL	Diagnostic	
miRNA-17-92 cluster (-18b, -20b, -106a	Increased	BL	Diagnostic	(14)
miRNA-155	Decreased	BL vs. DLBCL	Subtype	
		ABC-DLBCL vs. GCB-DLBCL		
miRNA-155, -200c, -130a,-125b, -21	Increased	DLBCL	Diagnostic	(57)
miRNA-451, and -145	Decreased	DLBCL	Diagnostic	
miRNA-9, -301, -338, and -213	Increased	FL	Diagnostic	(78)
miRNA-150, -550, -124a, -518b, -539	Increased	MALT	Diagnostic	(125)

Abbreviations: *miRNA, microRNA; DLBCL, diffuse large B-cell lymphoma; ABC-DLBCL, Activated B-cell like diffuse large B-cell lymphoma; GCB-DLBCL, Germinal center diffuse large B-cell lymphoma; FL, follicular lymphoma; MCL, mantle-cell lymphoma; HL, Hodgkin lymphoma; MALT, mucosa-associated lymphoid tissue lymphoma.*

indication. Their expression level is very good indicator for prognoses of lymphomas. For example, low miRNA-324a levels could serve as an indicator of poor survival (126). Circulating miRNAs have the potential to assist clinical decision making because they are highly stable in blood. They are overexpressed in cancer and are quantifiable within the diagnostic laboratory. They can be performed at each consultation to assess disease response and detect relapse.

Currently, there are so many miRNAs having therapeutic roles in BCNHL patients, particularly patients suffering from DLBCL disease have

been identified. A set of miRNAs, consisting of miRNA-222, miRNA-181a, miRNA-129-5p, and miRNA-18a, has been shown to have prognostic value for DLBCL patients (127). On the other hand, eight miRNAs were found to correlate with patient survival. Patients with down regulated miRNA-21, miRNA-23A, miRNA-27A, and miRNA-34A expression had an inferior overall survival (OS), while patients with low levels of miRNA-19A, miRNA-195, and miRNA-LET7G had a shorter event-free survival (EFS). Patients with low expression of miRNA-127 had low OS and

Table 2 Summary of miRNAs having prognostic role in BCNHLs*

MiRNA	Subtype	Expression level	Role	Reference
miRNA-21	de novo DLBCL	Increased	Prognostic-longer relapse-free survival	(69, 76)
miRNA-155	ABC-DLBCL	Increased	Prognostic-treatment failure	(14)
miRNA-125b, -130a	DLBCL	Increased	Prognostic-poor outcome	(57)
miRNA-18a, -181a and -222	DLBCL		Prognostic	(128)
miRNA-106b, -1181, -124, -1299, -25, -33b, -432, -551b, -629, -652, -654-3p, -671-5p, -766, -877, -93, -93	DLBCL	Increased	Prognostic -Predictive of response to treatment	(130)
miRNA-223, -217, -222, -221	FL	Increased	Prognostic	(123)
miRNA-29	MCL	Increased	Prognostic	(110)
miRNA-21, -23a, -27a and -34a	DLBCLs	Down regulated	Poor OS time	(15)
miRNA-19a	DLBCLs	Decreased	Shorter EFS time	(15)
miRNA-195, -let7g	DLBCLs	Decreased	Longer EFS time	(15)
miRNA-92a	DLBCLs	Decreased	A high relapse rate	(69)
miRNA-127	DLBCLs	Decreased	Poor OS and EFS	(15)

**Abbreviations: miRNA, microRNA; DLBCL, diffuse large B-cell lymphoma; GCB, germinal center B-cell; ABC, activated B-cell like; OS, overall survival; EFS, event free survival; RCHOP, rituximab, cyclophosphamide; RFS, relapse free survival*

EFS. Increased expression of miRNA-18a was associated with shorter OS whereas increased expression of miR-181a was seen in patients with longer EFS. In contrast, higher expression of miANA-222 was associated with shorter EFS (128, 129).

MiRNA-21, has been detected in the sera of DLBCL patients and has been suggested as an independent prognostic indicator in primary DLBCL (76, 78). Patients with down regulated miRNA-21, miRNA-23a, miRNA-27a and miRNA-34a expression levels had inferior overall survival (OS). In contrast, event free survival (EFS) was found influenced by low expression levels of miRNA-19a (shorter EFS), miRNA-195 and let-7g (longer EFS, respectively). A poor OS is most strongly correlated with decreased expression of miRNA-21 and miRNA-27a. In addition to miRNA-127, EFS is most strongly influenced by let-7g and miRNA-19a. A reduced expression level of let-7g is contributed to significantly longer EFS whereas a reduced expression level of miRNA-19a correlated with significantly shorter EFS. In conclusion, reduced expression levels of six miRNAs (miRNA-19a, miRNA-21, miRNA-23a, miRNA-27a, miRNA-34a and miRNA-127) identified as poor EFS and/or OS indicators, whereas the opposite is true for miRNA-195 and let-7g. A down-regulated expression level of this miRNA correlates with poor survival prognosis (78). (Table 2)

CONCLUSION AND RECOMMENDATIONS

The importance of miRNAs in cancer biology is through controlling expression of their target mRNAs to facilitate tumor growth, invasion, angiogenesis and immune evasion. MiRNAs are very important molecule in the pathogenesis, diagnosis and prognosis of BCNHL patients since they are easy to detect, are relatively stable during sample handling. They are important determinants of cellular processes controlling pathogenesis, progression, and response to treatment of several types of cancers including B-cell malignancies through translational repression and transcriptional degradation. As such, they can be taken as one of the important diagnostic and prognostic biomarkers available so far. However, integrating these biomarkers into clinical practice effectively and precisely in daily practice is challenging. Despite these challenges, there are many reasons to be optimistic that novel biomarkers will facilitate better algorithms and strategies as we enter a new era of precision medicine to better refine diagnosis, prognostication, and rational treatment design for patients with lymphomas.

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Abbreviations

***ABC**: Activated B-cell*

***AID**: Activation-Induced Cytidine Deaminase*

***BCNHL**: B-Cell Non-Hodgkin Lymphomas*

***BCL**: B-Cell lymphoma; BCR: B-Cell Receptor*

***BL**: Burkitt's lymphoma*

***CCND**: Cyclin D1*

***CSR**: Class Switch Recombination*

***DLBCL**: Diffuse Large B-Cell Lymphoma*

***DNA**: Deoxyribonucleic Acid*

***EFS**: Event free survival*

***FL**: Follicular Lymphoma*

***GC**: Germinal Center*

***HL**: Hodgkin's Lymphoma*

***IgH**: Immunoglobulin Heavy Chain Genes*

***LP**: Lymphocyte-Predominant*

***MCL**: Mantle Cell Lymphoma*

***miRNA**: microRNA*

***miRISC**: miRNA-associated RNA-Induced Silencing Complex*

***MYC**: Myelocytomatosis*

***NMZL**: Marginal Zone Lymphomas*

***NHL**: Non-Hodgkin Lymphoma*

***OS**: Overall survival*

***RNA**: Ribonucleic Acid*

***RAG**: Recombination Activating Gene*

***SHM**: Somatic Hypermutation*

***V(D)J**: Variable, diversity and joining*

***WHO**: World Health Organization*

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Authors' contributions

ZG and MM drafted the manuscript. ZG, FA and MM participated in the design of the study. ZG conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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In this issue: Non-coding RNAs as potential laboratory biomarkers

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Cell-free nucleic acids in prenatal diagnosis and pregnancy-associated diseases

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ABSTRACT

There is a great effort to find out the biological role of cell-free nucleic acids (cfNAs). They are considered very promising targets in the diagnosis of genetic diseases. Non-invasive sampling (liquid biopsy) has recently become a very popular method, and new molecular biological techniques have been developed for these types of samples. Application of next-generation sequencing (NGS) and massively parallel sequencing (MPS) is spreading fast. These are the part of the arsenal of the modern prenatal genetic diagnostic laboratories by now. Cell-free DNA based non-invasive prenatal testing accounts for more than half of the prenatal genetic tests performed, it is gradually replacing the invasive amniocentesis or chorionic villus sample-based diagnostics. Besides that, new non-coding RNAs are taking more attention: microRNAs (miRNAs), long non-coding RNAs (lncRNAs), circular RNAs (circRNAs) are in the focus of the clinical research to detect the most common pregnancy-associated diseases, like preeclampsia, fetal growth restriction, congenital heart diseases and gestational diabetes. The research is at advanced stage on the

use of microRNAs, while lncRNAs and circRNAs are still promising targets. In this review, comprehensive information is given about the recent developments on this field.

