UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO:

MicroRNAs in body fluids: The role and importance in the development and diagnosis of diseases

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniera Biomédica

Autor:

Solórzano Lucio Abigail Lisseth

Tutor:

Ph.D. Nelson Santiago Vispo

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DEDICATION

I especially dedicate this thesis and my entire career to my friend, partner, counselor, and mother, for being the unconditional person who has provided me with emotional, physical, spiritual, and financial support over the last five years. Every effort in this thesis is dedicated to my older brother, Gabriel Solórzano, because he has been my inspiration and, above all, my greatest motivation never to give up and always advance in the face of obstacles. To my sister and brother, Raquel and Robinson, for teaching me to be brave and strong, for their unconditional support and never losing faith in me. To my entire family for being a fundamental pillar in my life.

This new achievement is dedicated to all friends and teachers because it would not be possible without them.

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Abigail Solorzano

ABSTRACT

According to biology's central dogma, DNA transcript into RNA, and then RNA is translated into proteins. However, non-coding RNAs (ncRNAs) are RNAs produced from transcription but do not encode proteins. ncRNAs have been shown in recent years to represent the majority of the human genome, performing functions in gene regulation. MiRNAs are 22-nucleotide short non-coding RNAs that regulate various processes such as differentiation, proliferation, apoptosis, hematopoiesis, and even tumorigenesis. The biogenesis of miRNA is conducted by RNase III enzymes, Drosha and Dicer, directed towards target mRNA to induce mRNA degradation and translation suppression. Still, in some cases, the miRNA is responsible for activating translation or regulating transcription, depending on the miRNA's interaction and affinity.

On the other hand, some miRNAs are secreted into the extracellular medium via microvesicles to bind to their target cell and interfere with the activation or inhibition of biological processes. Circulating miRNAs, like intracellular miRNAs, participate in various physiological functions, including abnormal or pathological processes such as cancer involvement. This review explains the canonical and non-canonical miRNA generation pathways, interaction with target molecules, and mechanism of action. Also, evidence is being gathered on the transport of miRNAs secretion to extracellular fluids, its mechanism of interaction with its target cell, and its role as a tool for diagnosing and predicting diseases, in other words, their potential as biomarkers. We focus on the miRNAs found in body fluids such as saliva, urine, blood, and especially in breast milk and its relationship to breast cancer prognosis.

KEY WORDS: miRNA, extracellular, breast milk, breast cancer

RESUMEN

De acuerdo al dogma central de biología, el ADN se transcribe en ARN y luego el ARN se traduce en proteínas. Sin embargo, los ARN no codificantes (ncRNA) son ARN producidos a partir de la transcripción, pero no codifican proteínas. En los últimos años se ha demostrado que los ncRNAs representan la mayor parte del genoma humano, desempeñando funciones en la regulación genética. Los miARNs son ARN cortos no codificantes de 22 nucleótidos que regulan varios procesos como diferenciación, proliferación, apoptosis, hematopoyesis e incluso tumorigénesis. La biogénesis del miARN es conducida por las enzimas RNasa III, Drosha y Dicer, dirigidas hacia el ARNm diana para inducir la degradación del ARNm y la supresión de la traducción. Aun así, en algunos casos, el miARN es responsable de activar la traducción o regular la transcripción, dependiendo de la interacción y afinidad del miARN.

Por otro lado, algunos miARNs se secretan en el medio extracelular a través de microvesículas para unirse a sus ARNm diana e intervenir en la activación o inhibición de procesos biologicos. Los miARNs circulantes, como los miARNs intracelulares, participan en diversas funciones fisiológicas, incluidos procesos anormales o patológicos como su implicación en el cáncer. Esta revisión explica las vías canónicas y no canónicas de la generación de miARNs, la interacción con las moléculas diana y el mecanismo de acción. Asimismo, se está recopilando evidencia sobre el transporte de la secreción de miARN a los fluidos extracelulares, su mecanismo de interacción con su célula diana y su papel como herramienta para el diagnóstico y la predicción de enfermedades, es decir, su potencial como biomarcadores. Nos centramos en los miARNs que se encuentran en los fluidos corporales como la saliva, la orina, la sangre y especialmente en la leche materna y su relación con el pronóstico del cáncer de mama.

PALABRAS CLAVES: miARN, extracelular, leche materna, cáncer de mama

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1. INTRODUCTION

1.1.Ribonucleic Acid

Ribonucleic acid (RNA) is a single chain molecule found in prokaryotes and eukaryotes and is the only genetic material of some viruses. Nucleotides are the building blocks of RNA; each nucleotide in the RNA chain contains one phosphate group, one monosaccharide sugar, in this case, ribose, and four nitrogenated bases (adenine, uracil, cytosine, and guanine) (1). Based on the position of the components, three structures can be formed.

Primary Structure

RNA has a primary structure similar to DNA, consisting of nucleotides arranged in a linear sequence on a single strand (2).

Secondary Structure

In this case, the simple RNA chain folds itself by intramolecular base mating, forming secondary RNA structures defined by the shape that occurs during folding, such as propeller, loop, and fork loop; even if the chain has several folds, the 3' and 5' ends are always free (2).

Tertiary Structure

It is a structure formed by the stacking of nitrogen bases and hydrogen bonds at different points along the chain. It has a three-dimensional design made up of a complex folding on top of the secondary structure (2).

It fulfills different cellular functions, including a molecule involved in protein synthesis (1). The central dogma of molecular biology indicates a process of protein synthesis from a DNA sequence. The DNA is read into RNA through the transcription process, and then the information is translated to obtain proteins as the final product (fig. 1) (3)

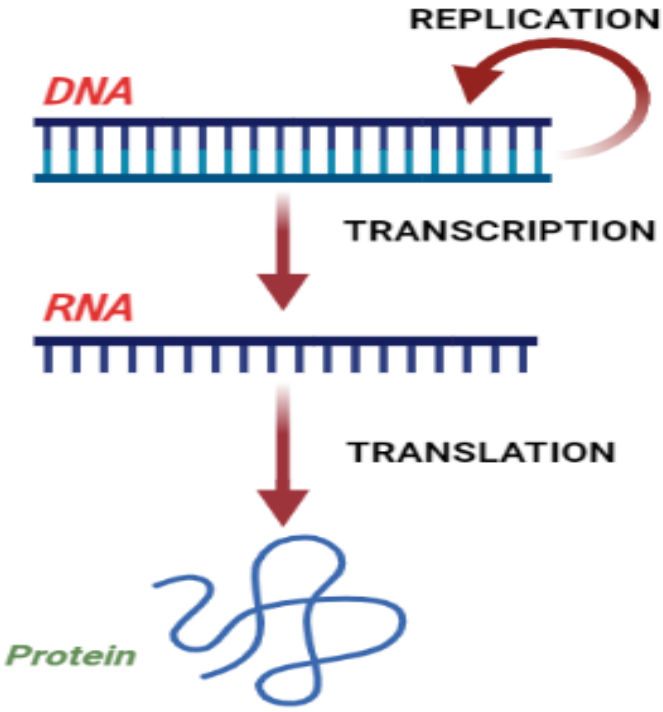

Figure 1. The central dogma of molecular biology is depicted graphically.

Although there is only one type of DNA, several types of RNA perform functions within the cell, and all contribute to protein synthesis in some way.

RNA Types	Name	Symbol	Function
Coding RNA	Messenger RNA	mRNA	Messenger RNA is a family of RNA molecules that complement DNA molecules and transport genetic information from DNA for translation into proteins through ribosomes. (4)
Non-Coding RNAs	Transfer RNA	tRNA	"Adapter function during translation of genetic information, providing the interface between nucleic acids and proteins during ribosome-dependent protein synthesis."(5)
	Ribosomal RNA	rRNA	It constitutes the most mass of the ribosome, is associated with ribosome proteins, and determines the ribosome structure and ribosomal protein position (6,7)

Ribonuclease P	*RNase P*	It is an endoribonuclease formed by protein and RNA subunits. It has catalytic activity and is involved in the processing of tRNA (8,9).
RNase Mitochondrial RNA Processing	*RNase MRP*	It is an endonuclease composed similar to RNase P and plays a role in mitochondrial processing, rRNA processing, and cell cycle regulation (10).
Telomerase	Ter/Tr	It is an enzyme responsible of the elongation of telomeres by adding guanine sequences for the proper functioning of DNA replication (11).
small interfering RNAs	siRNAs	They are short non-coding RNA with 20 to 25 nucleotides involved in post-transcriptional gene silencing. (12).
Piwi-interacting RNAs	piRNAs	They are small RNAs with approximately 24 to 31 nucleotides. They bind to PIWI proteins and play an important role in silencing transposons (13).
microRNAs	miRNAs	They are RNA non-coding 20 to 25 nucleotides in length. They contribute to gene expression regulation through mRNA degradation or inhibition of translation (1).
Small nuclear RNA	snRNA	It's a small RNA class that plays a fundamental role in cleavage and splicing introns (14).
Small Nucleolar RNAs	snoRNA	They are small non-coding RNAs involved in rRNA processing in the nucleolus (1).
Small Cajal body-specific RNAs	scaRNAs	They are non-coding RNAs that are found in the Cajal body and perform methylation and pseudouridylation functions on rRNA and snRNA (15).

Table 1. RNAs types and their main function

1.2. RNAs Types

1.2.1. Coding RNA

Messenger RNA (mRNA)

mRNA is single-stranded and accounts for approximately 3 to 5% of total RNA. It serves as a messenger, copying and transporting genetic information from DNA to cytoplasmic ribosomes for protein synthesis. In the nucleus, mRNA is synthesized using DNA as a template by a process known as transcription. The formed mRNA then exits the nucleus into the cytoplasm and determines the aminoacids that will bind to form proteins from a sequence of three nucleotides known as a codon and a complementary sequence known as an RNA anticodon. Proteins are assembled in the ribosome from mRNA for its production, a process called translation (1,16,17)

1.2.2. Non-coding RNA (NcRNAs)

Once the proportion of 98% of the human genome occupied by ncRNAs has been affirmed, the big question raised about this type of RNAs is whether they are just "transcriptional noise" or play an important role (18). In a comparison between the nematode worm *Caenorhabditis elegans* and humans, it could be found that the nematode with ~100 cells had ~ 20.000 protein-coding genes, the same amount of protein-coding gene as humans, which have 100 trillion cells and physiological complexity and development much greater than *C. elegans*. So, where is the part that sets us apart from the other species or even the rest of mammals? (19)

This difference in complexity is based on the amount of non-coding RNA of proteins that each species possesses. Affirming the deduction that ncRNAs are what differentiates one organism from another, Geisler & Coller (20) explain that non-coding RNA expression diversity and size are related to organism complexity.

Anastasiadou et al. (21) demonstrated that some ncRNAs are RNA molecules that play a role in biological activities such as chromatin remodeling, transcription, post-transcriptional changes, and signaling. The networks in which ncRNAs interact can impact a variety of molecular targets, causing distinct biological responses and cell fates. So it cannot be said

that all ncRNA is just "transcriptional noise," because they are responsible for performing different cellular functions that differentiate one species from another.

Non-coding RNA plays a role in regulating gene expression in animals, plants, and fungi. As an example of the significance of this type of RNA in species, studies are found less complex eukaryotes, like the nematode *Caenorhabditis elegans* where ncRNA called lin-4 and let-7 could be identified, which were responsible for controlling the larval development of the worm. (22)

The ncRNA can be divided into ribosomal (rRNA), transfer (tRNA), ribonuclease P (RNase P), RNase Mitochondrial RNA Processing (MRP), and telomerase (23). The regulatory ncRNA can be divided according to their length into two subclasses: short non-coding RNA with less than 20 nucleotides and long non-coding RNA (lncRNAs) with more than 200 nucleotides (24). These subgroups have been studied for their intervention in gene expression regulation, a process that is maintained at the transcriptional or post-transcriptional level. (3)

Transfer RNA (tRNA)

It is the smallest RNA, with approximately 75 nucleotides in its chain, and folds into a three-dimensional structure resembling a folded cloverleaf. tRNA accounts for approximately 15% of total RNA. They transport amino acids from the cytoplasm to the ribosome, resulting in the formation of a new protein chain, tRNA has a triplet of complementary bases of a specific codon known as an anticodon, and it captures the correct amino acids by reading the mRNA and indicating the information to recognize the amino acid to be included in the new protein chain (1,16,25)

Ribosomal RNA (rRNA)

It is the most abundant RNA, accounting for roughly 80% of total RNA in the cell. RNA binds to proteins to form ribosome subunits. The ribosome comprises four ribosomal RNA molecules, which account for 60% of the mass of the ribosome, and 70 to 80 proteins, which account for 40% of the mass of the ribosome. Ribosomes (ribosomal RNA + proteins) participate in protein synthesis, require components for aminoacid recognition

from messenger RNA, and act as a welding mechanism, providing chemical energy to form aminoacid bonds (1,16,17,25)

Ribonuclease P (RNase P)

It is an endoribonuclease responsible for processing the 5′- leader sequence of tRNA. In bacteria, RNase P comprises two subunits, a single protein subunit, and another RNA subunit; the RNA subunit shows catalytic activity. In archaea and eukaryotes, most RNase P consists of an RNA chain and a high content of associated proteins; for example, RNase P in humans is associated with ten proteins. As in bacteria, the RNA chain has a small catalytic activity (9,10)

RNase Mitochondrial RNA Processing (MRP)

It is a ribonucleoprotein with a composition related to RNase P. It contains 8 to 10 essential proteins, which can be found in RNase P. It participates in regulating the cell cycle through degradation of the mRNA and the maturation of the rRNA (10,26)

Telomerase

It is a ribonucleoprotein component essential for telomere elongation during DNA replication. Telomerase contains a reverse transcriptase function, which uses RNA as a template to synthesize the RNA chain, using the 3 'OH of the G T telomeric chain for DNA synthesis. It also contains proteins necessary for the functioning of telomerase, influencing elongation and binding to telomeres (10,11).

1.2.2.1. Long non-coding RNA (lncRNAs*)*

While research of non-coding RNAs was being carried out in different organisms, with the help of developing new sequencing techniques, lncRNAs have been shown to occupy about 65% of the human genome. LncRNAs are RNA's transcripts with around 200 nucleotides of longitude, and although they cannot produce proteins, investigations had seen that some lncRNAs produce small functional peptides. (19,27)

The number of functions of the lncRNAs has steadily increased by the number of investigations carried out. Some proven functions include that lncRNAs are involved in

imprinting genomic loci. The lncRNAs are often used to distinguish the paternally and maternally inherited alleles during imprinting (28). On the other hand, the expression of lncRNAs is involved in the development and configuration of cell type, the study, and understanding of how lncRNAs act within cells could reveal new functional knowledge and structural elements about cellular activities, such as cell differentiation or disease development. (29)

Another function is performed by the long-non coding RNA called HOTAIR, HOTAIR is a predictor of breast cancer, citing to Rinn & Chang (28) “enforced expression of HOTAIR was sufficient to drive breast cancer metastasis.” Besides, HOTAIR and other lncRNAs have been proposed as scaffolds to coordinate the orientation of different repressive histone-modifying complexes to the target loci. (20)

Among other functions and in a more general way is that lncRNAs called XIST are involved in many physiological processes such as inactivation of the X chromosome in mammals. XIST and HOTAIR are associated with polycomb repressive proteins resulting in a common silencing route (30). As Esteller M. (31) notes that the X chromosome is silenced by attachment of the X-inactivation specific transcript (XIST) lncRNA with the polycomb complex.

It is now attributed to several functional roles, and probably as research continues, many more will be discovered that contribute to the advancement of knowledge about the cellular activities and physiological processes of organisms.

1.2.2.2. Short non-coding RNA (sncRNA)

In recent years, RNAs called small or short non-coding RNAs (sRNAs or sncRNAs) have revolutionized the world because these small RNAs are involved in various biological functions such as developmental timing, cell differentiation, cell proliferation, cell death, metabolic control, transposon silencing, and antiviral defense (32) and are considered the main gene expression regulators capable of regulating more than 40% of known genes. (33,34) They are defined by their small size (20-30 nucleotides), by their association with Argonaute proteins, and by the function of gene regulation/silencing. (35)

With the advantage of natural RNA silencing, specific gene silencing can be achieved by arranging unnatural RNA precursors. Farazi T., Juranek S., and Tuschl (36) states that at the transcriptional and/or post-transcriptional stages, sRNAs control sequence-specific gene silence.

The pathway by which this gene silencing occurs is different in each species, usually depends on the associated proteins and the different mechanisms, such as the degree of mating between the sRNAs. However, they all work with a general action strategy, where sRNAs bind to their protein complexes and interact with their targets mostly in partial or total complementary form. (33,34)

To this day, three subclasses of sRNAs are known and divided according to their biogenesis mechanism and the type of Argonaute protein with which it is associated: microRNAs (miRNAs), small interfering RNAs (siRNAs), Piwi-interacting RNAs (piRNAs), Small nuclear RNA (snRNA), Small Nucleolar RNAs (snoRNA), and scaRNAs (small Cajal body-specific RNAs) (32).

In general, miRNA, its main function is the regulation of gene expression at the post-transcriptional level, are generated by two RNase III-type proteins, Drosha and Dicer, then binds to mRNA in such a way that it functions as post-transcriptional regulators. (32)

The small interfering RNAs (siRNAs) are fragments of 21-25 nucleotides; although they share similarities with miRNA, they differ in that siRNAs are derived from bi-catenary chains, biogenesis depends on endoribonuclease III, but only on the Dicer type and not on Drosha. The siRNAs can be endogenous and exogenous; endogenous are present in plants and animals, and exogenous are introduced either experimentally or by a virus. The double-stranded RNA is processed by Dicer, a fragment of ~22 nucleotides is generated and then loaded into a RISC multiprotein complex (RNA-induced silencing complex) by interacting with an Argonaute protein. However, some eukaryotes need RNA-dependent polymeric RNA proteins (RdRPs) to form double-stranded RNA and then be processed by Dicer in siRNAs biosynthesis (33,34). As points out Farazi T., et al (36), the siRNAs play a key role in protecting genomes from transgenes and transposons, in addition to foreign nucleic acids like viruses.

Piwi-interacting RNAs (piRNAs) interact with PIWI subfamily proteins, but unlike the previous two classes of sRNAs, piRNA biogenesis is independent of Dicer. It is the longest class, containing approximately 24-31 nucleotides. Its principal function is “the silencing of transposable elements in the germ line” (34); also, it performs functions as epigenetic regulation, gene and protein regulation, genome rearrangement, spermatogenesis, and germ stem-cell maintenance (37). PiRNA binds to PIWI proteins and is then directed to the target transposon, thereby promoting genetic diversity and instability (32).

Small nuclear RNA (snRNA) is a small non-coding RNA with approximately 150 nt in length, located in the nucleus. It performs essential functions in the cleavage and splicing of RNA introns. snRNA is associated with proteins and ribonucleoprotein particles (snRNPs), resulting in a spliceosome complex (14,38).

Small Nucleolar RNAs (snoRNA) are small non-coding RNA about 60 to 300 nucleotides in length and are found in the nucleolus. Its principal function is related to rRNA modifications. The two leading families of snoRNA, the first called box C/D snoRNAs and works on 2’O ribose methylation and box H/ACA snoRNAs, are responsible for converting uridine to pseudouridine (pseudouridylation). The two families work in rRNA modification and processing processes (39,40).

scaRNAs (small Cajal body-specific RNAs), located in the Cajal body, are part of the family of snoRNAs and fulfill the same functions, such as Methylation and pseudouridylation rRNA and snRNA; therefore it is essential for the functioning of the spliceosome (15,41).

Finally studies of small non-coding RNAs' expression profiles have revealed that any alteration leads to a cell disorder, leading to neurological, metabolic, or cardiovascular diseases, and chronic diseases such as cancer. For this reason, it is necessary to keep under control of specific signals and thus maintain good cellular function (33,34).

1.3. Application of RNAs

Different studies carried out around RNA and different biological applications of RNA have been identified and investigated. One of the most studied applications in recent years has been the use of RNA in vaccine development; RNA has meant a new therapeutic tool to activate the organism's immune response.

RNA as a new era in vaccinology

The World Health Organization (WHO) describes the vaccines prevent and reduce the risk of disease. As a result, mortality from several diseases, such as the smallpox virus, has been eradicated or reduced, such as measles or influenza, allowing people to live for many more years (42,43). A vaccine can be made from inactivated or dead viruses, bacteria, or germs, or purified microorganism products (43). Vaccines have been a great success in human survival; however, vaccine requires a short development time and large-scale implementation (42).

Because RNA is unstable, it has been considered a therapeutic tool. mRNA was first proposed as a therapeutic method in 1989 (44), and in 1990, in vitro mRNA tests on mice were performed, demonstrating the success of in vivo mRNA expression. This demonstrated the viability of mRNA in the development of vaccines (45). Because it offers advantages that other vaccines do not, mRNA has been studied and researched as a vaccine for various diseases. First, mRNA is effective in working as a vaccine. When compared to other vaccine modifications, changes to the mRNA sequence make it more stable and translatable. Furthermore, mRNA would be formulated into carrier molecules to achieve rapid absorption and expression in the cytoplasm through in vivo administration.

On the other hand, mRNA has its adjuvant properties responsible for activating adaptive immune responses, whereas vaccines made of proteins and polypeptides require additional adjuvants (42,46). Second, mRNA was proposed as a non-infectious medium without risk of infection or insertion mutagenesis (42). Due to mRNA's chemical constitution decreasing the probability of “mRNA to integrate into host DNA genome and induce a smaller immune rejection reaction” (46). Furthermore, by modifying the mRNA sequence and delivery method, they could control the expression and half-life of mRNA in vivo

(42,46) Third, RNA production is faster, cheaper, and, most importantly, scalable. In vitro transcription contributes and facilitates the production of mRNA vaccines, also, "most of the mRNA vaccine production and purification processes are quite similar despite different encoded antigens, so it is the potential to be retained or even standardized to develop other similar mRNA vaccines" (42,46)

The advantages of mRNA vaccines, such as their high efficiency and low cost, have enabled a continuous study of vaccine prevalence in clinical and pre-clinical trials against infectious diseases and cancer.

mRNA Vaccines Against Infectious Diseases

Vaccines have been the most effective method for combating infectious pathogens; however, conventional vaccines have not been successful in diseases caused by viruses that cause chronic or recurring infections, such as HIV; additionally, vaccine development and production have prevented vaccines from functioning in outbreaks of acute viral diseases, such as Zika or Ebola (42,46). As a result, mRNA vaccines were proposed as a viable and rapidly evolving method of combating infectious diseases.

Because of their numerous advantages, such as rapid virus development, mRNA vaccines have successfully established promising targets against infectious diseases, also with only one or two low-dose immunizations, mRNA can generate potent neutralizing antibody responses in animals (42).

Vaccines against infectious diseases such as influenza have been developed using technological advances; mRNA vaccines against influenza have proven to be a more effective method of prevention and treatment than other types of vaccines due to their ability to boost the generation of disease-specific antibodies (46). The past year has seen intense research into the rapid and effective development of a coronavirus vaccine. mRNA vaccines against SARS-CoV-2 have been developed. The mRNA vaccines contain mRNA that is read by ribosomes to produce spikes that are characteristic of the virus then, they appear on the surface of the cells to activate the immune response and generate antibodies to fight Covid-19 (46).

mRNA Vaccines Against Cancer

mRNA vaccines against cancer have been proposed as a new therapy for the treatment of malignancies. These vaccines are intended to target tumor-associated antigens such as antigens expressed in cancer cells, growth factors, or neoantigens (proteins that occur in the presence of cancer cells or during mutations in tumor DNA (47)). Because of their promising function in assisting the body in recognizing cancer cells as foreign and thus activating the immune system, especially T cells' response, neoantigens have been considered a target molecule for developing mRNA vaccines (42,46,47). For the time being, mRNA vaccines against cancer are therapeutic, causing cell immunity to reduce or eliminate the number of cancer cells or the tumor's size (46). Several personalized vaccines have been developed to treat various solid tumors, such as pancreatic cancer. A personalized mRNA-5671 vaccine was administered intramuscularly to patients with pancreatic cancer in phase 1. Because the vaccine elicited an anti-tumor immune response with specific T-cell responses against cancer-specific neoepitopes, it was accepted as a complementary therapeutic method to T-cell therapy (48)

The function of RNAs is widespread because, as shown in Table 1, there are RNAs involved in protein synthesis such as mRNA, tRNA, rRNA, but there are also RNAs not involved in protein synthesis called non-coding RNA (ncRNAs), and current studies have shown that the proportion in the human genome of ncRNAs is more significant than protein-coding RNA. (24). As Trovero & Geisinger state (3) it has been revealed that, surprisingly, the proportion of transcript genome to RNA but that it does not produce proteins is much higher than previously estimated.

2. OBJECTIVES

2.1. General Objective:

Investigate miRNAs' presence in extracellular fluids using bibliographic evidence and clinical trials and introduce a review of miRNAs' role as biomarkers in cancer diagnosis, prognosis, and therapy.

2.2. Specific Objective:

- To detail the sequential processes in the canonical and non-canonical pathways to generate mature miRNAs.
- To describe the mechanisms of action during the interaction of miRNAs with their mRNA target to understand their role in mRNA cleavage and translational repression.
- To determine the mechanisms of miRNA secretion into the extracellular medium.
- To identify circulating miRNAs present in saliva, urine, plasma/serum, breast milk, and their association with various cancer types.
- To relate circulating breast milk miRNAs to the development of breast cancer.

3. MicroRNAs

MicroRNAs (miRNAs) are single-stranded RNA, belong to the short non-coding RNA family because their length is ~19-25 nucleotides and originate from endogenous hairpin-shaped transcripts (32). Present in animals and plants, RNA molecules bind to target mRNA to prevent protein production (49).

3.1. Discovery of miRNA

The first miRNA was discovered in *Caenorhabditis elegans* called lin-4 in 1993 by Victor Ambros, Rosalind Lee, and Rhonda Feinbaum. In *C. elegans*, the lin-4 gene is necessary for the normal timing of various postembryonic development events (50). They found that the lin-4 gene acting on the larval development of *C. elegans* did not encode proteins, instead of producing two small RNAs approximately 22 and 61 nucleotides in length. Feinbaum and colleagues noted that these RNAs contained complementary sequences to multiple sites in the UTR'3 zone of the lin-14 gene, suggesting that lin-4 regulates lin-14 translation through an RNA-RNA interaction (35,51).

The second miRNA called let-7 was found seven years later, an RNA of 21 nucleotides in length in *Caenorhabditis elegans* Let-7 is complementary to the elements of the 3'UTR of the lin-14, lin-28, lin-41, lin-42, and daf-12 genes, thus indicating that let-7 can control the gene expression of these genes (35). Like lin-4, let-7 acted in *C. elegans*, promoting early development, moving from a late larva (L4) to adult form (51).

Stefani & Slack (52) remarks that larval development time in *C. elegans* is controlled by lin4 and let7. Following the discovery of let-7 and lin-4, more miRNAs in other genomes were reported and could function as a class of gene regulatory molecules in different species (51).

3.2. Function

MicroRNAs have different regulatory functions in organisms during cell development and proliferation. Studies demonstrated that different classes of miRNAs are involved in various development processes of the species, for example, miR2, miR6, and miR14 allow

the larval development in the *C. elegans*, these miRNAs "regulate tissue growth through modulation of both apoptosis and cell proliferation" (52). Likewise, in *Drosophila melanogaster*, the miRNA BANTAM promotes cell proliferation while suppressing apoptosis (53). Similarly, microRNAs are involved in tumorigenesis, inflammation, and their expression related to specific diseases. It is found that miRNAs also act as oncogenes and tumor suppressor genes; for this reason, miRNAs are being studied for the great potential they would have as therapeutic targets and biomarkers and thus give a new therapeutic tool for cancer diagnosis from these tiny RNAs (54).

miRNAs	Target Molecule	Function	Species	Reference
Lsy-6	Cog-1	Neuronal patterning	*Caenorhabditis elegans*	(55)
miR-273	Dap	Affect the division of germ line stem cells	*Drosophila melanogaster*	(56)
miR-14	sugarbabe	Acts on neurosecretory cells that produce insulin	*Drosophila melanogaster*	(57)
miR-132-3p	Btg2	Changes in alpha-cells	mammals	(58)
miR-18a-5p	syndecan4	Promoted the differentiation of vascular smooth muscle cells	mammals	(59)
miR-143	ERK5	Adipocyte differentiation regulator	mammals	(60)
miR-375	Myotrophin (Mtpn)	Insulin secretion regulator	mammals	(61)
miR-208	CDKN1A	Cell cycle regulation	mammals	(62)
miR-155	SHIP1	Involved in the control of hematopoiesis	mammals	(63)
miR-150	c-Myb	Differentiation of B Cells (has an effect on lymphocyte development and response)	mammals	(64)
miR-20b	HIF-1α	Hypoxia-induced cell response	mammals	(65)
miR-133	CTGF	The regulation of structural changes in the myocardium's extracellular matrix.	mammals	(66)

Table 2, Examples of miRNA functions and mRNA targets in different species

3.3. Location of miRNAs in the genome

MiRNAs are divided into different categories depending on where or region they are located:

1) Intronic miRNAs located in non-coding transcripts
2) Exonic miRNAs located in non-coding transcripts
3) Intronic miRNA located in protein-coding genes
4) Exonic miRNA located in protein-coding genes

MiRNAs have also been found in the 3-UTR region of protein-coding genes or in repetitive regions of DNA. (35)

Berezikov (67) explains that introns contain around half of the human miRNAs and 20% of *C. elegans* miRNAs. Introns are thought to be an "optimal point" for the emergence of new miRNAs because they already provide material for the evolution of a fork structure from transcribed RNA (67). Also, the expression of these miRNAs may be associated with the host gene's transcriptional regulation (51).

On the other hand, exons encoding proteins are taken as less essential sources for the new genes of miRNAs, because not enough miRNAs have been found in this region; for example, in studies of the species, *D. melanogaster* was found nine miRNA loci were identified in the coding sequences of genes (67). The expression of exonic miRNAs in coding gene proteins is regulated by independent or regulatory elements (51).