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INTRODUCTION

There is a great effort to determine the biological role and the clinical applicability of the cell-free nucleic acids. These molecules could be DNA, mtDNA, mRNA, miRNA, lncRNA, circRNA and other nucleic acids. They are present in different body fluids and offer the possibility to use them in diagnosis of different diseases. Liquid biopsy became very popular sampling method recently. The first non-invasive method in prenatal diagnosis was introduced by Dennis Lo from the University of Hong Kong, he was able to detect the fetal sex and RhD blood group in 1997 (1). He introduced the real-time PCR technique at that time for that purpose. Researchers tried to detect genetic diseases from maternal plasma applying similar technique (trisomies), but they were not very successful. There was a real breakthrough in 2011, when the first report was published on determination of trisomy 21 using massively parallel sequencing (MPS) (2). Newer genetic diseases were then detected prenatally. The results of a special interesting prenatal case called the attention for the possibility of diagnosis of oncological diseases by this technique. The next-generation sequencing (NGS) reading pattern warned for the mother's hemato-oncological disease, which was not diagnosed yet (3). This observation opened the door for new clinical applications of MPS in the field of oncology, and later for cardiovascular diseases, neurological diseases, infectious diseases, etc. The application of cfDNA is already

Table 1 The type of cell-free nucleic acids molecules with present and possible prenatal diagnostic application

Full name	Abbreviation	Size	Function	Prenatal application
Genomic DNA	gDNA	166 - >10,000 bp	unknown	trisomy, mutation, deletion, microdeletion
Mitochondrial DNA	mtDNA	20-100 bp; <1 – 21 kbp	unknown	preterm prelabour rupture
Messenger RNA	mRNA	varies	coding	not used
MicroRNA	miRNA	18-25 bp	regulation	preeclampsia, congenital heart diseases, gestational diabetes
Circular RNA	circRNA	varies	regulation	congenital heart diseases, gestational diabetes
Long non-coding RNA	lncRNA	over 200 bp	regulation	congenital heart diseases

a success story in prenatal diagnosis of genetic diseases, while there are other non-age-related pregnancy-associated diseases, which cause high maternal and fetal mortality and morbidity. It seems that non-coding RNAs could help to solve these problems. Table 1 shows those cell-free nucleic acids that have prenatal diagnostic potential.

CELL-FREE FETAL DNA (cffDNA)

The presence of cell-free DNA (cfDNA) was observed by Mandel and Metais in the sera of cancer patients in 1948 (4). Tan *et al.* reported a higher concentration of cfDNA in samples of cancer patients in 1966 (5). Later the first clinical application was introduced by Leon *et al.*, but did not get larger attention because of technical reasons (6). They measured the concentrations of cfDNA in blood samples from oncological patients. The real clinical application started when Dennis Lo detected the fetal gender and RhD group in the maternal plasma in 1997 (1). The maternal blood contains cfDNA molecules which originate in 90-95% from the mother and in 5-10% from the fetus. Introduction of the massively parallel sequencing made wider prenatal clinical application possible from 2011 (2). The number of the performed non-invasive prenatal tests is growing steadily, at least 50% of the genetic tests are made by this method nowadays. They have a very high sensitivity and specificity. There are several reports showing high number of non-invasive tests performed in different countries (7,8). It is possible to detect trisomies, mutations, deletions, etc. with NGS.

However, there are a few drawbacks in non-invasive prenatal testing (NIPT). Low fetal DNA content in those patients who are having high body mass index (BMI), or in the case of early pregnancy, these could cause false negative results. Placental mosaicism, vanishing twin could cause false negative or false positive results. These factors should be considered during the evaluation of the NIPT results.

CELL-FREE FETAL MITOCHONDRIAL DNA (cffmtDNA)

Higher level of nuclear DNA (nDNA) was observed in several pregnancy-related complications like in preeclampsia, fetal growth restriction and preterm delivery. Little is known about cfmtDNA in these syndromes. Recently, Kacerovsky *et al*. studied the nDNA and mtDNA levels in amniotic fluid samples obtained from preterm prelabor rupture of membrane cases (9). They observed higher levels of these DNA molecules in these cases. They suppose these are connected to the intra-amniotic inflammatory response. There are only a few studies related to the mtDNA and prenatal diagnosis of diseases.

CELL-FREE RNA (cfRNA)

mRNA

According to the latest results, there are about 23,000 genes in the human genome, which encode several types of RNAs, including mRNAs. Different tissues have their own mRNA profile. They are present in the serum and in other biological fluids and could be measured. During the pregnancy, placental markers are detectable in the maternal circulation. A paper was published on the application of single nucleotide polymorphism (SNP) for the detection of trisomies using allelic ratio of specific heterozygous SNP on cffmRNA. There are altered ratios in the case of trisomies, 1:1 shows diallelic, while 1:2 or 2:1 shows trisomic sample (10). Somehow mRNAs are not used in prenatal diagnosis of genetic diseases as yet.

miRNAs

miRNAs are short ribonucleic acid molecules with the size of 18-25 bp. They belong to the non- coding RNAs, produced from longer precursors. They

play a pivotal role in gene regulation. miRNAs are present in different biological fluids (plasma, liquor, saliva, seminal fluid, etc.) and they are considered as ideal molecules from the laboratory point of view: they are stable following freezing and thawing cycles, and it does not have an effect on their quality and concentration. Their encapsulation into extracellular vesicles during apoptosis and necrosis allows these stabilized miRNAs to reach any part of the body. The other possible way for their transport, formation of macromolecule complexes with Argonaute2 (Ago2), LDL and HDL (11).

Genomic studies identified several hundreds of miRNAs in the placenta (12), some of them expressed only in that tissue, while some in other tissues. Their role and function are not well known, probably they take part in the regulation of placentation (13).

Most key molecules from the biogenesis of miRNAs are detectable in the placenta (14, 15, 16). Placenta specific miRNAs appeared in the latest time of the evolution and they are present only in mammals (17). They are expressed differently in the certain parts of the placenta and secreted from the trophoblast layer in different concentrations during the periods of pregnancy (18, 19). This concentration depends on the signal transduction cascades and environmental factors (hypoxia, oxidative stress, etc.) (13).

There are placenta-specific miRNAs that are not expressed in other tissues, these are located on chromosome 14 and 19 in clusters (C14MC, C19MC and miR-371-3). The C14MC includes 34 mature miRNAs and these are evolutionarily conserved in mammals having placenta (20). The C19MC has 46 different spin-like structure miRNAs and from these 59 mature miRNAs are formed, this is the biggest known cluster in placental mammals (21). Both clusters are imprinted, while they show altered expression during the pregnancy. The C14MC miRNAs expressed from the maternal allele and their level is the highest in the first trimester and it decreases later (21). The C19MC is the opposite, the paternal allele is active (22) and the expression is increasing during the pregnancy (21). It is detectable even in the maternal circulation (23, 24). Less is known about the miR-371-3 cluster, which is located also on chromosome 19 (25).

They are expressed in the placenta and in embryonic stem cells (26). There is a great effort to find out the biological function of miRNAs and their diagnostic applicability in clinical practice. They could be classified as placenta specific, placenta-associated and placenta-derived miRNAs (27).

There are pregnancy-related complication and an intensive research performed to find out the utility of miRNAs in the diagnosis of preeclampsia, congenital heart diseases, gestational diabetes and fetal growth restriction.

PREECLAMPSIA

Preeclampsia is a serious pregnancy-associated disease and occurs in about 3-5% of the pregnancies. This is the main cause of maternal, neonatal morbidity and mortality. There is no reliable biomarker for the prediction of the development of this disease. There are numerous publications on the determination of miRNA expression in preeclampsia, even from the Central-Eastern European region several groups performed active research in this field (28). There is an agreement that placental dysfunction is the main cause of the development of this disease, while the pathogenesis is not clearly understood yet. Genetic predisposition, immune factors, and inflammation related causes are well studied. A number of research groups reported abnormal expression of miRNAs in the pathophysiological process of the disease (29, 30). These are involved in metabolic changes, immune function, cell adhesion, cardiovascular development, etc.

miR-210 is widely studied in preeclampsia as it is proved to be induced by hypoxia. It is upregulated in various tumors and cardiovascular diseases, and similarly in pregnancies with preeclampsia. miR-155 is upregulated along with transcription factor 1 and NF-κB protein, it may also inhibit trophoblast proliferation and invasion (30).