4. BIOGENESIS OF miRNAs

Thousands of miRNAs can be generated from endogenous transcripts, adopting secondary characteristics and then binding others such as Argonaut proteins (33). The biogenesis of miRNAs begins with its transcription by RNA polymerase II (Pol II) and is regulated by two RNases III proteins: Drosha acting in the nucleus and Dicer acting in the cytoplasm, by the so-called canonical pathway of miRNAs generation. However, miRNAs have been identified through new biogenesis pathways called non-canonical pathways, but many of these pathways do not include RNases III, Drosha, and Dicer proteins (54,68).

4.1.Canonical pathway of miRNA biogenesis

The canonical pathway of biogenesis begins with transcription. RNA Pol II transcribes the primary miRNAs (pri-miRNAs). The pri-miRNAs contain the 7-methylguanosine cap and a poly(A) tail, which are unique for Pol II transcripts (69). But there is a possibility that RNA Pol II does not transcribe the pri-miRNA; at first, it was commented that RNA Pol III could transcribe it because most of the small RNAs were transcripted by it. However, Lee et al. (70) demonstrated that miRNAs genes are transcribed by pol II. Pol III transcribes some pri-miRNAs as the C19MC (chromosome 19 miRNA cluster) (35).

Once the primary miRNAs transcript is formed, a pri-miRNA is observed with several kilobases (< 1Kb) in length and with a hairpin stem-loop structure that is formed of a stem and loop (54), then RNAse III Drosha splits the stem-loop structure and allows the release of the precursor of miRNA (pre-miRNA) (53) This reaction occurs at the nucleus where RNAse III Drosha DiGeorge syndrome critical region gene 8 is required as a cofactor (DGCR8) in humans and *Drosophila* and *C. elegans* it is called Pasha (partner of Drosha). The cofactor DGCR8/Pasha in its N terminal region contains the nuclear localization signal, also contains two double-stranded RNA-binding domains (dsRBDs) responsible for assisting Drosha in substrate recognition (35). The RNAse III Drosha and DGCR8/Pasha form a microprocessor complex of ~ 650 kDa in humans and ~ 500 kDa complex in *D. melanogaster* the terminal region C (69). The main objective for this interaction is that "DGCR8/Pasha helps in accurate Drosha processing by acting as a molecular ruler to measure and determine the Drosha cleavage site, which is at the 11-nt position from the

base of the stem structure" (35). As we mentioned, the primary miRNAs transcript is formed by a fork-shaped loop. A stem formed by single-stranded RNA (ssRNA) con ~ 33bp, DGCR8/Pasha then interacts with pri-miRNA and helps Drosha split ~ 11 bp stem-ssRNA junction to obtain a microprocessor called pre-miRNA with 70 nucleotides (54).

The miRNA result (pre-miRNA) is exported to the cytoplasm via one of the Ran-dependent nuclear transport receptors called Exportin 5 (EXP5). Like the other nuclear transport receptors, the EXP5 binds to the pre-miRNA and the GTP-bound form to export from the nucleus to the cytoplasm, and the load is released into the cytoplasm when GTP hydrolysis occurs (32). Then the pre-miRNA is processed by the RNase III endonuclease Dicer, Dicer as Drosha is associated with a cofactor containing dsRBD, in case of Dicer for human beings, it interacts with two proteins, the HIV-1 TAR RNA-binding protein (TRBP) and PACT (also known like PRKRA) and Dicer-1 in *D. melanogaster* interact with Loquacious (also known expert R3D1) (32,53). This interaction cuts to pre-miRNA near the terminal loop, and a mature miRNA duplex of ~ 22 nucleotides (microRNA: microRNA *) is obtained, the microRNA represents the "guide chain," and microRNA * represents the "passing chain" (54,69).

Once the duplex miRNA is formed, it is assembled with the ARGONAUTE (Ago) protein to form the so-called miRNA-induced silencing complex (miRISC), the complex selects the guide chain, and in turn, this chain is responsible the silencing (54). The miRISC is formed by several proteins such as Dicer, several Ago (Argonaute) proteins, TRBP, PACT, Gemin3, and KSRP (35). Dicer binds to the miRNA duplex to split the duplex through its RNA helicase activity, the unrolling starts on end with lower thermodynamic stability. The chain with the 5 "less stable" or 5 ′ uracil end is determined as a guide chain and the other as the passing chain through thermodynamic stability. The guide strand is then loaded into the Ago protein or miRISC complex, and the passing strand is degraded and eliminated by the Ago 2 protein (71). Kim et al. (32) argue that Ago protein has endonucleolytic enzymatic activity (slicer activity), and it is responsible for eliminating the passenger strand from some miRNA duplexes. Once Dicer cuts the duplex and the guide chain remains, the stable end binds to TRBP, and the other end binds to the Ago protein in the RISC complex. Once the miRISC complex has been formed, the guide chain directs the complex towards

the target mRNA due to complementarity (35).

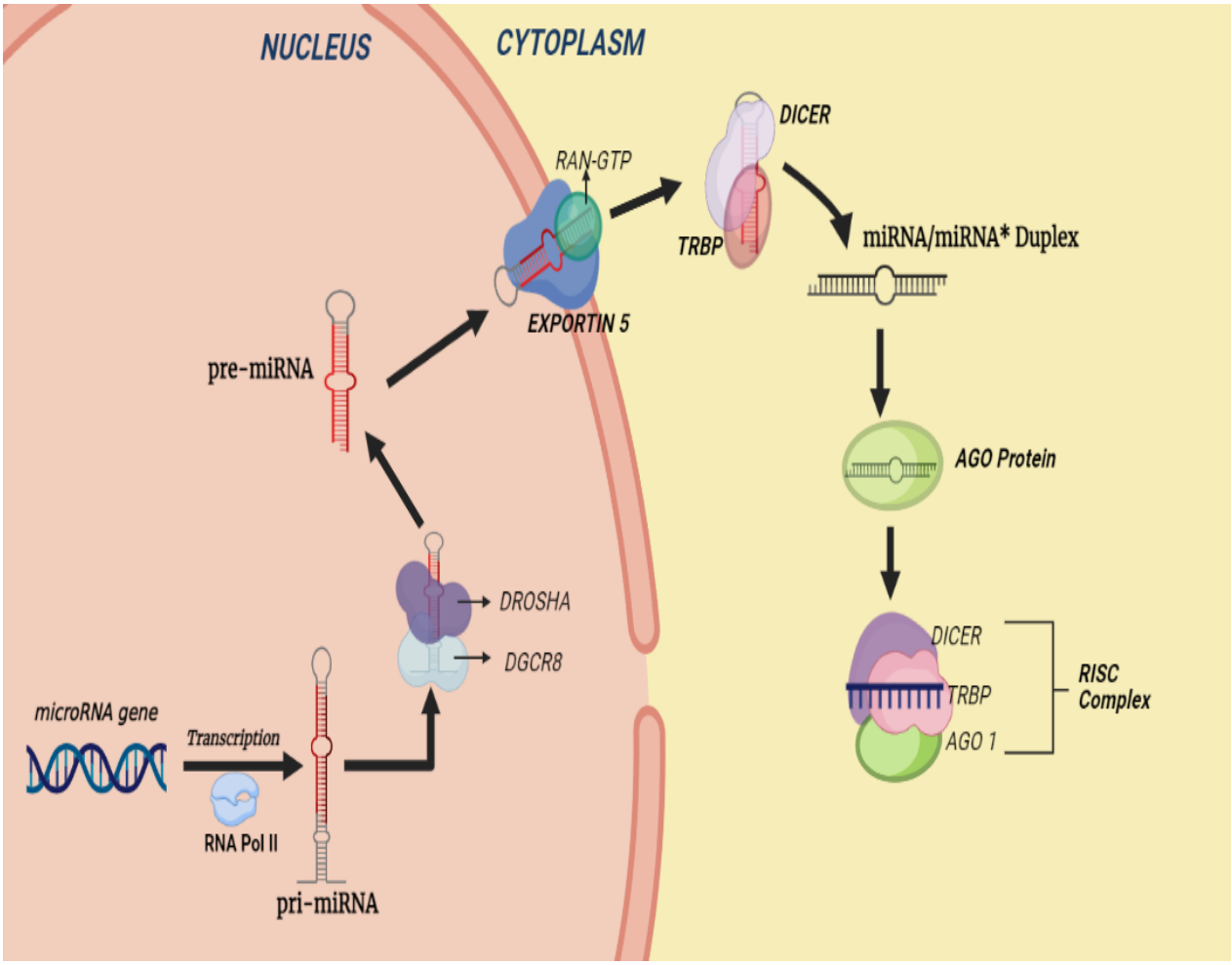

Figure 2. The Canonical MicroRNA (miRNA) Biogenesis Pathway. *RNA polymerase II (RNA Pol II/III) transcribes microRNAs generated by the canonical pathway. The DGCR8/Drosha complex processes the primary miRNA (pri-miRNA) transcript, forming a stem-loop structure. XPO5 transports the pre-miRNA to the cytoplasm, where Dicer processes it into a mature miRNA duplex. Finally, the mature miRNA associates with an AGO family member and enters the RISC complex.*

4.2. Non-canonical miRNA biogenesis pathway

The non-canonical pathway is one of the broad mechanisms to produce a functional miRNA. The canonical pathway works with two Dicer and Drosha proteins; however, the non-canonical pathway does not use the two proteins during the same process. This means that independent routes produce the biogenesis of non-canonical miRNAs; in other words, there are microRNAs produced by non-canonical pathways Drosha/ DGCR8-independent and microRNAs produced by non-canonical pathways Dicer-independent (72).

4.2.1. Drosha/DGCR8 independent pathways

4.2.1.1. MIRTRON

An example of Drosha's independent pathway is "the via mirtron". This non-canonical way was one of the first discovered, and as explained above, this mechanism uses the Dicer protein in the cytoplasm but does not use Drosha/DGCR8 in the nucleus pre-miRNA was produced (72). Instead, it was found "pre-miRNA-sized short introns" known as mirtrons (73). These introns are processed by spliceosomes and debranching enzymes to produce miRNA hairpins in the nucleus and be suitable for Dicer cleavage (72). These introns are then considered to be pre-miRNA imitators, which then go out into the cytoplasm with the help of EXP5 and continue with the process similar to canonical via (74).

The mirtrons were discovered in *D. melanogaster* and *C. elegans* and are produced from the mRNA introns, taking advantage of the large number of short introns possessed by these species (71,74).

According to their transcription mechanism, the mirtrons have been classified into conventional mirtrons and mirtrons with "tailed" (with unstructured 3' and 5' ends) (73). Conventional mirtrons omit the cleavage by the Drosha protein, and instead, they are derived from splicing and lariat debranching (75). At first, the intron splicing result is not linear; instead, it has a loop-shaped where its 3' end binds to the 5' end of the intron; however, the lariat debranching converts it to pre-miRNA fold to be transferred to the cytoplasm via EXP5 (76).

The mirtrons with "tailed" are mirtrons are splicing and debranching and then require additional trimming because they have extra nucleotides at the 3' or 5' (75). For example, *D. melanogaster* discovered 5' hairpin ends, whereas vertebrate species discovered 3' hairpin ends. Each end is preceded by a section of nucleotides or tail between the hairpin base and the splice site (72,74).

So for the mirtrons with tail at the 3' end, exosome (the central eukaryotic 3'–>5' exonuclease complex) is used to trim this end; however, for mirtrons with tail at the end 5', the enzyme responsible for trimming is still unknown, it is assumed that might be XRN1/2,

the primary 5'->3' exonucleases in eukaryotes, although it is still in studies this option (73,76). The additional trimming is necessary to form a temporary structure enough to bind to exportin 5; therefore, it is believed that the trimming is given in the nucleus and then transported, in the form of Dicer substrate, by exportin 5 to the cytoplasm (75,76).

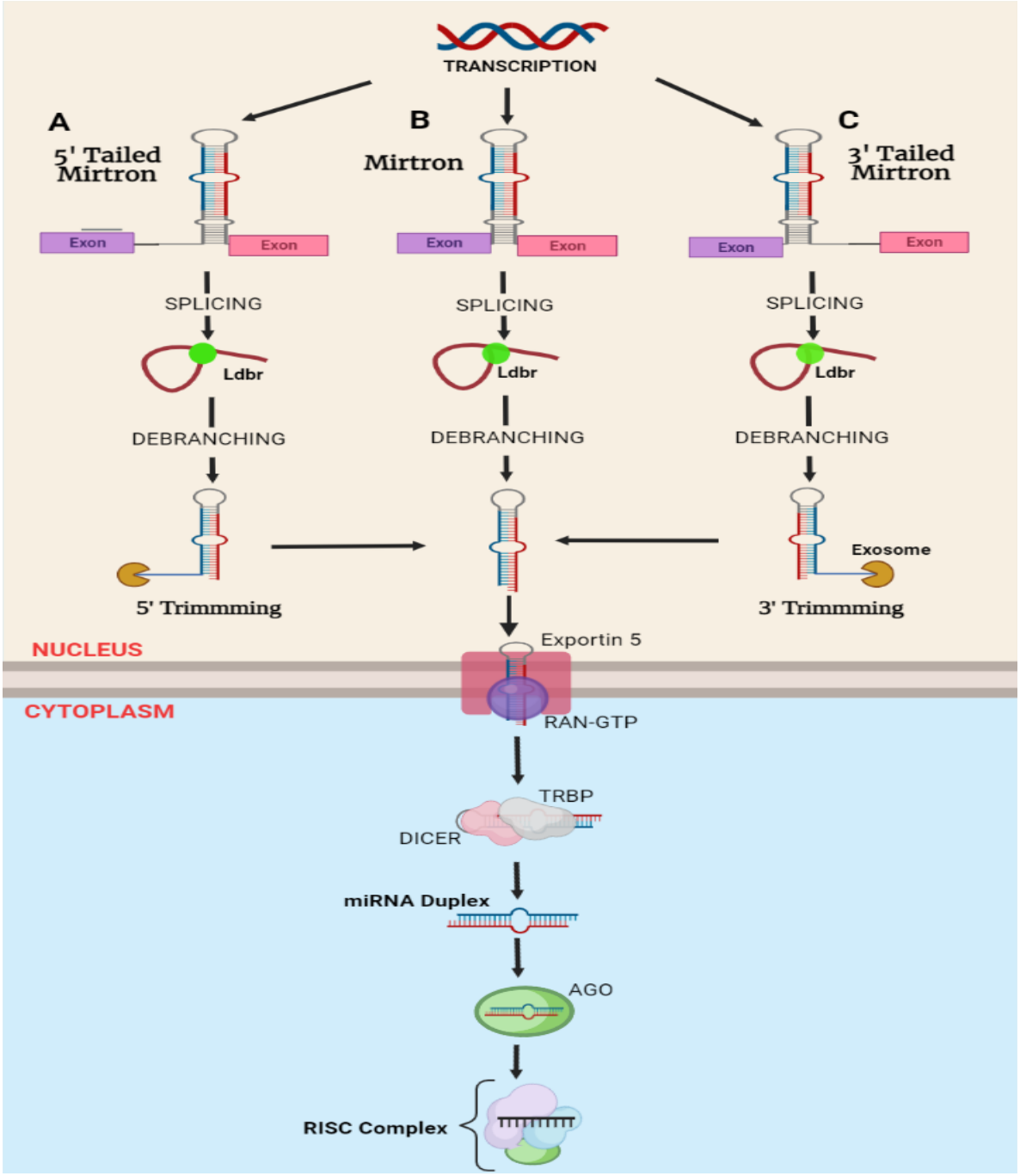

Figure 3. Drosha/DGCR8-independent, Dicer-dependent biogenesis. *Mirtrons, a type of miRNA derived from introns, are processed by the spliceosome and a debranching enzyme in the noncanonical pathway. B)*

Lariat debranching enzyme (Ldbr) splices and debranches introns entering the mirtron pathway, after which they fold into pre-miRNA hairpins. A) and C) Tailed mirtrons are a subset of mirtrons produced from longer introns, and consequently, require 3' or 5' exonucleolytic trimmings after debranching to generate the pre-miRNA hairpin structure. The RNA exosome trims 3' tails, but the enzymes responsible for 5' trimming are unknown. Following that, the spliced RNA takes on a pre-miRNA-like form; then, it shares by the subsequent steps of nuclear export to continue the canonical pathway.