Skallis *et al.* published a review recently on miRNAs in preeclampsia. They divided their effect according to play a role in impaired trophoblast migration and invasion (miR-195, miR-276C, miR-278a-5p, miR-210), impaired angiogenesis (miR-210, miR-21, miR-22) and dysregulation of maternal immune system (miR-223, miR-148a, miR-152) (31).

CONGENITAL HEART DISEASES (CHD)

This is the most common congenital malformation with an incidence of 4-5% in the general population (32). The exact etiology of this disease group is not known yet. CHD causes a serious health issue accounting for 30–50% of mortality among newborns and infants (33). Unfortunately, the misdiagnosis is very high besides the use of fetal ultrasound echocardiography, the diagnostic efficiency is about 6-35% (34). There are other not very specific biomarkers in the clinical practice, like acylated ghrelin, beta human chorionic gonadotropin and pregnancy-associated plasma protein A (PAPP-A). Early prenatal diagnosis of CHDs may reduce postnatal morbidity and mortality (35-39). Zhu et al. performed a SOLiD sequencing for comparison of miRNA profile from women having a fetus with CHD and healthy pregnant women. These were ventricular septal defect, atrial septal defect, or teratology of Fallot cases. They found miR-19b, miR-22, miR-29c, and miR-375 significantly upregulated in the patient group (40). Our research group found elevated miR-99a level as a possible biomarker for the detection of CHD by analyzing maternal plasma samples (41). The miR-99a/let7c miRNA cluster is located in the chromosome region 21q21.1 and has been shown to control cardiomyogenesis in embryonic stem cells (42). We analyzed also let-7c expression in the maternal circulation and found that similarly to miR-99a, it is also overexpressed in cases of fetal cardiac malformations (43). CHDs are the most common cause of birth defects; however, present prenatal screening methods are not able to detect high-risk cases effectively. MiRNA studies in the maternal circulation could improve the efficacy of diagnosis and give new opportunities for CHD research and diagnosis.

GESTATIONAL DIABETES (GDM)

Another serious pregnancy-related complication is gestational diabetes (GDM). Early and effective diagnosis of the disease is an urgent need. About 7% of pregnancies effected by GDM and the number of cases growing year by year (44). Even some calculations predict 25% prevalence in the USA (45, 46). The screening of GDM is performed between the 24th-28th gestational weeks all over the world. Naturally it means late diagnoses, so the treatments usually do not start before the end of the first trimester. Screening strategy involving earlier detection could help in the proper diagnosis and treatment of the GDM.

Irregular expression of circulating miRNAs has been associated with GDM. They could serve as potential early biomarkers. The miR-518d was the first recognized miRNA showing altered expression in GDM, it belongs to the C19MC cluster (47). It seems that this miRNA regulates peroxisome proliferator-activated receptor α (PPARα) gene. Microarray analysis performed on GDM and non-GDM placentas showed dysregulation of miR-508-3p, miR-27a, miR-9, miR-137, miR-92a, miR-33a, miR-30d, miR-362-5p and miR-502-5p. Interestingly these miRNAs

target genes involved in the epidermal growth factor receptor (EGFR) signaling, which could cause e.g. macrosomia (48). Wander et al. reported recently an altered expression of 10 microRNAs, including miR-155-5p and miR21-3p, which showed higher plasma levels in GDM (49).

More extensive research is needed on microRNAs to introduce them as biomarkers in the early GDM diagnosis.

Circular RNAs (circRNAs)

Circular RNAs are special non-coding RNAs with evolutionary conservation, structural stability, and tissue specificity. They act like miRNA sponges and regulate the expression of different genes (50). CircRNAs are present in the placenta and could be involved in pregnancy related pathological processes (51). They have role in the development of tumors and other diseases (52, 53).

A recently published study measured the level of three lncRNAs in GDM and combined these with the expression of 99 miRNAs, however, circ_5824, circ_3636 and circ_0395 levels were significantly lower in GDM (54). CircRNAs could be other interesting molecules for functional studies in GDM.

Long non-coding RNAs (lncRNAs)

LncRNAs are a kind of non-translating RNA having the length of over 200 nucleotides. They are stable in plasma and other biological fluids; they show disease and tissue specificity. There are more than 1,000 lncRNAs which are involved in different biological processes (55). Recent studies call attention for their potential role as biomarkers or prognostic markers.

LncRNAs have a role in the development of the heart and CHD related lncRNAs could be detected in placental tissues and even in the maternal circulation (31). Gu *et al.* performed a study on 62 CHD patients and 62 healthy controls by using microarray and determined 3694 up-regulated and 3919 down-regulated genes. They validated the CHD-associated lncRNAs and found *ENST00000436681*, *ENST00000422826*, *AA584040*, *AA706223* and *BX478947* suitable to use as biomarker (31). There is intensive research to find out the role and clinical applicability of lncRNAs.

CONCLUSION AND FUTURE PERSPECTIVES

Cell-free nucleic acids have a special role in the normal physiological processes and in the development of diseases. CfDNA was the first clinically applicable non-invasively obtained sample type which is already widely used in the prenatal detection of genetic diseases using NGS. The application of different cfRNAs are in experimental phase now, with research groups performing studies to find out their role and clinical utility, like miRNAs, lncRNAs and circRNAs. Cell free miRNAs have a potential diagnostic, prognostic and therapeutic applicability.

They are expressed in all cell types and changes in their expression patterns could call the attention for pathological conditions. We know less about lncRNAs and circRNAs they have a potential for clinical use in the next couple of years. Several pregnancy-associated diseases are in the focus of the research, like preeclampsia, gestational diabetes and congenital heart diseases. Epigenetic changes causing fetal-maternal complications is not well known, additional studies are necessary to provide insight into the molecular pathological mechanisms. There is a critical issue related to the lack of standardized protocols on sample processing, expression profiling, and data analysis.

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In this issue: Non-coding RNAs as potential laboratory biomarkers

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Exosomal long non-coding RNAs as biomarkers in human diseases

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ABSTRACT

The intensive study of extracellular vesicles was started about a decade ago revealing alterations of their amount and content to several cellular stimuli, highly depending on the releasing cell type. Exosomes, a type of extracellular vesicles, are released by every cell type and are present in most body fluids, what makes them attractive targets of biomarker research. Several studies have indicated that their content – including proteins and coding, as well as non-coding nucleic acids – could represent the disease state and serves as specific disease biomarkers. Out of these molecules, a special interest was gained by long non-coding RNAs (lncRNAs). Just as exosomes, lncRNAs are specific to their cell of origin and often specific to diseases, also found extracellularly, mainly contained in extracellular vesicles. Thus, recent efforts in biomarker research has turned to circulating exosomal lncRNAs, which might lead to the development of highly specific disease markers.

Here we summarize the current knowledge on disease-associated exosomal long non-coding RNAs. The

intensive studies in this area have revealed numerous potential targets for biomarkers, and highlighted the potential of their combination with other exosomal markers to represent a highly sensitive and specific diagnostic tool. However, we believe that additional functional data on both exosomes and lncRNAs are necessary for understanding their deregulation in diseases and developing their use as diagnostic approaches.

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INTRODUCTION

The detection of soluble biomarkers from biological fluids — referred to as liquid biopsy — is a method that is more and more frequently applied for the early diagnosis of several diseases. It is believed that altered levels or the appearance of several circulating molecules potentially are indications of disease and can be detected at early stages for which other — often invasive — diagnostic tools are ineffective. Since extracellular vesicles are present in body fluids and their content depends on the cell of origin, circulating extracellular vesicles could potentially serve as early diagnostic markers for malignant diseases (1).