4.2.2. Dicer-independent pathways

This pathway is dependent on the Drosha microprocessor but independent of the Dicer protein. A clear example was miR-451, it maturing across the independent pathway of Dicer, miR-451 splits into the nucleus for the protein Drosha/DGCR8 to produce the pre-miRNA of ~18-bp, once produced it is directly loaded in the protein Ago-2, and with its catalytic slicer activity, it slices the 3p strand of pre-miR-451, to bind later the complex RISC (74).

4.2.2.1. SIMTRONS

We mentioned earlier the mirtrons produced in the absence of the protein Drosha, pro splicing, and debranching enzymes. However, during studies, mirtrons called miR-1225 and miR-1228 were found to occur in the absence of splicing and a Drosha-dependent pathway. The biogenesis of a simtron does not change when it is removed from the process to Dgcr8, Dicer, Exportin-5, or Ago; however, if it is affected with the Drosha knockout (72)

On the other hand, the critical point was that mirtrons and simtrons are involved in silencing their mRNA target when interacting with the RISC complex, resulting in a functional miRNA (73).

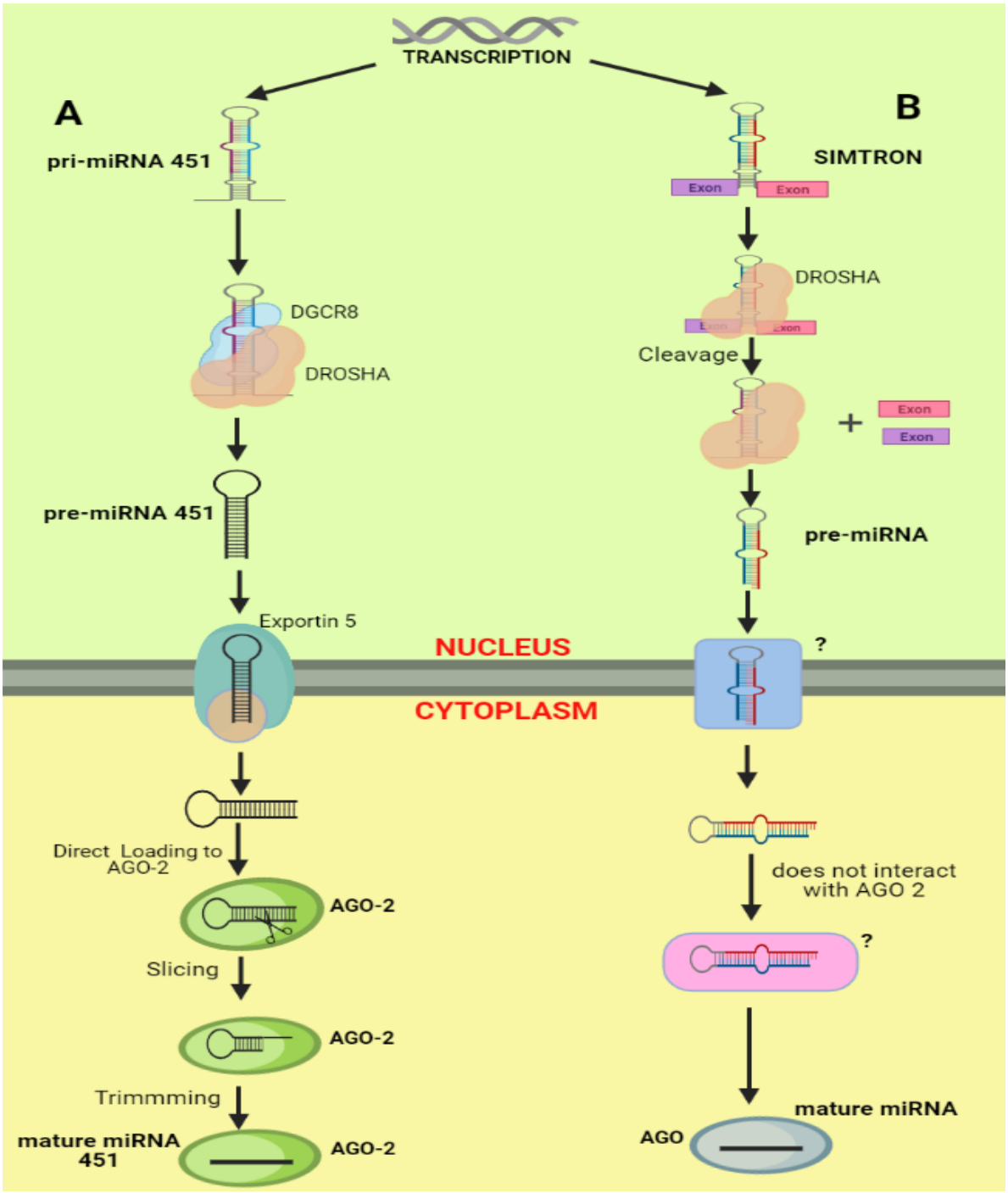

Figure 4. Dicer-independent, Drosha/DGCR8-dependent biogenesis. *A) Drosha produces a short pre-mir-451, to be exported by EXP5 to the cytoplasm and loaded without Dicer processing on Argonaute 2 (AGO2). The stem of pre-mir-451 is cleaved by AGO2, resulting in AGO-cleaved pre-mir-451 (ac-pre-mir-451). Then, miR-451 is subjected to a trimming reaction to produce a mature miR-451. B) The simtron pathway is a non-canonical pathway independent of Dicer and involves Drosha but not DGCR8. Unknown factors process simtron before they enter the RISC complex with any of the four human Argonaute proteins.*

4.3. The machinery involved in miRNA biogenesis

miRNAs need several proteins for processing and play a fundamental role in their biogenesis. The main proteins involved are Drosha, Dicer, Argonaut 2. In addition to additional proteins involved such as DGCR8, Exportin 5 and TRBP

4.3.1. DROSHA

It belongs to the endonuclease RNAsa III family associated with the protein DGCR8 in mammals or PASHA in *D. melanogaster* and *C. elegans.* It plays an essential role in miRNA biogenesis's initial process, forming part of the microprocessor complex. Drosha in the nucleus asymmetrically cuts the double chain of the primary miRNA (pri-miARN) at sites near the fork to form the so-called pre-miRNA with about 60-70 nucleotides. It consists of a Proline-rich domain, an Arginine-Serine-rich domain, two RNase III catalytic domains, and a double-stranded RNA binding domain (dsRBD) (54,77).

4.3.2. DICER

Endoribonuclease type III is associated with TRBP and PACT and is responsible for the production of mature microRNAs. Once the pre-miRNAs are transported to the cytoplasm by EXP5, Dicer cuts the single pre-miRNA chain's loop zone, resulting in a short duplex (~21 nucleotides) of miRNA with a guide and passenger strand. Furthermore, DICER is responsible for processing dsRNA in small fragments of siRNA (54,78).

4.3.3. ARGONAUTE 2

Argonaute proteins are responsible for post-transcriptional silencing guided by small RNAs. These proteins are characterized as being formed by two domains, PIWI and PAZ (by piwi-Argonauta-zwille). The PAZ domain has the function binding to the 3’ end of the RNA either from miRNAs or siRNAs. On the other hand, the PIWI domain is responsible for giving an endonuclease activity to the protein; however, this function by the PIWI domain only presents Argonaute 2 (AGO 2) proteins responsible for cutting the miRNA* in the miRISC complex. Once the Dicer protein generates the miRNA duplex with a guide and passenger strand, the guide strand binds to AGO while the passenger is degraded. Also, AGO2 to fulfilling its endonuclease activity, is responsible for binding the miRNA with its target and, in this way triggering post-transcriptional silencing (54,79)

4.3.4. DGCR8

It is a binding protein for the initial process of biogenesis, Drosha needs to be associated with the DGCR8 protein in mammals; however, its specific function is still unknown, but it is responsible for anchoring to Drosha to be part of the microprocessor complex and cutting the pri-miRNA. DGCR8 contains three domains, one WW domain and two dsRNA binding domains (dsRBDs). (80,81)

4.3.5. EXPORTIN 5

XPO5 (Exportin 5) is a gene that codes for proteins and is part of the karyopherin β family of transport factors. These proteins are responsible for transporting proteins from the nucleus to the cytoplasm and vice versa. The XPO5 plays a vital role in the miRNAs transport and uses the Ran-GTPase cofactor to transport the pre-miRNA through a nuclear pore from the nucleus to the cytoplasm. Once in the cytoplasm miRNA is released from XPO5-RanGTP when GTP is hydrolyzed (54,82). Another feature of XPO5 has recently been discovered Wu et al. states that "XPO5 has been recently shown to regulate the expression of Dicer, an RNase III required for pre-miRNA maturation". Then exportin 5 is responsible for the miRNA transport and intervenes in the expression of Dicer (54,82).

4.3.6. TRBP

TAR RNA-binding protein (TRBP) is a protein required to form the RISC complex, involved in miRNA's biogenesis. TRBP and PACT are two proteins with double-stranded RNA-binding domains (dsRBDs) acting as Dicer's cofactors to stabilize Dicer. It intervenes in the cleaves pre-miRNA hairpin substrates to generate a miRNA dúplex (microARN:microARN*) (54,83). TRBP and PACT relate to Dicer mediate miRNA isoform (isomiR) processing, resulting in related miRNAs of varying lengths and targeting specificity (83).

5. MECHANISM OF ACTION

As explained above, the miRNA can bind to its target depending on complementarity to directly degrade its mRNA target or suppress protein translation. After removing the miRNA passing chain, the RISC microprocessor and guide chain search for their mRNA target and come together to direct their post-transcriptional repression (68).

The mechanisms of action of miRNAs vary by their complementarity with mRNA, in other words, they come together depending on base-pairing interaction. Usually, miRNAs bind to the region 3-UTR to inhibit protein translation; also, it is in charge of mRNA deadenylation and decapping. On the other hand, when miRNA binds to the 5-UTR region, it can silence gene expression, but it is also responsible for interaction between the promoter region to induce transcription (71,84,85)

5.1. Silencing

The RISC complex and the miRNA guide strand bind to the mRNA target through Watson–Crick base pairing of the 3' UTR region of the target and miRNA. The miRNA's binding and recognition region comprises a highly conserved area called the "seed region", which is formed of 6 nt in length and is located around nucleotides 2 and 7 in direction 5'-3'. This region is responsible for recognizing the 3'UTR region of the mRNA target molecule, thus causing protein translation inhibition (68,86).

As explained above, the mechanism of action of miRNA will depend on the degree of complementarity of the base pairing, so if perfect or near-perfect complementarity occurs between miRNA and mRNA, the mRNA is split and degraded, which would determine the gene's silencing mechanism (86).

For miRNA to cut the mRNA target, the RISC complex must contain AGO2 protein, the only protein with endonuclease activity that will cut the mRNA. A perfect or almost perfect base pairing should be given for the protein and miRNA to act effectively (86). On the other hand, AGO proteins are involved in the inhibition of mRNA translation. Martinez & Dinkova (87) comments that in eukaryotic messenger RNAs, the eIF4E translation initiation factor is responsible for recognizing the 5' CAP structure at the 5' end and

activates the protein synthesis machinery to begin translation. Hence, AGO proteins are responsible for competing with factor eIF4E, inhibiting translation through interaction with various factors that will start the translation (73).

On the other hand, an imperfect base pairing, which occurs in most animals, could help translational repression through the union ARNm-miRNA's destabilization due to deadenylation and subsequent decapping (86). Several studies have shown that Ago proteins in miRNAs have achieved deadenylation in mRNA, which does not always cause the degradation of the target mRNA, but if its translational repression, on the other hand, when the shortening of the poly (A) tail followed by unpacking can lead to a degradation of the mRNA. For example, in *D. melanogaster*, the miRNA's Ago1 protein interacts with GW182 to recruit the CCR4 – NOT complex (consisting of two deadenylases, CCR4, and CAF1), which is responsible for promoting shortening of the poly(A) tail or deadenylation. This could be the end of the process causing a weak translation silencing; however, decapsulation could be given in charge of the DCP2 enzyme, followed by degradation of mRNA target by XRN1 (88,89)

5.2. Induction

Most studies related to miRNAs function focus on gene inhibition or silencing; however, several studies are on the regulation or induction of gene expression by miRNAs. Induction involves two protein complexes called miRNPS (microRNA ribonucleoprotein complex) to activate mRNA translation; although the specific process that occurs during activation of miRNAs-mediated gene expression is still unknown, the protein machinery involved in the process has been studied in certain species, for example, in *D. melanogaster*, the Ago2 protein together with the RISC complex bind to the eIF4E factor inducing the activation of translation directly. Another example of induction the transcription is through the binding of miRNA to the 5'UTR region of mRNA; it is responsible for encoding ribosomal proteins during amino acid starvation (71,73,90).

New forms of miRNAs-mediated gene expression induction are discovered every day and this makes the study on miRNA mechanisms of action more complex. However, it has become clear that gene expression induction occurs under specific conditions and specific

components of miRNPs (73).

6. MiRNAs ARE DIFFERENTIALLY EXPRESSED IN CANCER

Cancer is the world's second leading cause of death and encompasses a large number of cancers; according to the National Cancer Institute, common cancers in the 2020 statistics are breast cancer, lung and bronchial cancer, prostate cancer, and colon and rectal cancer, and women diagnosed with breast, lung, and colorectal cancer account for about 50% and men diagnosed with prostate, lung, and colorectal cancer account for about 43% (91). Every year there is an increase in people diagnosed with cancer; however, new early diagnosis methods have been found, increasing people's survival (92).

Cancer is formed by uncontrolled cell proliferation and exceeds the number of standard or healthy cells. Normal cells are damaged and die and replaced by new cells; however, damaged cancer cells survive, and new cells are produced, although they are not needed, and the accumulation of all of these cells combine to form a mass known as a tumor, and if malignant, they can invade other tissues in the body (92,93).

Cancer is developed by several processes that produce genomic alterations, affecting different molecular pathways, including alteration or deregulation of miRNAs (94). In recent years, miRNAs have been involved in various diseases, including cancer (95), “miRNAs are post-transcriptional regulators, and therefore mediate different tumorigenesis processes; inflammation, cell cycle regulation, differentiation, apoptosis, etc.” (96)

miRNAs in cancer were evaluated for the first time in B-cell chronic lymphocytic leukemia (B-cell CLL). CLL is classified as the most common leukemia; blood cancer results from a proliferative disorder of lymphocytes that accumulate in the blood, bone marrow, and lymphoid tissues. Its pathogenesis has been related to an inherited factor because several cases have been shown where it is common to find several people in the same family with this disease (97).

50% of CLL cases are caused by genetic abnormalities related to 13q14 chromosome deletions, but within the same loss of chromosomal material is the deletion or downregulation of two tumor suppressor miRNAs miR15 and miR16 (97). Cailin and

collaborates study peripheral blood samples from CLL patients showed that miR15 and miR16 are inside a small portion of the 13q14 chromosome part selected, thus influencing a downregulation expression of miRNAs (98). Besides, miR15 and miR16 are related to BCL-2 (the most crucial oncogene in CLL, anti-apoptotic protein) in reverse regulation, i.e., decreased expression of miRNAs would mean unregulated overexpression of BCL-2 (96,97).

After this evidence, several studies conducted using Northern blot or quantitative real-time polymerase chain reaction (qRT-PCR) to know the alterations or deregulation of miRNAs expressions in human cancer (92,94). Deregulation of miRNAs would be one factor that directs tumorigenesis; depending on the effect they generate on the target molecule; miRNAs can be oncogenes or tumor suppressors.

6.1. Oncogenic miRNAs (oncomirs)

Oncomirs are overexpressed miRNAs, so-called because they act similar to oncogenes. These miRNAs are responsible for promoting tumor development by causing negative regulation or inhibition of tumor suppressor genes (85,92).

MiRNAs are overexpressed in several tumors due to post-transcriptional deregulation. Among the oncomirs studied is miR-21; this miRNA is involved in cancer processes such as invasion and migration (85).

MiR-21 is oncomir in several cancers; for example, tissues with glioblastoma multiform (brain tumor) were studied for miRNAs profiles, their data showed that miR-21 is significantly overexpressed in tissue samples with glioblastoma, associated with blocking essential genes for apoptosis. That is, miR-21 plays a role as an oncogene in brain cancer (99). On the other hand, the overexpression of miR-21 was demonstrated in hepatocellular carcinoma, this miRNA regulates cell proliferation, apoptosis, and invasiveness by targeting PTEN, PDCD4, and RECK in HCC in carcinoma; therefore, miR-21 is an oncomir responsible for regulating the formation and progression of hepatocellular cancer (100). In breast cancer, the presence of miRNAs was studied, the miR-21 overexpression was found in breast cancer samples compared to standard samples, TGFB was predicted to

be the target gene of miR-21 (101), other breast cancer studies concluded that miR-21 is one of the miRNAs differentially expressed in Stage III breast cancer (102).

Similarly, there are other oncomirs already recognized as miR-155 or miR-27a. MiR-155 was investigated on lymphoblastic leukemia y high-grade lymphoma, demonstrating that this miRNA's overexpression suggests involvement in the two diseases' initiation and progression (103). MiR-27a was overexpressed in prostate cancer tissues and is responsible for the proliferation and cell cycle of prostate cancer by binding with the SPRY2 target molecule (104).

6.2. Tumor Suppressors MicroRNAs (Anti-oncomirs)

Tumor suppressor miRNAs suppress their expression in tumors and are named for their similar function to tumor suppressor genes (92). Anti-oncomirs inhibit tumorigenesis through oncogene repression (85).

Let-7 represents an anti-ocomirs, this miRNA was investigated in lung cancer. The first research into the level of expression of let-7 resulted in a reduced expression in lung cancer samples, associated with short survival (105), and similarly, another study reported that the expression of two members of the let-7 family (hsa-let-7a-2 and let-7f-1) was reduced in lung cancer, and to hsa-let-7a-2 was correlate with poor survival (106)

MiRNAs, miR-125a, and miR-125b were investigated in colorectal cancer. Their results were a downregulated expression in tissue samples with colorectal cancer. They studied hyper methylation of the two miRNAs and correlated inversely to the two miRNAs with CpG island methylation in colorectal cancer, suggesting miR-125 as a tumor suppressor of this type of cancer (107). Besides, low regulation of miR-125 was found in hepatocellular cancer, allowing control of abnormal regulation of SIRT7 (contributes to the proliferation and transformation of malignant hepatocellular tumor) (108).

All types of cancer have a genomic alteration of miRNAs, which may suppress expression (anti-oncomirs) or overexpression (oncomirs); therefore, it points to a link between miRNAs and the development and progression of cancer. Knowing the altered miRNAs and their role in cancer would help identify appropriate treatment. Besides, miRNAs will

potentially develop therapies by identifying target molecules and finding new biomarkers for cancer.

miRNA	Target	Types of Cancer	Characteristic	Reference
miR-196-5p	HMGA2	Hepatocellular Carcinoma	tumor suppressor	(109)
miR-375	KLF4	Colorectal Cancer	tumor suppressor	(110)
miR-141-3p	ATF5	Glioma	tumor suppressor	(111)
miR-499-5p	VAV3	Lung Cancer	tumor suppressor	(112)
miR-205	MED1	Prostate Cancer	tumor suppressor	(113)
miR-433	Rap1a	Breast Cancer	tumor suppressor	(114)
miR-181	SPRY4	Breast Cancer	Oncogene	(115)
miR-486-5p	PTEN	Cervical Cancer	Oncogene	(116)
miR-155	SOCS1	Thyroid Cancer	Oncogene	(117)
miR-21		Glioblastoma	Oncogene	(99)
miR-15 and miR-16	Bcl2	Chronic lymphocytic leukemia	Oncogene	(118)
miR-372 and miR-373	LATS2	Testicular germ cell tumors	Oncogene	(119)

Table 3. The list of miRNAs that have experimental evidence supporting a tumor suppressor or oncogene function in cancer.

7. CIRCULATING MiRNAs

Studies have revealed extracellular miRNAs or circulating miRNAs. Various tissue and cell miRNAs are found in extracellular fluids such as saliva, plasma, serum, urine, tears, breast milk, peritoneal fluid, and cerebrospinal fluid, and these are secreted via extracellular vesicles (EVs) or protein complexes. Unlike cell RNA, extracellular miRNAs are resistant to degradation caused by RNAsas (71,120,121).

Because miRNAs have been considered easy to detect by non-invasive methods, it has been possible to study miRNAs in body fluids and associate them with the progression of diseases such as cancer. Even Kosaka et al. stated that miRNA expression regulation has been shown to contribute to tumor progression via various mechanisms including genetic modifications in microRNA such as mutations involving miRNA loci, also epigenetic silencing, the dysregulation of specific miRNA-targeting transcription factors, and the inhibition of processing (108) For this reason, the expression profile of extracellular miRNAs is considered a helpful tool to achieve disease diagnosis and prognosis.

As a result, miRNAs have been postulated as possible biomarkers to diagnose diseases such as cancer, inflammatory diseases, autoimmune, intestinal diseases, etc. Studying miRNAs as biomarkers were the main objective that has led many scientists to investigate the mechanisms by which extracellular miRNAs are transported. So, two theories have been produced to explain the secretion of miRNAs, the first is focused on transport through vesicles and the second theory is the association with proteins. In addition to explaining extracellular transport, it also explains miRNAs' stability in an extracellular environment, protecting itself from endogenous RNAse activity (123,124).

8. SECRETION OF EXTRACELLULAR MiRNAs

Cellular communication of miRNAs to an extracellular environment occurs through two mechanisms: (A) Vesicles traffic, through multivesicular bodies (MVB) b) Transport as part of protein-miRNA complexes (123).

8.1. Transport Via Extracellular Vesicles

Several studies have focused on the transport of miRNAs through vesicles such as microvesicles, exosomes, and apoptotic bodies; although it is not known for sure what the vesicular transport is for each miRNA, some research is carried out on the size, density, method of purification, surface markers, the process of formation, and release mechanism to differentiate each extracellular vesicle (123).

First, we have smaller vesicles with a size of 40-100 nm called exosomes. The exosomes are vesicles generated by the fusion of multivesicular bodies (MVBs) and the plasma membrane (123), and they are found in most biological fluids. The membranes of the exosomes are mainly made up of lipids and proteins and are originated by most cells through an endosomal pathway; therefore, it has given the exosomes a fundamental role in cell-cell communication (125).

The beginning of the exosome biogenesis occurs by invagination of the inner membrane of the endosomes. The exosomes enter the cell again, and here they are called endosomes and form intracellular multivesicular bodies (MVB) rich in cholesterol, then fuse with the cell membrane and leave the cell as exosomes (125,126). Enzyme activation and energy (ATP) are required to form and release exosomes (124). On the other hand, there are three mechanisms of interaction between exosomes and receptor cells; the first mechanism is a direct interaction between transmembrane proteins in exosomes and signaling receptors in target cells, the second mechanism involves the transfer of exosome content to the cytosol through fusion with the plasma membrane of the target cell and the third mechanism is the internalization of exosomes within target cells; however, the exosomes can have two paths from here. The first path is the fusion of the exosomes to become endosomes and, through a process of transcytosis, will release the content in neighboring cells; in the second path, the exosomes can suffer degradation when they mature into lysosomes (127).

There are several evidence to test the exosomes as an intercellular transport medium for miRNAs; also, the exosomes protect miRNA from degradation by remaining stable in the extracellular environment and achieving communication with the target cell (125). Some profiles of miRNAs that are incorporated into the exosomes have been demonstrated; for example, studies by Guduric-Fuchs et al. concluded that miR-150, miR-142-3p, and miR-451 enter the exosomes (128). Exosomes are related as a means of cancer proliferation; Zhang et al. argues that exosomal miRNAs are involved in the development of diseases, induce angiogenesis, and intervene in cancer metastasis (127), for this reason, one study found that miRNA let-7 is abundant in cancer cell exosomes of the AZ-P7a family, but when analyzed in other exosomes of cancer families there were no let-7 (127).

Although exosomal miRNAs' functions are still under investigation, some studies have been obtained showing that exosomal miRNAs could be important in tumor cell expression. One study has shown miRNA-210 as the main exosomal miRNA expressed by hypoxic tumor cells, with the potential to influence angiogenesis or the tumor microenvironment of cancer cells. Exosomal miRNA, miRNA-210, derived from hypoxic tumor cells, influences tube formation in human umbilical vein endothelial cells (HUVECs); for this reason, miR-210 has been seen as a way to inhibit angiogenesis or elimination of tumor-initiating cells (129). On the other hand, exosomal miRNAs have also been shown to intervene in an immune response, the miRNAs, miR-21, and miR-29a in tumor-secreted exosomes act as a ligand to bind to toll-like receptors (TLRs) in immune cells to trigger a prometastatic inflammatory response. In this way, it would aid the immune system's communication, representing a new form for cancer treatment (130).

Second, vesicles with a size 100 to 1000 nm in diameter called microvesicles (MV) or ectosomes are found, they are originated and give off from the plasma membrane through gemmation and fission outward; therefore, the membrane consists of a lipid bilayer with lipids similar to the plasma membrane of the original cell (124,131). Microvesicles can be found in various biological fluids such as blood, urine, saliva, tears, etc, and inside can be found mRNA, miRNA, and cytoplasmic proteins (132). Initially, MVs were believed to be a cellular waste; however, over time, it was shown to help cell-cell communication; therefore, they intervene in a variety of biological processes (124,133). The mechanism of

communication of the microvesicles with the target cell is through the target cell receptors activated by the MVs ligands (134).

The miRNAs have been packaged in microvesicles, for example, in cells such as mesenchymal, cancerous, endothelial, mast cells, and platelets. Studies demonstrated that mir-143 was secreted in microvesicles from THP-1 cells of human monocytic leukemia (135). Microvesicles have also been found with miRNAs in peripheral blood, or various types of cancer such as lung, breast, ovarian, etc., Dahiya and col. Performed studies were showing that expressed miRNAs existed in patients with ovarian cancer (136). On the other hand, microvesicles have been a way to transfer the content to embryonic cells; one study found that microvesicles designed can bind to embryonic stem cells and transfer their contents, and joining this technique with miRNAs content can be a valuable tool to control signaling within embryonic cells (134).

Third, vesicles larger than 1μm in diameter are found and are known as apoptotic bodies; these vesicles are originated from cells in apoptosis (programmed cell death) (133). When cells suffer from apoptosis, they release vesicles exposed to phosphatidylserine (PS) and form in the late stage of the cell death process. Like extracellular vesicles, apoptotic bodies carry molecules such as miRNA, mRNA, and DNA fragments (124).

Concerning miRNAs, a study by Alma Zernecke and collaborators (137) observed an enriched presence of miR-126 in apoptotic bodies derived from human umbilical vein endothelial cells (HUVECs). They also demonstrated that the miR-126 triggered CXCL12 production (C-X-C Motif Chemokine Ligand 12, is a protein-coding gene), resulting in a reduction in atherosclerosis in mice. Apoptotic bodies revealed a new tool in tissue repair and angiogenesis (137).

The three types of extracellular vesicles provide new tools to help control, diagnose and predict diseases such as cancer, and take a step toward a new method to solve questions that have occurred for years. However, to achieve progress, it is necessary to fully understand the mechanisms of formation, secretion, and release of content to know at what point it can interfere with the inhibition or activation of processes.

8.2. Transport via RNA-binding proteins

As we observed, miRNAs can be transported by extracellular vesicles, however, it has been found that miRNAs can also be transported with the association to lipoproteins into extracellular fluids. Lipoproteins are composed mainly of lipids and proteins and are responsible for transporting fats throughout the body. Some lipoproteins transport cholesterol from the liver to tissues called low-density lipoproteins (LDL) or the lipoproteins that transport cholesterol from tissues to the liver for excretion are called high-density lipoproteins (HDL) (124). Among the lipids that form lipoproteins are phosphatidylcholine, which forms stable complexes with nucleic acids, and interaction through divalent cation bridges between lipids with RNA and DNA has been proven (138).

For this reason, miRNAs were associated with lipoproteins to be transported to the extracellular medium, especially HDL. Vickers et al. observed miRNAs in HDL and LDL derived from blood plasma but in greater HDL quantity. The miRNAs identified were miR-223, miR-105, and miR-106a, and it was observed that mice with hypercholesterolemia and dyslipidemia have a significant HDL-miRNA profile compared to healthy models (138).

The mechanism of exporting miRNAs through HDL is not yet entirely clear, the components of miRNA such as GW182 and AGO2 are necessary for its operation, and they are involved in extracellular transport (131). It is known that GW4869 is involved in the inhibition of neuronal sphingomyelinase 2 by increasing the export of miR-223 to HDL (139) Then the mechanism of release of miRNA cell content by HDL could be mediated by SR-BI (lipoprotein receptor), delivering to miRNA in the cytoplasm, which would help prevent its degradation by making them more stable (131,139).