Extracellular vesicles are omnipresent in human tissues and many types of biological fluids, including blood, breast milk, urine, sperm, amniotic fluid, saliva, bronchoalveolar lavage, cerebrospinal fluid, synovial fluid, pleura effusions and ascites (2). Initially, extracellular vesicles were considered a mechanism by which cells rid themselves of cytoplasm and membrane proteins (3); however, not long after their discovery, they were found to be truly functional and actively released from cells for a variety of reasons. Extracellular vesicles are generally classified by their size, morphology and biochemical composition as well as their biogenesis as apoptotic bodies, microvesicles and extracellular vesicles (2). Apoptotic bodies are the largest — with diameters of up to 5000 nm — and are released during apoptosis by direct budding of the membrane. These vesicles contain nuclear content, cell organelles, DNA, ribosomal RNA and messenger RNA (mRNA). Microvesicles or microparticles are in the mid-range of extracellular vesicles with diameters of 100–1000 nm and are formed by direct shedding of the plasma membrane through outward invaginations. Microvesicles contain plasma membrane and cytosolic proteins as well as nucleic acids. Exosomes are small membrane nanovesicles released from various cells (B and T cells, dendritic cells, mast cells, mesenchymal stem cells, epithelial cells, astrocytes, endothelial cells and cancer cells) into the extracellular environment. Exosomes are the smallest extracellular vesicles, with diameters of 30–150 nm, and have a lipid bilayer containing various proteins, coding and non-coding RNAs and bioactive lipids, depending on their cell of origin. Exosomes are formed by inward budding of endosomal membranes that produce multivesicular bodies in which intraluminal vesicles develop. Intraluminal vesicles are subsequently secreted into the extracellular space as exosomes when multivesicular bodies fuse with plasma membrane (2). Exosomes have a plethora of biological functions. In addition to mediating cell-to-cell communication, signal transduction and transport of genetic material, exosomes play important roles in immune modulation and are involved in the pathogenesis of various human diseases, such as cancer and autoimmune inflammatory diseases (4-6).

During infection, exosomes carry pathogen-derived proteins, nucleic acids, lipids and carbohydrates and, thus, serve as antigen presenters, activating innate immune receptors to induce host defense (2). Exosomes shuttle both coding and non-coding RNAs, which maintain their function when transferred to recipient cells. This epigenetic signaling has an important role in cell-to-cell communication (7).

Long non-coding RNAs (lncRNAs) are longer than 200 nucleotide and lack protein-coding potential. These transcripts are involved in many cellular processes, including the regulation of chromatin modification, gene transcription, mRNA translation and protein function. The expression of lncRNAs is generally tissue- or cell-type specific and malignant cells have been shown to have a specific lncRNA signature (8). Since these molecules have a role in the pathogenesis of many diseases, they might be used as biomarkers for detection of disease at early stages (6,9); however, the biological functions of circulating lncRNAs and the mechanisms regulating the levels of circulating lncRNAs still need to be evaluated. Numerous studies have confirmed that lncRNAs play crucial roles in various multifactorial human diseases, such as cancer and neurological diseases, and that they also have an impact in the differentiation and activation of immune cells. These results together suggest that lncRNAs contribute to the pathogenesis of human inflammatory diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and psoriasis (10).

LncRNAs have been found to be enriched in exosomes compared to the cell of origin (11, 12). Of the circulating extracelluar vesicles, exosomes are the richest reservoirs for almost all lncRNAs (13). When using lncRNAs as biomarkers, the circulating exosome fraction is more useful than whole body fluid, as exosomal lncRNAs are protected against RNases, they are enriched in the exosome fraction compared to the whole-body fluid and their exosomal expression levels is dependent on the cells of origin (14). Several hypotheses have been proposed for the mechanism by which molecules are loaded into exosomes. Most probably, structural motifs in the lncRNAs interact with the proteins responsible for RNA localization and exosomal loading. As certain RNAs are enriched in exosomes, it is likely that these RNAs are actively loaded into extracellular vesicles (2,12).

Most studies that investigate exosomes as biomarkers have focused on cancerous diseases (15), and the study of exosomal lncRNAs as biomarkers is more advanced for cancer than any other disease. Numerous studies indicate that the expression pattern of circulating lncRNAs probably carries information about the size of the tumor or malignancy or other important characteristic of the disease, and, therefore, the presence of circulating and exosomal lncRNAs may reflect disease progression in particular cases. The use of circulating lncRNAs as biomarkers is also emerging for chronic inflammatory diseases, as many lncRNAs associated with these diseases are found in circulating exosomes. In this review, we summarize our knowledge about exosomal lncRNAs and their potential use as biomarkers for several human diseases, including chronic inflammatory and cancerous diseases.

EXOSOMAL lncRNAs IN CHRONIC INFLAMMATORY DISEASES

Rheumatoid arthritis

RA is a common chronic inflammatory autoimmune disease that is characterized by the infiltration of lymphocytes and macrophages into the synovial fluid, hyperplasia of the synovial membrane, degradation of cartilage and bone erosion (6,9). LncRNAs seem to be implicated in the development of the disease, as altered expression is associated with the severity and activity of the disease (for an extensive review see reference (16)). The first lncRNA that was associated with RA was the 2.3 kb H19 RNA, which exhibited significantly higher expression in the synovial tissue of RA patients compared to the tissue of healthy individuals (16,17). Since this discovery, numerous high-throughput analyses were completed to describe the lncRNA profile

of RA from cells as well as from serum. Xu et al. investigated the expression of lncRNAs in serum samples from RA patients and healthy donors and identified 5 lncRNAs that were expressed at significantly higher levels in RA samples than controls: RNA143598, RNA143596, HIX0032090, IGHCgamma1 and XLOC_002730 (18). Comparing the lncRNA expression in serum exosomes, Song et al. found significantly higher expression of HOTAIR, LUST, anti-NOS2A, MEG9, SHNG4, TUG1 and NEAT1 and significantly lower expression of mascRNA, PR antisense transcripts, PRINS, and HOXA3 expression in RA patients than in healthy individuals (19). These molecules are likely to serve as biomarkers for RA, and combined analysis of these molecules may be a robust method to determine disease prognosis. However, to date there is a lack of information about the sensitivity and specificity of these molecules in RA, therefore, until further studies elucidate these characteristics, the diagnostic application of exosomal lncRNAs is greatly limited.

Systemic lupus erythematosus

SLE is a chronic autoimmune disease, characterized by the production of multiple autoantibodies against nuclear auto-antigens and double-stranded DNA. Abnormal interaction between the innate and adaptive immune system and activation of the complement system leads to tissue or organ damage (10,16,20). Lupus nephritis (LN) is one of the most serious manifestations of SLE, and 10–30% of LN patients progress to end-stage renal disease. Renal biopsy is still the “gold standard” to predict renal outcome, and a non-invasive method to assess glomerular damage would be major improvement. Recent results suggest that exosome-derived markers, especially from urine samples, might be appropriate for such an assay (9,21,22). Circulating exosomes in the plasma of SLE patients are derived from platelets, endothelial cells and leukocytes and have clinical and serological correlations (22). Circulating exosomes are also involved in the pathogenesis of SLE, since serum exosomes isolated from SLE patients were able to induce cytokine production in peripheral blood mononuclear cells of healthy donors (23).

LncRNAs are strongly associated with susceptibility to SLE. The gene for the GAS5 lncRNA is located in the SLE-susceptibility locus of chromosome 1q25 and is closely linked to SLE susceptibility (10). The GAS5 lncRNA is also implicated in RA pathogenesis (24), and GAS5 levels are also downregulated in the serum of SLE patients (25). Moreover, serum GAS5 level could be a highly specific but not sensitive biomarker for SLE, and in combination with linc0597 lncRNA could be used as a highly sensitive (83.44%) and specific (93.75%) biomarker (25). Expression of another lncRNA, NEAT1, is increased in the peripheral blood cells of SLE patients, as it is in RA patients (19), and is positively correlated with disease activity (26). NEAT1 often colocalizes with MALAT1, which is also an abnormally upregulated lncRNA in both RA and SLE. MALAT-1 is a key factor in the pathogenesis of SLE, as it regulates the expression of IL-21 and SIRT1 in monocytes from SLE patients (16,27). These studies on lncRNAs in SLE focused on either circulating lncRNAs (25) or cellular lncRNA expression. The fact that the circulating and cellular lncRNAs in SLE are also present in the serum exosomes of RA patients (19) suggests that these lncRNAs might also be present in serum exosomes of SLE patients. Future studies on exosomal lncRNAs in SLE could provide the necessary information to include circulating exosomal lncRNAs as disease specific markers in SLE.