High-density lipoproteins are composed of apolipoproteins (apo), non-polar lipids (triglycerides and cholesterol esters), and amphipathic lipids (phospholipids and free cholesterol) (140). The apolipoprotein component of HDL is necessary for the systemic release of small interfering RNA (siRNA) (138). On the other hand, miRNAs were associated as components affecting HDLs, in their biogenesis, cellular cholesterol efflux, selective cholesterol uptake from HDL, and bile transport; for example, miR-33a and miR-33b are involved in HDL production through binding to the molecule ATP-binding cassette

transporter A1 (ABCA1). ABCA1 is responsible for controlling the HDL output rate to apolipoprotein A-I (apoA-I); when the miRNA binds ABCA1, it regulates HDL plasma levels (141).

With all the studies and research, it could be said that an HDL-miRNA relationship could mean an essential biological relevance through different mechanisms and functions compared to extracellular vesicles; however, studies on HDL-miRNA profile-related mechanisms are still needed, the understanding could provide a helpful tool for the detection of diseases as potential biomarkers.

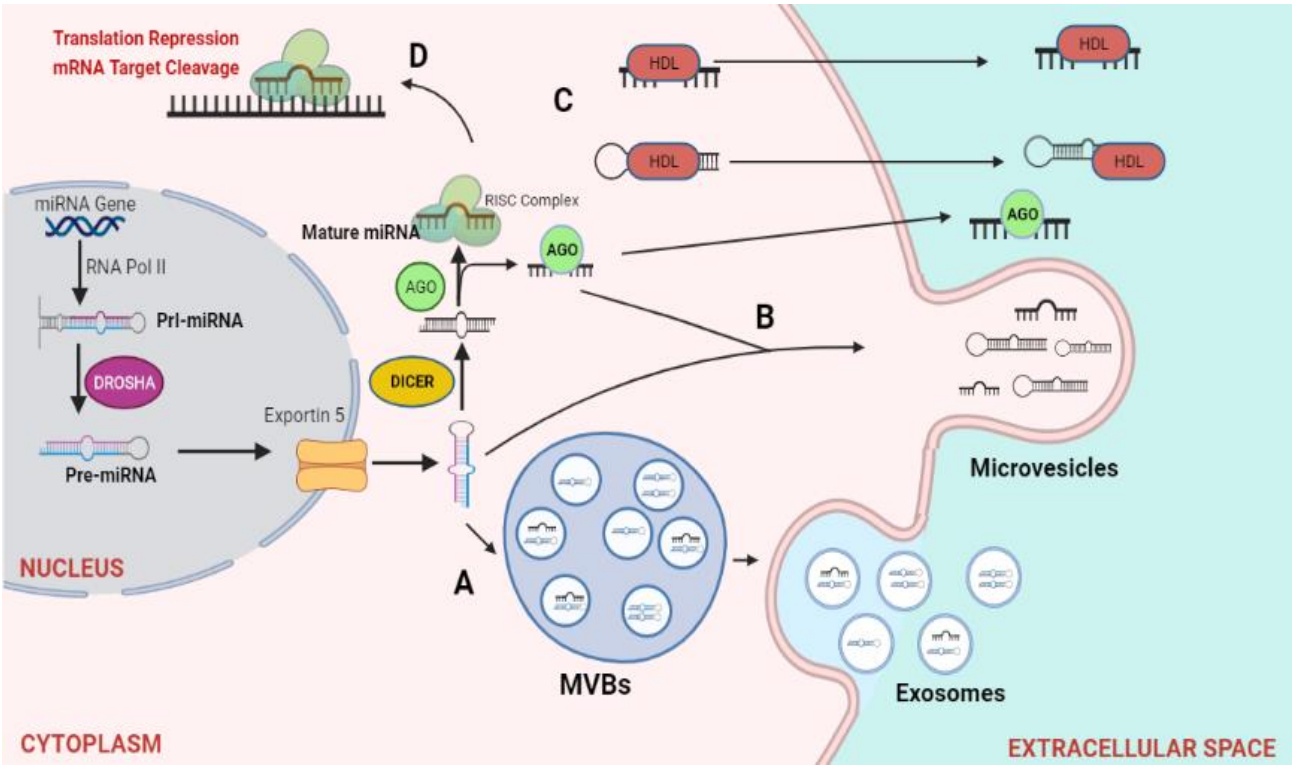

Figure 5. Methods of microRNAs release into the extracellular space. *The miRNAs (pre-miRNA or mature miRNA) can be incorporated into A) small vesicles that originate from the endosome and are discharged when multivesicular bodies (MVB) fuse with the plasma membrane, called exosomes, B) or can be released by microvesicles, C) can be coupled with Ago2 protein or HDL. D) The mature miRNA guides RISC complex to silence the target mRNA through mRNA cleavage, translational repression.*

9. MiRNAs AS BIOMARKERS

As mentioned above, miRNAs are molecules that mediate signaling and communication between cells; for this reason, they have been considered as potential biomarkers for the diagnosis and prognosis of diseases. Also, the discovery of miRNAs in biological fluids gives an extra benefit to apply as biomarkers of different diseases such as autoimmune diseases, cardiac diseases, diabetes, cancer, etc.

A biomarker is an acronym for "biological marker," this is only a marker or substance used as a biological indicator, which could reflect the risk or the presence of a disease. With technological advances, biological indicators have become more precise, reliable, and useful as a tool to prevent health risks.

For the selection and validation of a biomarker, it must meet some criteria such as: 1) The biological process must be identified, in other words, knowing the specificity of the pathology investigated. Previous studies should be carried out that reveal the characteristics of the biomarker, the exposure agent, and the pathology. 2) A selection of the evaluation procedures available for analysis, with the reliability of sample integrity. 3) It should be reproducible for the clinic's investigation and have a relatively cheap, simple, and fast detection method. 4) It should be obtained from an easily obtained biological source (biological fluids) (142,143)

MiRNAs play an essential role in cellular processes and regulatory functions such as modulation of myogenesis and carcinogenesis, and they are involved in the differentiation, regulation, and replication of cells. Due to miRNAs' participation in the development of neoplasms such as cell proliferation, apoptosis, and angiogenesis, and even on organogenesis and hematopoiesis, they have been proved to mediate different processes of tumorigenesis (54,144).

There are numerous studies on the potential of miRNA as a biomarker, the evidence suggests that it could be an important biomarker in diseases with late diagnosis potential. The potential of miRNAs originates from the discovery of circulating miRNAs in different biological fluids, where the number of miRNAs profiles was observed to be higher in

cancer patients than in healthy patients (144). MiRNAs have a wide variety of advantages that make them good biomarker candidates. 1) Biomarkers should be easy to obtain, and miRNAs can be obtained from body fluids, and their process of obtaining is non-invasive and does not require tissue samples. 2) MiRNAs have high specificity and sensitivity due to tissues or cells of origin, therefore, they are sensitive to the development or progression of diseases. 3) Detection methods such as nucleic acids have advanced their technology, making miRNAs easy and fast to detect and quantitatively (131,143)

Detection of diseases such as cancer requires invasive or painful methods for diagnosis or prognosis; however, new non-invasive forms were found, such as circulating miRNAs biomarkers.

10. TYPES OF FLUIDS WITH MiRNAs

10.1. Saliva

Saliva is a secretion from salivary glands and moistens the entire oral cavity, is made up of water, enzymes, proteins, antibodies, hormones, and cytokines (145,146). The presence of miRNAs in saliva has meant an advance in the research of oral diseases, cancer, and systemic diseases through salivary diagnoses. Research has shown the application of circulating miRNAs saliva as potential biomarkers to detect diseases related to tissues near the salivary gland (147). Several studies are presented showing the presence of miRNAs in saliva and the use of them as a non-invasive marker of disease detection

Research by Noh Jin Park and colleagues showed the presence of miRNAs in saliva and saliva supernatant. Their studies were conducted on 12 participants, and 47 miRNAs were detected in saliva and 52 miRNAs in saliva supernatant. At least half of the miRNAs of saliva and saliva supernatant are share in 11 participants. On the other hand, they tested whether the miRNAs found served as biomarkers in Oral squamous cell carcinoma (OSCC); their results showed miRNAs: miR-200a and miR-125a as possible biological markers of this type of cancer because they observed that the amount present in OSCC patients was much lower than in healthy participants (148).

Lirong Wu et al. showed studies of miRNAs in saliva; their research was based on finding a diagnosis and prognosis method for Nasopharyngeal carcinoma (NPC). They found 1064 miRNAs in saliva samples with NPC, of which 51 were highly expressed compared to samples from healthy people; for example, miR-3679-3p, miR-574-5p, miR-205-5p, and miR-6131 increased their expression in patients with NPC. On the other hand, they also found 47 miRNAs such as miR-30b-3p, miR-575, and miR-650, which complied with negative regulation in NPC samples. They concluded that miRNAs could serve as non-invasive diagnostic biomarkers to detect nasopharyngeal carcinoma (147)

On the other hand, studies have investigated the relationship between saliva miRNAs and pancreaticobiliary tract cancer, looking for miRNAs as diagnostic biomarkers of this disease. The report made by Tatsuya Machida and colleagues showed results where two miRNAs (miR-1246 and miR-4644) in salivary exosomes could be biomarkers of diagnosis

for pancreaticobiliary tract cancer. They were based on previous studies where miRNAs, Mir-1246, Mir-3976, Mir 4306, and Mir 4644 had higher expression levels in serum exosomes in patients with pancreatobiliary tract cancer; they concluded that saliva like a final product of blood circulation, then blood molecules could be present in saliva. For this reason, they evaluated the presence of the 4 miRNAs in saliva exosomes of patients with this type of cancer; however, they found only two miRNAs that showed high expression and would serve as biomarkers (149).

10.2. Urine

Urine is liquid excrement secreted by the kidneys and excreted through the urethra, composed of 91-96% water and the rest by inorganic salts, urea, organic compounds, and organic ammonium salts and has a light yellow color due to the presence of cells, crystals, cylinders, detritus, proteins, fats and mucus (150). The appearance, color, and smell of urine may define or indicate the presence of disease; similarly, it has been found that the urine components could be used as biomarkers or diagnostic tools for a various of diseases (151). For years urine has been used as the body fluid that allows non-invasive sampling to detect various diseases, especially urological; for this reason, the presence of miRNAs in urine has been considered possible biomarkers. Several studies have shown miRNAs in the urine and their importance for diseases related to oncology, nephrology, and cardiology (152).

Pospisilova S. and colleagues studied the possibility of using miRNAs as biomarkers for the diagnosis of bladder carcinoma; based on previous studies, they tested with known miRNAs profiles; for example, miR-99a was detected in tissues and blood of bladder cancer patients. They found miR-125b, miR-204, miR-99a, miR-30b, and miR-532-3p, with the down-regulated urine supernatant levels in patients with bladder cancer. Also, the researchers were able to discover one of the target molecules of miR-125b, the oncogene E2F3; it is bind to miRNA to produce inhibition of cell growth in bladder cancer. The research resulted in a group of miRNAs that could mean a new non-invasive method to detect bladder cancer in its early stage (153).

Studies showed that miRNAs are involved in kidney cancer expression; for example, they

have been reported to have an oncogenic effect on renal cell carcinoma (RCC). For this reason, White and his collaborators performed tests on miRNAs of the urine to check if they can intervene in RCC metastasis and thus have a therapeutic application in kidney cancer. Their results demonstrated to the miR-215 joined its target molecule to act as an oncogene or tumor suppressor. The miR-215 binds SIP1/ZEB2 to suppress the epithelial-mesenchymal transition; also, a high expression of miRNA leads to a decrease in cell migration of cells in RCC. Overexpression of Mir-215, on the other hand, was used to investigate genes involved in cancer metastasis and progression, and genes such as MMP7 and MMP13 were discovered (involved in the degradation of the extracellular matrix and the deregulation of miRNA); their interaction can affect cell invasion. Their findings suggest that Mir-215 plays an important role in cancer development and metastasis, and as a result, it will be a step forward in our understanding of metastasis processes, and it may be a biological predictor of prognosis for RCC patients (154)

For early detection of urothelial carcinoma (UC), cystoscopy is used; however, this procedure may cause pain and discomfort. As a response, there is a need to find a biomarker that can help diagnose UC with high sensitivity and specificity. Mir-96 and Mir-183 may be indicators of UC tumors; according to studies by Yasutoshi Yamada et al., their findings revealed that UC patients had higher levels of expression of miR-96 and miR-183 than healthy controls, they also demonstrated that the two miRNAs could be used as biomarkers for urothelial carcinoma detection because they have high sensitivity and specificity. With their studies detecting two significant miRNAs in 27 patients with UC, the patients were initially negative for cystoscopy, underwent surgery, and then had their samples reanalyzed, with the latest tests showing a decrease in the amount of Mir-96 and Mir-183. The two miRNAs were found to be important markers of UC and to be a less invasive and painful diagnostic tool than cystoscopy (155).

10.3. Plasma/serum

New cancer diagnosis types were pursued using miRNAs circulating in plasma and serum as a non-invasive diagnostic tool. According to several studies, the epigenetic and genetic features of cancer can be found in serum and plasma; for this reason, one of the first experiments to discover circulating miRNAs was done on serum (156). Around 10% of

human miRNAs are present in plasma, and the majority are found in cancer-related genomic sites; therefore, they can play an essential role in tumorigenesis (157).

While miRNAs in plasma have been demonstrated, further research is needed to determine each miRNA's function in cancer. As a result, Charles H. Lawrie et al. researched to see whether miRNAs MIRN155 (miR-155), MIRN210 (miR-210), and MIRN21 (miR-21) can be used as diagnostic markers for diffuse large B-cell lymphoma (DLBCL). In comparison to healthy control samples, the three miRNAs serum samples with DLBCL had higher expression levels. Furthermore, a high serum MIRN21 expression in DLBCL patients has been associated with a more extended survival period. The three miRNAs could then be used as a biomarker for DLBCL diagnosis and prognosis, demonstrating that circulating miRNAs can be used as a non-invasive process (156).

Since MiR-19a is expressed in esophageal squamous cell carcinoma (ESCC) tissues, researchers looked into whether miRNA might be a new non-invasive biomarker with higher sensitivity and specificity for detecting ESCC in its early stages. Yongying Bai and colleagues' findings revealed that miR-19a expression in plasma was higher than in control samples, and its sensitivity was also tested. Although they had higher sensitivity and specificity (89.34 and 52.50 percent, respectively) than existing biomarkers, it was decided to improve sensitivity by binding a biomarker called Cyfra21–1 with Mir-19a detection accuracy. It achieves a higher sensitivity for early diagnosis of ESCC due to a high level of diagnosis. Patients with esophageal squamous cell carcinoma are diagnosed after 5 years, so attempting to find a biomarker that can help define an early cancer stage is fundamental. Their research identifies a new biomarker that, when combined with Cyfra21–1, can be used to aid in the early detection of ESCC (157).

Aysegul Gorur et al. conducted another study to determine miRNA profiles that influence or diagnose gastric cancer (GC). Numerous studies have revealed miRNAs in gastric cancer patients, with some regulating negatively and others positively. As a result, the study discovered 27 miRNAs expressed in gastric cancer samples, 14 of which were negatively regulated, and 11 were positively regulated. For example, it was discovered that miR-195-5p has a negative regulation in serum samples from patients with gastric cancer compared

to control samples, indicating that this miRNA is a tumor suppressor and can aid in gastric oncogenesis (158).

11. BREAST MILK

Breast milk is milk produced by the mammary glands and comprises the gland's components. It is essential for newborn mammals, including humans. Breastfeeding refers to a feeding system that protects the newborn. Given the importance of keeping infants safe in humans, the composition of breast milk is significant due to the reduction in infant morbidity and mortality, human breast milk is classified into stages based on volume, duration, and composition (159,160).

11.1. Breast Milk Classes

11.1.1. Colostrum

It appears 5 to 7 days after birth, is yellowishly caused by carotenes and has a sticky consistency due to its high density. The volume gradually increases early stages with 2 to 20 ml/day to satisfy the newborn and increasing according to the need, intensity, and frequency of the suction stimulus, until reaching volumes of 500-750 ml/day in the final days. Colostrum contains proteins (particularly immunoglobulin A (IgA)), fat-soluble vitamins (E, A, K), carotenes, some minerals such as sodium and zinc, lactoferrin, growth factor, and Bifidus lactobacillus, and less fat, lactose, and water-soluble vitamins. Its composition protects the newborn, through immunoglobulins, against environmental germs that can lead to infections or allergies (159,161,162).

11.1.2. Transitional Milk

Its production begins after colostrum and lasts between five and ten days. Increased levels of fat, lactose, and hydrosoluble vitamins due to higher cholesterol and phospholipids and decreased levels of protein, immunoglobulins, and fat-soluble vitamins, because the volume of production is increased and diluted, its volume reaches 400-600 ml/day by day 15. It has white coloration due to fat emulsification and the presence of calcium caseinate (159,161,162).

11.1.3. Mature Milk

Its production is from the 15th day after birth and could last for the next 15 months. Its volume can reach 700-900 ml/day in the first semester and 600 ml/day over the next few months. It contains fats, proteins, and carbohydrates, ensuring efficient absorption and digestion, resulting in a more efficient immune system. It also contains an osmolarity of 287 to 293 mOsm and an energy content of 670 to 700 kcal/L provided by carbohydrates and fats (159,162).

11.1.4. Pre-Colostrum

Since the 16th week of pregnancy, it is a plasma exudate produced by the mammary gland. When a baby is born before 35 weeks of gestation, it contains immunoglobulins, proteins, fatty acids, total nitrogen, magnesium, iron, sodium, and chlorine; however, it has low lactose concentrations to low lactase activity in the preterm infant (161,162).

11.2. Benefits of Breastfeeding

Breastfeeding has numerous advantages for infants, and the World Health Organization (WHO) recommends breast milk feeding exclusively for the first six months, without solids or liquids, and as a complementary diet until two years of age. These recommendations are based on much research on the multiple benefits of breast milk (160,162).

11.2.1. Benefits for the newborn

Breastfeeding has benefits such as immunity to environmental germs during the first six months of life, as the infant's immune system is lower than that of adults; therefore, breast milk should be regarded as the "first vaccine" that protects the child against infection. Breast milk contains a variety of humoral and cellular immune components that aid in the formation of a barrier against viruses, bacteria, and parasites, as a result, breastfeeding has been shown to delay the onset of asthma, allergic rhinitis, atopic dermatitis, and food allergies, also, decreases the onset of several diseases, including rheumatoid arthritis, ulcerative colitis, multiple sclerosis, and Crohn's disease (161)

Children who feed breast milk for the first six months are less likely to develop or die from diseases such as diarrhea, pneumonia, or allergies. In 1987, the research showed that infants

who only fed breast milk had a lower risk of dying from diarrhea and pneumonia (163). Furthermore, data from infants up to 8 months old in the UK revealed that exclusive breastfeeding protects newborns from hospitalization for diarrhea or respiratory tract infection (164).

Human milk serves as nutritional support for infants, adapting to their needs. In newborns under one-year-old, the nutrients in the milk provide maximum bioavailability. Lipids are the primary source of energy in breast milk; on the other hand, lactose is the most abundant carbohydrate in breast milk and aids in the growth and development of the central nervous system; besides, its presence in the small intestine promotes intestinal transit, which helps to avoid constipation. Casein, a protein, is responsible for milk's easy digestion because it forms soft clots in the stomach; cysteine and taurine promote brain myelination. The osmolarity of breast milk and its content in modulating factors and digestive enzymes allow for digestive system development and more excellent digestibility (160,162,165).

11.2.2. Mother's Benefits

Breastfeeding immediately after delivery releases oxytocin through the newborn, sucking the nipple, which aids in uterine contractions and the rapid expulsion of the placenta, it also helps prevent anemia postpartum bleeding by reducing vaginal bleeding (160,162).

Oxytocin secretion helps prevent postpartum depression because it acts as a calming and relaxing effect on the mother. Furthermore, breastfeeding secretes endorphins that cause pleasure sensations, which benefits the baby because it is transferred through the milk, resulting in an extraordinary mother-child bond (160).

Breastfeeding helps mothers quickly regaining their pre-pregnancy weight. Exclusive breastfeeding secretes prolactin, which increases lipoprotein lipase activity in the mammary gland but inhibits it in the subcutaneous cell tissue, resulting in a decrease in fatty tissue deposit; women can restore their pre-pregnancy weight in this manner (160).

A long-term advantage for the mother, still under study, is breast cancer protection. According to research, women who have breastfed at least once have a lower risk of developing breast cancer. A study was conducted, and an inverse relationship between breastfeeding and breast cancer was discovered, particularly in women under 40 (162,166).

12. MicroRNAs IN BREASTMILK

Breast milk is regarded as the ideal food for an infant's proper development; therefore, its composition is being studied to answer questions about its benefits to both the newborn and the mother. Human milk contains essential nutrients such as carbohydrates, proteins, and lipids, and bioactive factors such as cells, anti-infectious and anti-inflammatory agents, growth factors, and prebiotics (167). Breast milk bioactive factors perform immunological functions that help protect the newborn (168). As a consequence, recent studies have concentrated on bioactive components, such as microRNAs (miRNAs) (169).

MiRNAs are found in biological fluids, but breast milk is the fluid that contains the highest concentration of miRNAs (1400). Exosomes, as previously stated, capture circulating or extracellular miRNAs to protect them from degradation into extracellular fluids (169). Because exosomes can be found in any biological fluid, the presence of miRNAs in breast milk was examined in three fractions: cells, lipids, and skim milk. When compared to cells and lipids, the skim milk fraction contains the most miRNAs (170).

According to Jessica A. Weber and colleagues' research, skim milk (mature) and skim milk (colostrum) contain approximately 429 and 386 miRNAs, respectively. In breast milk, miRNAs were identified as miR-193b, miR-10a, miR-924, miR-518c *, also found in the placenta, in colostrum miRNAs were identified as miR-130a *, miR-193b *, miR-192 *, miR-18a *. A study on 12 bodily fluids discovered common miRNAs among most of these fluids as miR-509-5p, miR-515-3p, and miR-335 *, implying a common origin for all of them (121).

Similarly, there is evidence that miRNAs appear in milk lipids, Munch Erika et al. analyzed breast milk samples and discovered 308 miRNAs enriched in breast milk fat globules, which are thought to target 9074 genes (171). The amount of miRNAs in human milk cells has been poorly studied; however, Alsaweed M. et al. performed a comparison between maternal peripheral blood mononuclear cells (PBMCs) and breast milk cells to observe the number of miRNAs for each, there were 292 miRNAs found in breast milk cells and 345 in PBMCs (169). These studies have found many miRNAs in all three fractions of breast milk, which could help identify miRNAs involved in various nutritional or immunological processes.

12.1. Human Milk miRNA from the mammary gland

According to research, the miRNAs found in breast milk are produced by the mammary gland (172). Their relation was demonstrated by studies in mammalian animals during their lactation period, which discovered that the same miRNAs found in breast milk were also found in animals' mammary glands.

A study in the tammar wallaby model detected the presence of miRNAs in milk for the first time during their lactation status. Among the most abundant miRNAs were miR-191, miR-184, miR-181, miR-148, miR-375, and the let-7 family (7f, 7a, and 7i) distributed in all three lactation phases (173). To determine whether the mammary glands produce miRNAs, it is first established that tammar wallaby lactation is divided into three phases: gestation, phase 2A (up to day 100), phase 2B (from day 100-200), and phase 3. (after day 200) (173) Based on tests performed on the same animal model, where baby exchanges were performed to feed them with breast milk from mothers with a more extended period of breast-feeding (170 days), the result was an acceleration of growth and ripening of the baby's stomach (174). Another study in bovine milk compared the presence of miRNAs in each milk fraction (fat, whey, and cells) to those in the mammary gland, revealing that fat miRNAs in milk were similar to those in the mammary gland (175).

Three approaches are being investigated. The first is that miRNAs may be involved in the development of animal breeding, Second, the types of miRNAs found in breast milk are similar to those found in the breast gland; and third, different types of miRNAs are detected during each lactation phase. The miRNAs discovered in breast milk could be markers for cellular activities in the mammary glands (172). As a result, studies of miRNAs in breastfeeding may represent a new tool for studying and understanding the mammary gland's biology and contributing to the development of biomarkers for pathology related to this gland.

12.2. Transmission of miRNAs from mother to newborn

Several studies have found that miRNAs are passed from mother to child via breastfeeding and are transported through exosomes, microvesicles, and somatic cells to avoid degradation in the infant's gastrointestinal tract (176).

Extracellular vesicles travel through the newborn's gastrointestinal tract and can withstand adverse conditions such as high temperatures and acid pH, endocytosis then captures these vesicles and internalizes them into the bloodstream, where they can reach different cells, tissues, and organs of the newborn, and participate in the epigenetic regulation of various functions such as immunity development or protection (170,176).

12.3. MiRNAs act on the immunity of the newborn

Immune regulation can lead to a variety of diseases, including autoimmune pathologies and cancer (177). Recent research has revealed that miRNAs play a role in immune regulation, contributing to monocyte and neutrophil, B and T cell development, and inflammatory processes. (178). As a result of these findings, miRNAs appear to support the differentiation and development of hematopoietic lineages (177). For example, the miRNAs, miR-181a in the thymus and miR-223 in the bone marrow; according to Lindsay Mark (178) these miRNAs are involved in the development of pluripotent hematopoietic stem cells (HSCs) such as B and T cells. The miR-17-92 cluster comprises six miRNAs (miR-17, miR-18a, miR-19a, miR20a, miR-19b-1, and miR-92–1) B-cell regulation; also mir-17-5p, mir-20a, and mir-106a are all involved in monocyte maturation and differentiation. In mouse studies, miR-181a plays a positive role in B cells; similarly, they participate in T cell differentiation; however, T cells' role is still being investigated (178).

Exosomes, on the other hand, contain immune-relevant structures, indicating their involvement in the immune system. Human milk contains exosomes, which help in the development of the newborn's immune system; according to one study, exosomes in breast milk contain MHC classes I and II, CD63, CD81, and CD86, which are responsible for inhibiting cytokine production as well as the regulation and development of T cells (179).

Once it was demonstrated that miRNAs play a positive or negative role in regulating immunity depending on the type of miRNA and the presence of exosomes in breast milk, we can conclude that miRNAs found in breast milk play a role in newborn immune development. Kosaka N. et al. conducted studies showing that miRNAs regulate the newborn's immune system; for example, miR-155 plays a role in the maturation of T and B cells, miR-223 regulates neutrophil activation and proliferation, miR-181a, miR-181b and

Mir-17-92 cluster involved in B-cell regulation. Simultaneously, they discovered miR-181a and miR-17 in the CD63-positive exosome fraction as a structure to provide stability to miRNAs in breast milk. However, they did not dismiss the possibility that miRNAs could find in other microvesicles or apoptotic bodies as anti-degradation mechanisms (180). Another study in breast milk samples revealed the presence of exosome-carried miRNAs. Four main types of miRNAs, such as miR-30b-5p, miR-182-5p miR-200a-3p, and miR-148a-3p participate as immune-related pre-miRNAs; they also discovered other miRNAs such as miR-29a-3p, miR-141-3p, and miR-378-3p, which are still being studied but are known to harm the immune system (181).

The role of miRNAs as immune cognition mediators has been demonstrated by their involvement in epigenetic regulation of development (170). All of these advances in understanding miRNAs with immunological properties lead to promising new therapeutic tools for immune-related diseases.

12.4. Milk microRNAs as Diagnostic Tools

The discovery of miRNAs in breast milk could mean the way for a new method of disease detection, thanks to their role in immune system regulation. As demonstrated, miRNAs can influence the immune system positively or negatively, so any alteration or change in immune-related milk microRNA should affect the mother's or child's health (170). Furthermore, miRNAs found in breast milk have been shown to originate from the mammary gland and to differ depending on the stage of lactation. Then, the miRNAs present in milk as biomarkers for the diagnosis of various diseases represent a new non-invasive, effective, and early detection method for pathologies related to immune system negative regulation.

Human breast milk miRNAs are still being studied as biomarkers; however, data on the types of miRNAs found in milk correspond to the same types of miRNAs found in another fluid that has already been studied. The possibility that a specific miRNA work or is involved in immune processes that involve or prevent a specific disease is being investigated, regardless of the fluid in which it is found.

Previous research revealed the presence of ten types of miRNAs related to immune system

regulation; however, there are still miRNAs that have not been tested for their function in breast milk, but if were examined in other fluids (181) Mir-141, for example, was discovered in breast milk as well as plasma, because of its high presence in cancerous tissues compared to healthy tissues, plasma miR-141 has been identified as a potential biomarker for the detection of colon cancer. Consequently, this miRNA is considered a possible biological marker, and clinical research into it could be very important (182). Its association with colon cancer sheds light on its negative role in the immune system, and its presence in breast milk would support the search for potential biomarkers.

Animal models are another form used to study miRNAs in milk as biomarkers. There is research being conducted on various mammalian species to test the presence and function of miRNAs; in this case, studies on breast milk samples were conducted to see if altering their immune system through the presence of various pathologies resulted in a high level of expression of specific miRNAs.

The presence of miR-21, miR-146a, miR-155, miR-222, and miR-383 in bovine milk meant potential biomarkers. The study was based on bovine milk tests from cows with mastitis, and the results showed that these miRNAs had altered expression levels in infected bovine milk, implying their involvement in mastitis. Lai Y. et al. demonstrated that miRNAs could be used as biomarkers of bovine mastitis with 80% sensitivity and specificity to distinguish mastitis milk from regular cow milk (183). Another study in bovines identified that miRNAs in milk exosomes could be used to detect Staphylococcus aureus infection in the mammary gland. The research discovered six miRNAs with significant expression in the exosomes of bacteria-infected milk; however, they proposed two miRNAs, bta-miR-142a and 223, as bacterial biomarkers infection in the mammary gland by Staphylococcus aureus (184).