Psoriasis

Psoriasis is a chronic inflammatory skin disease, characterized by the abnormal proliferation and

differentiation of basal keratinocytes and their deregulated interplay with professional immune cells (28). Many non-coding RNAs, including microRNAs (miRNAs) (29) and lncRNAs (30), have been found to be deregulated in this disease and implicated in disease pathogenesis.

One of the first lncRNAs to be associated with psoriasis was described by our research group in 2005: psoriasis associated non-protein coding RNA induced by stress (PRINS). The PRINS lncRNA is upregulated in non-lesional skin samples of psoriatic patients (31) and functions as a regulator of cellular apoptotic functions by interacting with nucleophosmin (32) and miR-491-5p (33), and effecting G1P3 gene expression (34). PRINS also regulates inflammatory cytokine expression through interactions with their mRNA (35).

Another regulatory lncRNA expressed in the epidermis, TINCR, is localized to the cytoplasm of differentiated human keratinocytes and is able to stabilize mRNAs linked to differentiation (36), many of which are implicated in psoriasis pathogenesis (30).

Approximately 10–30% of psoriatic patients are also affected by psoriatic arthritis (PsA), which causes skin symptoms as well as joint erosion and new bone formation. The analysis of lncRNAs in blood samples from PsA patients indicate that lncRNAs are involved in disease pathogenesis (37) and that these molecules could be used as new biomarkers and possibly therapeutic targets for PsA.

Although lncRNA expression in psoriatic skin and immune cells was described by numerous research groups, circulating lncRNA levels and exosomal lncRNAs have not received much attention to date. Importantly, it has been suggested that circulating exosomes could serve as a tool for prognosis and for monitoring therapy efficiency (5).

EXOSOMAL lncRNAs AS BIOMARKERS IN CANCER

Early diagnosis of malignant neoplasms is important for successful treatment and survival of patients. Cancers have a high mortality rate due to the lack of suitable, specific and early detection of diagnostic tumor biomarkers. Tumor cells release exosomes, which facilitate communication within the local environment and primary tumor cells, supporting tumor-cell growth, tumor-associated angiogenesis and tissue inflammation in both autocrine and paracrine manners. Numerous studies show that cancer-derived exosomes activate signal-transduction pathways involved in cancer cell proliferation and survival (38,39). Exosomes regulate immune modulation, including immunosuppression that supports the growth of the tumor (4). Moreover, as exosomes are known to alter cellular functions, they have been intensively studied for their potential in metastasis formation, especially through the mechanism of epithelial-to-mesenchymal transition (40).

The peripheral blood of cancer patients contains significantly more exosomes than blood samples from healthy individuals (41), as tumorous cells release higher amounts of exosomes. Circulating exosomes support the dissemination of the tumor, are involved in the initial events of metastasis, and carry a unique molecular fingerprint from their cell of origin. The high number of circulating exosomes and their molecular content makes them ideal candidates for tumor biomarkers and for predicting the metastatic potential of a tumor in liquid biopsies (38,42,43). However, these biomarkers have not been integrated into clinical routines, as their isolation is expensive and time consuming (44).

Downregulation of tumor-suppressive miRNAs and upregulation of oncogenic miRNAs have been described for various human cancers.

Cancer-associated miRNAs regulate tumorigenesis, survival, angiogenesis, migration and invasion of tumors (45); moreover, clinical studies have correlated dysregulated expression of particular miRNAs with tumor responsiveness to chemotherapies (46).

Similarly to microRNAs, lncRNAs have been shown to play a fundamental role in cancer cell growth, proliferation, cell death, invasion and formation of metastasis (47); however, the majority of these genes have not yet been characterized functionally. Currently, many attempts focus on the use of lncRNAs as prognostic markers for cancer patients, as tumor cells can be characterized by their distinct lncRNA profile (48). These lncRNAs can be packaged within exosomes and, as such, offer great potential for use as markers for specific tumors (Table 1), especially as exosomal release rate is increased in cancer cells compared to healthy cells (41).

The use of the exosomal lncRNAs as cancer biomarkers is also supported by the high sensitivity (70% to 94%) (49–60) and specificity (72% to 94%) (49–60) of these markers. Moreover, the combination of either the lncRNA markers (58–60) or lncRNAs with miRNAs (61) or already used protein diagnostic markers (56,60) can increase both the sensitivity and specificity of these markers.

This possibility was tested in prostate cancer, where the level of prostate specific antigen (PSA) is used for screening for prostate cancer with high specificity (~93%) but low sensitivity (~20–25%) at a cutoff value of 4 ng/ml (62), however it can be also modestly elevated in benign prostatic hyperplasia. Wang *et al*. suggest, that exosomal lncRNA expression could help differentiation between prostate cancer and benign prostatic hyperplasia in cases, where PSA levels (4–10 ng/ml) alone have little diagnostic value (60).

EXOSOMAL lncRNAs IN OTHER HUMAN DISEASES

Bacterial and viral interaction with host cells

Extracellular vesicles are produced by both Gram-negative and Gram-positive bacteria (73) and have several functions, including molecular transport, mediation of stress response, biofilm formation and influence on hosts cells (2). Both normal human flora and pathogenic bacteria communicate with the host cells through extracellular vesicles, and infected host cells respond by releasing exosomes to alert surrounding cells (74). The extracellular vesicles released by infected cells contain both pathogen- and host-derived factors and play key roles in pathogen-host interactions, including pathogen uptake and replication and regulation of the host immune response (75). Viruses also modify the number and content of exosomes released by infected cells, which often contain virus-associated miRNAs, as in the case of Epstein-Barr and human immunodeficiency viruses, or parts of the viral genome (2).

Monoclonal gammopathies

Multiple myeloma is a heterogeneous disease with focal lesions in the bone marrow, and analysis of a biopsy specimen obtained from a single site in the bone marrow is not sufficient for the prediction of disease outcome. However, circulating molecules (DNA, miRNAs and lncRNAs) in peripheral blood could serve as potential diagnostic, prognostic and predictive markers. PRINS, for which exosomal expression correlates with characteristic chromosomal aberrations in the disease, is one such candidate molecule (76).

Neurodegenerative diseases

LncRNAs are expressed in the central nervous system, which allows the possibility that they play roles in normal neurological development

and growth and, possibly, in tumorigenesis. Circulating and cerebrospinal-fluid-derived exosomes could be used to detect biomarkers for neurodegenerative diseases and for tumors of the central nervous system; however the lncRNA content of such exosomes has not yet been thoroughly investigated (77).

Osteoarthritis

By analyzing the lncRNA profile of exosomes derived from plasma and synovial fluid, Zhao and Xu found that, whereas plasma-derived exosomal lncRNAs had no diagnostic value in the disease, PCGEM-1 was significantly upregulated in the synovial fluid of late-stage osteoarthritis patients.

They hypothesize that PCGEM-1 expression can be used to distinguish early osteoarthritis from the late-stage disease (78).

Chronic kidney disease

In urine-derived exosomes of patients suffering from chronic kidney disease 30 differentially expressed non-coding RNAs were identified as suitable biomarkers for early diagnosis, of which the most powerful disease marker is miRNA-181a, while lncRNAs were found to be less than 1% of all deregulated RNA molecules in the disease (79).

DISCUSSION

Liquid biopsies represent a non-invasive and painless method for monitoring health and disease states of individuals. Protein and RNA content of extracellular vesicles, which are present in all body fluids, is receiving increasing levels of attention for their potential as biomarkers. Although it seems that expression levels of many lncRNAs within exosomes are sufficient to serve as disease markers that can potentially be used in diagnosis or prognostic tools for human diseases, most research studying lncRNAs in body fluids has not determined whether the transcripts are freely circulating in the body fluid or are contained in exosomes. Moreover, most reports about exosomal lncRNAs show that their expression is deregulated in several diseases (Table 1), indicating that these molecules might not be sufficiently disease specific to be used as biomarkers (19,24–26).

To overcome this issue, some studies used a combined analysis of exosomal lncRNAs, miRNAs and proteins as biomarkers increasing the sensitivity and specificity of the diagnostic test (56,58–61). Studies using such combined analysis are also helpful for building interaction networks and databases of exosome-derived molecules and support the functional study of these molecules necessary to understand their role in

Table 1 Exosomal lncRNAs as potential biomarkers in cancer

Exosomal lncRNA	Cancer type	Reported findings
CRNDE-h	Colorectal cancer	High levels correlate with poor prognosis (49)
ENSG00000258332.1	Hepatocellular carcinoma	Higher levels in serum exosomes compared to liver cirrhosis and chronic hepatitis B (50)
H19	Bladder cancer	High levels in serum exosomes associated with poor disease prognosis (51)

HOTAIR	Bladder cancer	High levels in urine-derived exosomes (63)
	Cervical cancer	High levels (64)
	Glioblastoma multiforme	High levels in serum and exosomes (52)
	Laryngeal squamous cell carcinoma	High levels in exosomes (61)
HOTTIP	Gastric cancer	Expression in exosomes is an independent prognostic factor (54)
HOX-AS-2	Bladder cancer	High levels in urine-derived exosomes (63)
LINC00161	Hepatocellular carcinoma	High levels in serum-derived exosomes (55)
LINC00635	Hepatocellular carcinoma	Higher levels in serum exosomes than observed for liver cirrhosis and chronic hepatitis B (50)
lncRNA 91H	Colorectal cancer	Early biomarker for colorectal recurrence or metastasis (65)
lncRNA-ATB	Hepatocellular carcinoma	Independent predictor of mortality and disease progression in combination with miRNA-21 expression (66)
lncRNA-HEIH	Hepatocellular carcinoma	High levels in serum and serum exosomes (67)
lncRNA-p21	Prostate cancer	Different levels in benign prostate hyperplasia and prostate cancer (56)
lncRNASNHG14	Breast cancer	High levels in patients resistant to trastuzumab (57)
lncUEGC1/2	Gastric cancer	Highly sensitive and stable biomarker (68)
MALAT1	Bladder cancer	High levels in urine-derived exosomes (63) associated with poor prognosis (58)
	Cervical cancer	High levels (64)
	Epithelial ovarian cancer	Correlated with an advanced and metastatic phenotype and independent predictive factor for overall survival (69)
	Non-small cell lung cancer	High levels (70)

MEG-3	Cervical cancer	Low levels (64)
PCAT-1	Bladder cancer	Expression in urine-derived exosomes associated with poor disease prognosis (58); serum exosomal expression used as biomarker (59)
SAP30L-AS1	Prostate cancer	Used in combination with SChLAP1 to differentiate between benign prostatic hyperplasia and prostate cancer (60)
SChLAP1	Prostate cancer	Used in combination with SAP30L-AS1 to differentiate between benign prostatic hyperplasia and prostate cancer (60)
SNHG16	Bladder cancer	High level in serum exosomes is a diagnostic marker (59)
SPINT1-AS1	Colorectal cancer	High level associated with poor prognosis (71)
SPRY4-IT1	Bladder cancer	Presence in urine-derived exosomes associated with poor disease prognosis (58)
UBC1	Bladder cancer	High level in serum exosomes is a diagnostic marker (59)
ZFAS1	Gastric cancer	High serum-exosomal level (72)

disease pathogenesis and promote their use as biomarkers.

The intensive study of both lncRNAs and exosomes only started a decade ago, thus there are plenty of open questions. Most studies on both fields are still descriptive, comparing expression levels of lncRNAs or number and contents of exosomes in diseased tissues, cells or liquid biopsies to healthy samples. But there is still a lack of knowledge on the function of both exosomes and lncRNAs in both healthy and diseased states. It is also debatable whether the identified differences are specific to one disease or general features of related diseases. The expression of the same lncRNAs in several inflammatory diseases (19,24–26) suggest that their expression is rather specific to chronic inflammation than the disease itself. There are plenty of open questions in this topic: What are the target cells of the circulating exosomes and how their cargo – especially their lncRNA content – alters the function of target cells? Are the targeted cells specific to the disease and have a function in the disease course? Although there are some evidences that the amount of exosomes and their content changes with therapy (46,57), whether their characteristics return to healthy state is unknown. Nevertheless, the lack of consensus on exosome isolation is one of the biggest issue to overcome (44,80–84). Most methods used so far were shown to have high laboratory-to-laboratory and method-to-method differences in the amount and quality of the isolated extracellular RNA (80), which is the biggest barrier before their implementation as routine biomarkers.

Taken these limitations into account we believe that the already described disease specific expression of one or more lncRNAs in exosomes should be the starting point to their functional

study, to provide the necessary information for future implications in detecting, monitoring and treating disease.

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In this issue: Non-coding RNAs as potential laboratory biomarkers

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Dysregulated expression profile of myomiRs in the skeletal muscle of patients with polymyositis

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ABSTRACT

MicroRNA (miRNA) research has intensively developed over the past decade. Characterization of dysregulated miRNA expression profiles could give a better understanding of the development of pathological conditions and clinical disorders, such as autoimmune diseases with polygenic etiology, including idiopathic inflammatory myopathies (IIMs). IIMs are a group of rare autoimmune disorders characterized by skeletal weakness and inflammation. Polymyositis (PM) is one of the conditions of autoimmune myopathies with proximal skeletal muscle weakness. A novel group of miRNAs, known as myomiRs are described as striated muscle-specific or muscle-enriched miRNAs. They are involved in myoblast proliferation/differentiation as well as muscle regeneration. To determine the role of myomiRs in the development and progression of PM, we performed an initial skeletal muscle miRNA profiling using microarray technique at diagnosis. The aim of the study was to examine myomiRs expression profile in patients with PM in order to remark the association between the dysregulated

myomiRs' expression and the development of the disease. As a results of microarray investigation, most of the myomiRs showed altered expression patterns in the muscle samples of PM patients compared to controls. These results suggest that myomiRs, especially miR-1, miR-133a, miR-208b, miR-486, and miR-499 function in a network, and are associated with the development of PM.

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INTRODUCTION

MicroRNAs (miRNAs) are endogenous non-coding RNAs, playing critical roles in regulating gene expression, and they are important in a wide range of physiological processes such as cell development, differentiation and function at transcription, post-transcription and translation level (1) (2). MicroRNA research has intensively developed over the past decade. The miRNA database, called miRBase (www.mirbase.org) provides increasing number of newly identified miRNAs with 1917 human miRNAs according to its current version. Some miRNAs are ubiquitously expressed in tissue, while others are tissue-specific or tissue-enriched. Alterations in the expression of miRNAs provide valuable information on the development of pathological conditions and clinical disorders. Changes in miRNA expression profiles have been identified in different autoimmune diseases such as multiple sclerosis (MS) (3), systemic lupus erythematosus (SLE) (4), rheumatoid arthritis (RA) (5) (6), Sjögren-syndrome (pSS) (7) (8). A recently published work, that examines the whole expression profile of miRNAs in both SLE and pSS instead of only certain miRNA (9). A certain miRNA may have hundreds of different mRNA targets and a target might be regulated by multiple miRNAs. Thus, characterization of dysregulated miRNA expression profiles could give a better understanding of the development of immunological disturbances in autoimmune diseases with polygenic etiology, including idiopathic inflammatory myopathies (IIMs). IIMs are a group of rare autoimmune disorders characterized by skeletal muscle weakness and inflammation (10) (11). PM is one of the five conditions of autoimmune myopathies predominantly with proximal skeletal muscle weakness. Not much was known about the pathogenesis of this condition. MiRNAs represent a new and potentially exciting pathway to the future research into idiopathic inflammatory myopathies as well. Identification of dysregulated miRNAs has led to a greater understanding of inflammation, muscle weakness/wasting and extra-muscular organ involvement in IIMs (12) (13) (14). For instance, five miRNAs, miR-146b, miR-221, miR-155, miR-214 and miR-222, have been found to be usually over-expressed across 10 primary muscle disorders including IIMs (12) (15). Up-regulation of immune-related miRNAs in muscle, for example, miR-155 and miR-146b, is closely related to autoimmunity (12). However, down-regulation of miRNAs such as miR-1 and miR-206 is associated with inhibition of muscle regeneration (16) (17). MiR-1 and miR-206 are members of a novel group of miRNAs, known as myomiRs (14) (18). MyomiRs are described as striated muscle-specific or muscle-enriched miRNAs (19). The group of myomiRs include eight miRNAs: miR-1, miR-133a/b, miR-206, miR-208a/b, miR-486, and miR-499 (Table 1). MyomiRs are expressed in both cardiac and skeletal muscle with the exception of miR-206, which is skeletal muscle-specific, and miR-208a, which is cardiac muscle-specific (14) (Table 1). Some studies have proved that not all myomiRs are exclusively expressed in a muscle-specific manner but may be detected in low levels in other tissues (20) (21). However, myomiRs main function is confined to muscle. MiR-486 is sometimes considered muscle-enriched rather than muscle-specific as it is also expressed in other tissues. Skeletal muscle development is a complex process requiring

coordination of multiple factors, which control the proliferation of myoblast, their exit from the cell cycle and subsequent differentiation into multinucleated myotubes (14). MyomiRs affluence is regulated by myogenic regulatory factors in a negative feedback loop, through influencing many aspects of myogenesis. For example, miR-1 stimulates differentiation of myoblast, miR-1 and miR-206 promotes myogenic differentiation (19). Their network has a main role in the regulation of skeletal muscle plasticity by organizing changes in fiber type and muscle mass in response to altered contractile activity (Table 1) (19). They are involved in myoblast proliferation/differentiation, muscle regeneration, or fiber type specification. The tissue specificity of myomiRs is scheduled either for the genomic location of their coding DNA within introns of myosin heavy