Potential biomarkers of miRNAs obtained from breast milk are effective in pathologies originating in the mammary gland. Then, breast cancer, the most common cancer in women, should be investigated for its origin in mammary gland cells (185).

13. BREAST CANCER

The World Health Organization (WHO) reports that breast cancer is the most common cancer in women in developed and developing countries (186). Breast cancer originates from the uncontrolled proliferation of cells in the mammary glands (187).

According to statistics, several factors, including influence breast cancer risk 1) age, as women's ages increase, so does their risk of cancer. 2) Gender, women, are the most affected. 3) Genetic, it could be hereditary or due to gene mutations. BRCA1 and BRCA2 are the most important genes that increase cancer susceptibility. 4) Environmental factors, radiation exposure. 4) Hormonal causes are related to sex hormones, early menarche, first delivery after 30, late menopause, and nulliparity (185,188)

13.1. Cancers, according to their histology

It is essential to understand where cancer begins to grow because this is how cancer's behavior is determined, and the best way to treat it is discovered. It may behave with "in situ" or non-invasive carcinomas and invasive carcinomas.

13.1.1. "In Situ" or Non-Invasive Carcinomas

- Carcinoma Lobular in situ, its development begins within the breast lobe and does not spread. A biopsy can detect it by chance, i.e., a biopsy is performed for some other reason (185,188)
- Carcinoma Ductal in situ, cancer begins in the milk duct (the pipes that carry milk from the lobules to the nipple) and does not spread to the breast tissues. There is a risk of developing invasive breast cancer later on (185).

13.1.2. *Invasive Carcinomas:*

Breast cancer that has scattered to other parts of the body.

- Invasive Lobular Cancer or known as infiltrating lobular carcinoma. This type of cancer accounts for 10% of all cancers that have infiltrated breast tissues. They pass through the wall of the lobulillo and invade the breast tissues. Invasive lobular cancer is homogeneously impregnated in the breast, making it technically hidden and difficult to detect using conventional diagnostic methods, and it is only detected

when the cancer is advanced (185).

- Invasive Ductal Cancer, or known as infiltrating ductal carcinoma. It is the most common cancer, accounting for 50% to 70% of invasive breast cancer. It is distinguished by crossing the milk duct wall and invading the breast tissue. Mammograms or palpation can detect it, revealing small lumps or abnormalities (185).

Breast cancer can also be classified based on the expression of estrogen, progesterone, and HER2 receptors. Breast cancer cells have receptors that allow them to bind to the hormones estrogen and progesterone, allowing them to grow. Endocrine treatments are selected to prevent cancer growth. On the other hand, cancer cells that produce a large amount of protein HER2 can be treated with drugs that aim to reduce HER2 and thus decrease cancer growth (188,189).

Other carcinomas that affect different types of cells, but are less common, include:

- Paget Nipple Disease
- Inflammatory Breast Cancer
- Filodes
- Angiosarcoma

13.2. Diagnostic Methods

Several breast cancer diagnostic techniques are available, including 1) Self-examination or clinical examination, patient examination, history, habits, or previous treatments. Breast palpation is used to detect lumps. 2) Mammography is an imaging technique used in patients with suspected cancer or as a preventative measure for early detection. 3) Ultrasound, which is used for young women as a supplement to mammography, allows for the differentiation of a liquid or solid tumor. 4) Magnetic Resonance Imaging (MRI), should not be a replacement for mammography or ultrasound and is used in complex cases. 5) A biopsy is the removal of cells and tissues for examination, allowing the doctor to determine the type of cancer and whether it is benign or malignant (185,188)

Despite the availability of multiple diagnostic methods, a rapid screening method that can provide an early diagnosis in the patient while being less invasive and painful than a biopsy

is still required. For this reason, tumor biomarkers, which are based on each tumor's molecular characteristics, are a new tool for detecting and determining any type of cancer. Circulating miRNAs have been proposed as new biomarkers of breast cancer through breast milk (185).

14. MiRNAs in Breast Cancer

Several miRNAs are significantly differentiated in some cancer (190); for example, Calin et al. (98) demonstrate that miRNAs called miR15 and miR16 are located at chromosome 13q14 and act in a negative regulation or are deleted in most cases with B cell chronic lymphocytic leukemia. Therefore, the presence of highly related miRNAs in breast cancer can be found both positively and negatively.

MiRNAs were discovered to play a role in breast cancer for the first time in 2005. Iorio M. and colleagues compared tissue samples from breast cancer patients to those from healthy people, found 29 miRNAs that were significantly deregulated, including the five most deregulated miRNAs in breast cancer. Three of them were down-regulated: miR-10b, miR-125b, and miR-145 indicating that they act as tumor suppressors, while miR21 and miR-155 were up-regulated, indicating that they act as oncogenes (101).

Deregulated miRNAs in breast cancer were thoroughly investigated. In breast cancer, MiR-155 is an oncogenic miRNA. Zhang et al. and colleagues discovered that MiR-155 promotes cell growth and inhibits apoptosis in MCF-7 cells, miRNA binds to TP53INP1 (MCF-7 cell inhibitor, induces cell apoptosis, and inhibits cell progression) to aid the survival and growth of breast cancer cells (191). MiR-21 is overexpressed in breast cancer and has been proven to be an oncogenic miRNA in several studies. One investigation identified nine miRNAs, including hsa-miR-21, hsa-miR-365, hsa-miR-181b, hsa-let-7f, hsa-miR-155, hsa-miR-29b, hsamiR-181d, hsa-miR-98, and hsa-miR-29c, participating in breast cancer. However, as in previous studies, it was identified that two of them, miR-21 and miR-155, were over-expressed in cancer. Moreover, miR-21 was found to be associated with cancer progression, and miRNA was found to be overexpressed in patients who had metastases (192).

Because of advances in technology in various techniques for identifying new miRNAs, such as qRT-PCR, microarray, and RNA-seq (92) MiRNAs miR-7 and miR-218 act as tumor suppressors; evidence suggests that the two miRNAs are inversely related to HoxB3 expression, influencing histone modification and DNA methylation, in this way, it acts as RASSF1A and Claudin 6 expression inhibitor (193,194). Similarly, miR-335 functions as a tumor suppressor in breast cancer, inhibiting tumor reinitiation and suppressing cancer

metastases. Silencing miR-335 inhibits the reactivation of breast cancer tumors by reducing its expression (195).

Further evidence shows the relationship between miRNAs and breast cancer, miRNAs may act as oncogenes or tumor suppressors in cancer (193); therefore, research into the role they play has become critical. However, detection methods or biomarkers are still invasive, so the idea of using circulating miRNAs as a new method for clinical needs has sparked a lot of research interest.

15. Breast Milk miRNAs and Breast Cancer

According to data collected, women between the ages of 25 and 40 are a vulnerable group to an asymptomatic period of breast cancer, and there is a peak in the same age range that corresponds with women in a period of fertility lactation. As a result, breastfeeding women can benefit from an early diagnosis by detecting miRNA expression levels in breast milk (196).

Currently, the presence of the same miRNAs in other body fluids can be used to study and evaluate the relationship of breast milk miRNAs with any pathology; for this reason, studies on breast cancer through breast milk are not yet sufficiently conclusive. However, analyzed data have been found comparing miRNAs obtained in women with breast cancer versus miRNAs found in breast milk.

The miR-181a has been extensively studied for its role in breast cancer, with several studies indicating that downregulation of miR-181a expression participates in advanced cancer stages. For example, studies on breast cancer tissue samples with and without lymph node metastasis revealed eight miRNAs, including downregulated miR-181a in lymph node metastasis compared to samples without metastasis (197,198).

On the other hand, the upregulation of miR-181a helps the invasion and metastasis of cancer. For example, in triple-negative breast cancers (TNBC), miR-181a was defined as a "metastamir" regulated by TGF- β, indicating that TGF- β is an essential regulator of miR-181a expression. Their findings show that this miRNA expression is upregulated in breast cancer metastasis samples, implying that miR-181a supports BC metastasis via epithelial-mesenchymal transition stimulation (198,199).

Do Canto et al. obtained 17 miRNAs differentially expressed between tumor samples and healthy samples; some function as oncogenes or tumor suppressors, while others serve to predict breast cancer, and still others are involved in cancer invasion metastasis. They discovered that miR-181a was upregulated in cancer-affected samples and was associated with BC invasion and metastasis. It was related to the 17 miRNAs in biological fluids such as plasma and serum, but it should be emphasized that miR-181a was found in breast milk (200). In breast milk, Mir-181a regulates the differentiation of B-cells and the selection of

CD4+ T-cells. (180).

Thus, high TGF-regulated miR-181 expression resulted in decreased survival time and increased BC metastasis. However, if miR-181a expression is reduced, it indicates that Bim expression is being regulated, which leads to sensitization of malignant mammary epithelial cells before anoikis. In other words, a decrease in miR-181a will contribute to cancer cell apoptosis and act as a regulator in response to chemotherapy (199).

Given that research on breast milk miR-181a in breast cancer patients is insufficient, the following studies should be based on the expression of this miRNA found in breast milk to understand its function during cancer cell development, and it will be known if future work as a biomarker is possible.

The let-7 miRNA was also found in breast cancer patients. This miRNA has been studied and found to be involved in the regulation of self-renewal and tumorigenicity of breast tumor-initiating cells (BT-IC), in other words, let-7 is almost not expressed in BT-IC, but a noticeable expression can be seen in differentiation, which involves let-7 working in key tumor differentiation processes. The investigation of this miRNA in BT-IC may help determine the likelihood of chemotherapy resistance (201). Tumor initiator cells are more resistant to chemotherapy compared to tumor differentiated cells (202). Then, Wu J. and colleagues demonstrated that low let-7 expression resulted in low chemosensitivity to breast cancer via increased cell apoptosis (203).

Let-7 family works for breast tumor differentiation and self-renewal of cancer cells (204). As a result, it is associated with let-7 as a response to therapy in breast cancer patients (200). So this miRNA could mean a new biomarker for early identification of breast cancer and prognosis to clinical response. The let-7 family of miRNAs is one of the most abundant in breast milk (172); therefore, let-7 present in breast milk could mean a better biomarker for detecting BC.

Consequently, Gu Y. et al. investigated the role of let-7 in mammary carcinogenesis and looked for the presence of this miRNA in milk stasis to propose a non-invasive biomarker. They compared milk stasis to milk stasis plus breast neoplasm samples and discovered differences in miRNA profiles between milk stasis plus breast neoplasm and milk stasis,

some miRNAs will be upregulated and other downregulated. They found that some members of the let-7 family functioned as oncogenes and others as tumor suppressors, so they investigated the presence of let-7a in milk stasis. Their findings revealed that milk has a higher expression of let-7a than blood and may be a more sensitive biomarker than blood. As a result, they concluded that miRNAs found in milk stasis play an important role in breast carcinogenesis and serve as better biomarkers of breast cancer than those found in blood (204).

In conclusion, miR-181 was discovered to be a biomarker for predicting breast cancer metastasis and thus aiding patient survival, in addition to being considered as a therapeutic target in BC (199). And the let-7 miRNA has been associated with cancer cell differentiation and self-renewal, indicating its potential as a significant biomarker more sensitive to BC's presence. The two miRNAs are among the most abundant in breast milk (172), and are secreted by the mammary gland (172,173). For these reasons, expression levels could be established as a biological marker of breast cancer cells' presence.

In 2019, breast milk tests were performed on healthy women to compare with reported breast cancer samples and determine if the presence of miRNAs and cell-free DNA could mean an early diagnostic factor for BC. They discovered a problem in their research that changes in miRNA expression levels at an early stage of cancer could go unnoticed; for this reason, Song et al. proposed a close relationship between changes in miRNA levels and baseline levels in samples. They then examined the miRNAs found in breast milk and discovered that up-regulated miRNAs had a lower baseline level in breast cancer while down-regulated miRNAs had a higher baseline level. MiRNAs with altered basal level abundance have been proposed as new biomarkers for BC early detection (196).

They proposed that breast milk be the primary method for rapid detection of BC as a non-invasive diagnosis due to the presence of miRNA and cell-free DNA in a stable form, and because these molecules come from breast-derived cells, they contain similar genetic information to that of breast cancer (196).

16. CONCLUSION

MiRNAs have been extensively researched to provide detailed information on their generating processes and mechanisms of action of miRNAs. MiRNAs produce in a series of sequential processes involving several protein factors such as EXP5, Drosha/DGCR8, Dicer, TRBP, Argonaute, and a union of several of these factors known as the RISC complex. However, in some biogenesis pathways, it has not yet been possible to study all of the factors involved in the generation of miRNAs.

Because evidence suggests that primary miRNA (pri-miARN) can be processed in different ways via canonical and non-canonical pathways, the steps and the machinery that guide the pri-miRNA to become a mature miRNA must consider. Because there are still unknown factors involved or factors that have not yet been directly studied, all of these processes have become a new study tool. Research into the biogenesis of miRNAs could provide new evidence on the machinery involved and its generation pathways and a better understanding of miRNAs' mechanism of action. MiRNAs play an essential role in gene regulation, and research has revealed several mechanisms of action in the target cell. Gene silencing via mRNA degradation or protein translation inhibition via mRNA deadenylation and decapping and induction of gene expression through mRNA translation are modes of action that rely on base complementarity between miRNA and the mRNA target.

As a result, miRNAs' role in regulating biological processes may express under abnormal or pathological conditions; thus, studies on miRNA-mRNA complementarity, miRNA levels, target mRNA, and miRNA location will help understand miRNA involvement in various diseases.

Because extracellular or circulating miRNAs are involved in various pathologies, their role as biomarkers has resulted in a new research tool for cancer diagnosis and prognosis. However, understanding the transport mechanism by which miRNAs release into biological fluids is required. Mature miRNAs and pre-miRNAs can be released into fluids such as plasma/serum, saliva, urine, or breast milk via microvesicles, exosomes, or protein association and their role involve cell-cell communication. Its role in each fluid has also meant studying various diseases such as cancer types and discovering new non-invasive biological markers for early diagnosis.

MiRNAs in breast milk are transported from mother to child and used to protect newborns through miRNAs' intervention in the newborn's immunity functions. However, because of the relationship between miRNAs found in the mammary gland and miRNAs found in breast milk, it has also meant a mechanism for detecting different types of cancer in the mother. Breast cancer is the leading cause of death in women with cancer; an early diagnosis could save hundreds of women's lives. Despite the current invasive, painful, and uncomfortable screening methods, a new, less invasive, and effective early detection tool is needed. MiRNAs in breast milk could be a new biomarker of breast cancer because the same miRNAs have been found in breast milk and cancer, indicating a regulatory association; additionally, they can be extracted in a non-invasive manner; and, most importantly, they could be an early detection method by reaching women aged 25-40 during an asymptomatic period of breast cancer.

17. RECOMMENDATIONS

- Future research should focus on new factors and their mechanisms of action during miRNA regulation to understand their specific function in the biogenesis and whether they could be a target for miRNA silencing or expression.
- Investigate circulating miRNAs to see if there is a relation between them and cancer diagnosis.
- To examine breast milk miRNAs in samples from women with breast cancer to find possible biomarkers for this type of cancer.

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