Table 1 Group of skeletal muscle myomiRs: locations, tissue specificity, summary the function of myomiRs, and their host genes*

MyomiRNAs	Location on chromosome	Genome context	Tissue specificity	Function	Host gene
miR-1	20q13.33 (miR-1-1) 18q11.2 (miR-1-2)	intragenic	heart/skeletal muscle	Stimulation of myoblast differentiation, regeneration, angiogenesis regulation	*MIB1*
miR-133a	18q11.2 (miR-133a-1)	intragenic	heart/skeletal muscle	Promotion of myoblast proliferation, differentiation, fusion, regeneration, muscle fiber shift	*MIB1*
miR-133b	6p12.2	intergenic	skeletal muscle	Promotion of myoblast differentiation and fusion, regeneration	intergenic
miR-206	6p12.2	intergenic	skeletal specific	Promotion of myoblast differentiation, regeneration, regeneration of neuromuscular synapses	intergenic

miR-208a	14q11.2	intragenic	heart	Muscle fiber shift, promotion of muscle growth	*MYH6* *MYH7*
miR-208b	14q11.2	intragenic	heart/skeletal specific	Muscle fiber shift, promotion of muscle growth	*MYH7*
miR-486	8p11.21	intragenic	heart/skeletal specific	Promotion of myoblast differentiation and fusion	*ANK1*
miR-499	20q11.22	intergenic	heart/skeletal specific	Muscle fiber shift, promotion of muscle growth	*MYH7B*

**Data are from M. Horak et al. (14).*

chain genes or for transcriptional factors such as MyoD, Mef2, or muscle-specific transcriptional factors such as MyoD, Mef2, or Srf (22).

To determine myomiRs role in the development and progression of PM, we performed skeletal muscle miRNA profiling using microarray technique in PM patients at diagnosis before treatment. Improved understanding of the role of miRNAs and their targets therefore could help to elucidate the pathogenesis of PM. Although, there are some reports about the changes of miRNA level in muscle of PM patients, microarray profiling of all muscle-related miRNAs, called myomiRs is not available in the literature.

The aim of our study was to examine myomiRs expression profile in patients with PM in order to remark the associations between the dysregulated myomiRs' expression and the development of the disease. Since the pathogenesis of PM characterized by symmetric muscle weakness, elevated serum creatine kinase levels, typical myopathic features, we paid a special attention on myomiR expression levels in PM patients compared to controls.

To our best knowledge, this is the first study to investigate all the eight myomiRs in the initial skeletal muscle of patients with PM.

PATIENTS AND CONTROL INDIVIDUALS

Out of five hundred and forty patients with IIM myositis patients, 4 patients were selected for this study at the Division of Clinical Immunology, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, Hungary (10) (23). They all were diagnosed with the clinicopathological subgroup PM and had a definitive diagnosis according to Bohan and Peter (11).

This means that they had progressive symmetric muscle weakness of the proximal upper and lower extremities and neck flexors; had no skin lesions pathognomic or characteristic for dermatomyositis (DM); had elevated serum creatine kinase levels; had typical myopathic features on electromyography; and had positive muscle biopsy features (endomysial infiltration of mononuclear cells surrounding, but not invading, myofibers). Muscle biopsies were taken from the weaker deltoid muscle or quadriceps femoris muscle, in local anaesthesia, by surgeon

specialists at the Surgical Department, Faculty of Medicine, University of Debrecen, Hungary. Muscle biopsy was taken in every single case before steroid or any other immunosuppressive therapy not to suppress inflammation. The biopsy-samples were frozen in liquid nitrogen until used for preparation. The average age of the patients was 60.75 years, while the female:male ratio was 3:1. No patient had any internal organ manifestations; their PM was localized only in muscle. The control group consisted of three age-matched (average age: 54.3 years, female:male ratio was 2:1) healthy female and male volunteers who underwent surgery for total hip replacement, and the muscle biopsy was taken and collected, and kept in liquid nitrogen then. No patients or controls enrolled in this study had ongoing infections, either viral or bacterial.

This study meets, and is in compliance with, all ethical standards of medicine. Informed consent was obtained from all of the subjects enrolled in the investigation, and the study has been approved by the Ethics Committee of our University and the Policy Administration Services of Public Health of the Government Office (protocol number: HBR/052/00766-2/2014.). This study is ethically compliant and was carried out in compliance with the Declaration of Helsinki.

METHODS

Sample handling

After taking the muscle biopsies of PM patients and healthy individuals samples obtained from each study subject were collected and kept in liquid nitrogen until used.

miRNA processing

MiRNAs were extracted from frozen biopsy samples, using the mirVana miRNA Isolation Kit (Ambion) in accordance with the manufacturer's instructions.

miRNA microarray and data analysis

Integrity of RNA samples was checked on Agilent BioAnalyzed using RNA Nano chips (Agilent Technologies), samples with > 7 RNA integrity number were used for the further experiments. To obtain global miRNA expression data Affymetrix miRNA 3.0 arrays (Affymetrix) were processed. Total RNA samples were labelled using Affymetrix FlashTag Biotin HSR RNA Labeling Kit according to the manufacturer's protocol. Briefly, 500 ng of total RNA samples were poly (A)-tailed using poly A polymerase enzyme and ATP at 37°C for 15 minutes, then biotinylated by ligating biotin-labeled fragment to the 3' end. Labeled samples were hybridized on miRNA 3.0 arrays at 48°C and on 60 rpm for 16 hours. After that, arrays were washed and stained by standard Affymetrix protocol using Affymetrix Hybridization, Wash and Stain Kit on FS 450 fluidic station instrument then the arrays were scanned on Affymetrix GeneChip Scanner 3000 7G instrument.

Quality of miRNA arrays were checked in miRNA QC Tool (Affymetrix) software and raw intensity values were exported with annotations as a text file. Further data analysis was performed in GeneSpring GX 12.0 software (Agilent Technologies). First, a custom technology was created based on the exported text file then data were normalized using quantile normalization algorithm. To determine differentially expressed miRNAs between diseased and healthy groups moderated T-test was executed and $p < 0.05$ was considered as statistically significant difference.

RESULTS

We carried out analysis to evaluate the expression patterns of miRNAs focusing on levels of myomiRs (miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486, and miR-499). For this purpose, initial skeletal muscle biopsies of 4

patients with active PM have been investigated and compared to muscle samples of healthy individuals. MiR-1, miR-133a, miR-208b, miR-486, and miR-499 were differentially expressed in PM skeletal muscle. All the myomiRs's expression were significantly altered in the muscle samples of PM patients compared to healthy controls (Table 2) ($P < 0.05$). In specific, miR-1, miR-499 and miR-208b were significantly down-expressed in the affected muscle of PM patients compared to controls. At the same time, miR-133a and miR-486 were significantly up-regulated in the same patient samples compared to control samples.

In contrary, miR-133b and miR-206 expression levels were the same in both groups. In PM patients both up-regulated myomiRs, namely miR-133a and miR-486 had less than 2-fold change in their expression compared to controls (Table 2). On the contrary, miR-1, miR-208b, and miR-499 were significantly down regulated in PM group (Table 2), miR-1 showed nearly 2-fold change, but miR-208b had over 2-fold change compared to controls. However, miR-499 expression exceeded 5-fold change in patients compared to controls (Table 2).

DISCUSSION

Recently, miRNAs have appeared as new elements in skeletal muscle myogenesis by participating in arranged gene regulation processes. They have essential role in skeletal muscle development. MicroRNAs which are exclusively or preferentially expressed in striated muscle are called myomiRs. The group currently includes eight miRNAs: mi-1, miR-133a, miR-133b, miR-206, miR-208a, miR-208b, miR-486, and miR-499 (Table 2). Some myomiRs are located on chromosomes in bicistronic clusters and are thus transcribed together; this includes miR-1-1/miR-133a-2, miR-1-2/miR-133a-1 and miR-206/miR-133b families placed in humans on chromosomal regions 20q13.33, 18q11.2 and 6p12.2, respectively (14). MiR-206/miR-133b is intergenic. Other myomiRs are monocistronic and situated in protein coding genes. MiR-208a, miR-208b and miR-499 are encoded within the

Table 2 Expression profile of myomiRs in muscle biopsies of patients with active PM*

miRNA	Fold change in expression	P-value	Regulation
miR-1	1.69	0.026	down
miR-133a	1.46	0.021	up
miR-133b	none	-	-
miR-208a	none	-	-
miR-208b	2.91	0.042	down
miR-486	1.8	0.036	up
miR-499	7.58	0.038	down

**Fold changes of myomiRs in PM patients compared to healthy individuals. MiR-1, miR-208b, and miR-499 were significantly down regulated, while miR-133a and miR-486 were significantly up regulated.*
$P < 0.05$ was considered as statistically significant difference.

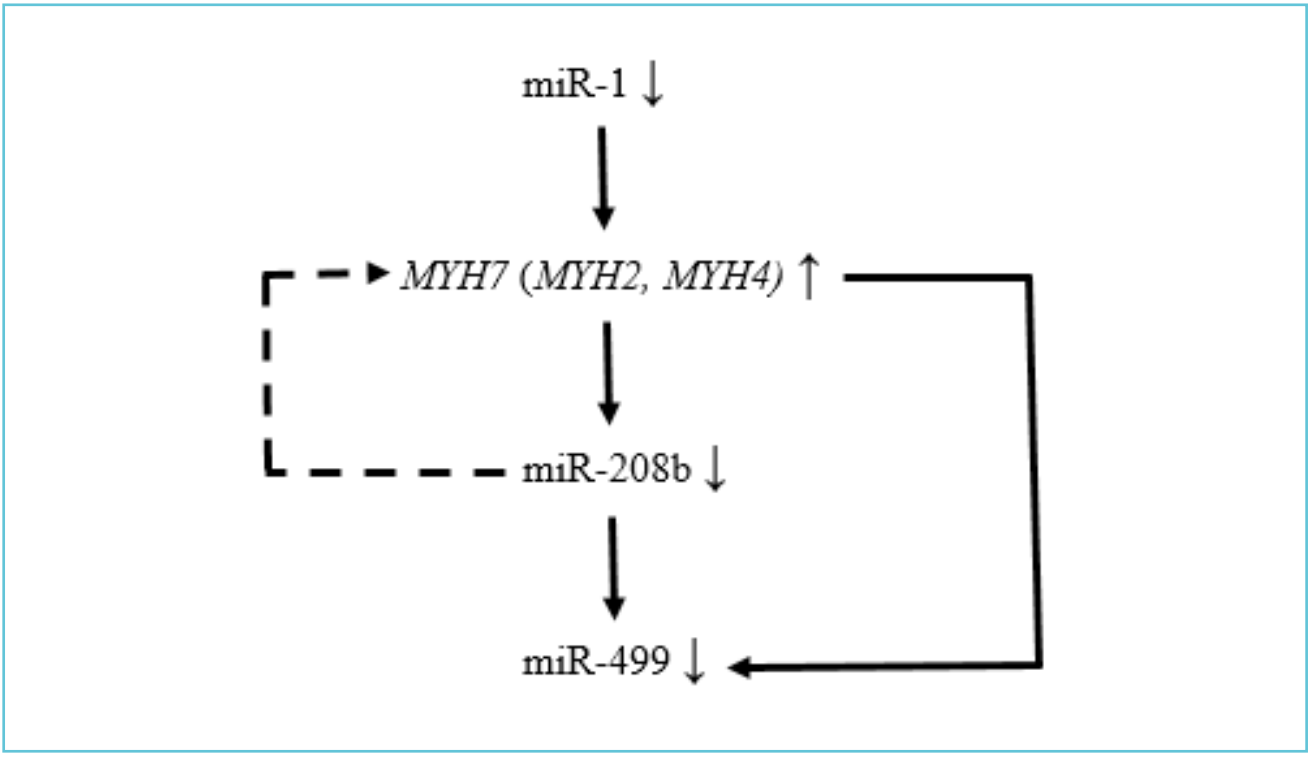
introns of the myosin heavy chain genes (*MYH6*, *MYH7*, and *MYH7B*). MiR-486 is encoded in the intronic region of the *ANK1* (14). MyomiRs arrange a subset of miRNAs participating in myogenesis as a network.

In our study, we revealed alterations in the expression patterns of myomiRs in patients with PM investigating initial muscle biopsies taken from the weaker deltoid or quadriceps femoris muscle. Analyzing myomiRs expression profiles including diseased muscle of patients and healthy skeletal muscle, we hypothesized to get better insights into the disease-specific changes. To determine differentially expressed miRNAs between diseased and healthy groups moderated T-test was executed and $p < 0.05$ was considered as statistically significant difference. In total, there were three myomiRs (miR-1, miR-208b, and miR-499) down regulated significantly, while miR-133a and miR-486 were up regulated significantly in the muscle biopsies of the PM patients investigated.

Based on work from our laboratory and results of published literature, we assume the hypothesis of a regulatory network including myomiRs and myosin heavy chain genes in PM. Based on our findings, dysregulated expression of myomiRs might be in association with the symptoms, the clinical conditions and the course of PM.

Reduced miR-1 expression is the starting point of our hypothesis. However, up to this point the reasons for why miR-1 expression level is reduced are unknown.

Figure 1 **Regulatory network of myomiRs and myosin heavy chain genes in PM***



**MiR-1 is a key element with reduced expression level in the regulation of myosin heavy chain genes' expressions (MYH7, MYH2 and MYH4) (24). MYH7 gene is over-expressed as well as the expression of MYH2 and MYH4 genes based on preliminary results. Expression level of myomiRs, which are located in intronic regions of MYH7 (19) and MYH7B has been changed; more precisely, expression level of miR-208b and miR-499 decreased. In addition, miR-208 also regulates stress-dependent myosin heavy chain gene (for example, MYH7, MYH2, and MYH4) expressions and down-regulation of miR-208b promotes further upregulation of myosin heavy chain genes by positive feedback (20).*

According to the literature data available, miR-1 is a key element in the regulation of myosin heavy chain genes' expressions (*MYH7, MYH2* and *MYH4*) (24). Literature has confirmed that the *MYH7* gene is over-expressed as well as the expression of *MYH2* and *MYH4* genes based on preliminary results (we have not yet published our own results). It is already acknowledged that miR-208b and miR-499 are placed in the intronic regions of *MYH7* gene (19). As a result of changes in the pattern of *MYH7* expression, the expression level of myomiRs, which are located in intronic regions of *MYH7* and *MYH7B* has been changed; more precisely, expression level of miR-208b and miR-499 decreased; measuring 2.91-fold change for miR-208b and 7.59 fold change for miR-499, respectively (Table 2). However, as far as we know, miR-208 controls miR-499 gene expression as well (25) and this condition makes miR-499 expression level even lower. In addition, miR-208 also regulates stress-dependent myosin heavy chain gene (for example, *MYH7, MYH2,* and *MYH4*) expressions (20) and down-regulation of miR-208b promotes further upregulation of myosin heavy chain genes by positive feedback.

There are several studies to date that reported the function of myomiRs. These reports established that miR-1, miR-208b, and miR-499 stimulate myoblast differentiation and regeneration; promote angiogenesis regulation; affect the muscle fiber shift; and encourage muscle growth in healthy conditions. Their reduced expression levels could influence their functions resulting in muscle weakness, muscle atrophy, which are typical symptoms of PM. Present data suggest that muscle levels of certain myomiRs might be associated with PM. However, the number of patients and controls are relatively low in our study and miRNA microarray results have not been validated yet on the tested and/or independent samples by real-time qPCR. Validation of miRNA microarray results as well as our hypothesis also need further studies.

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