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Circulating Noncoding RNAs as Candidate Biomarkers of Endocrine and Metabolic Disease

Lead Guest Editor: Guido Sebastiani
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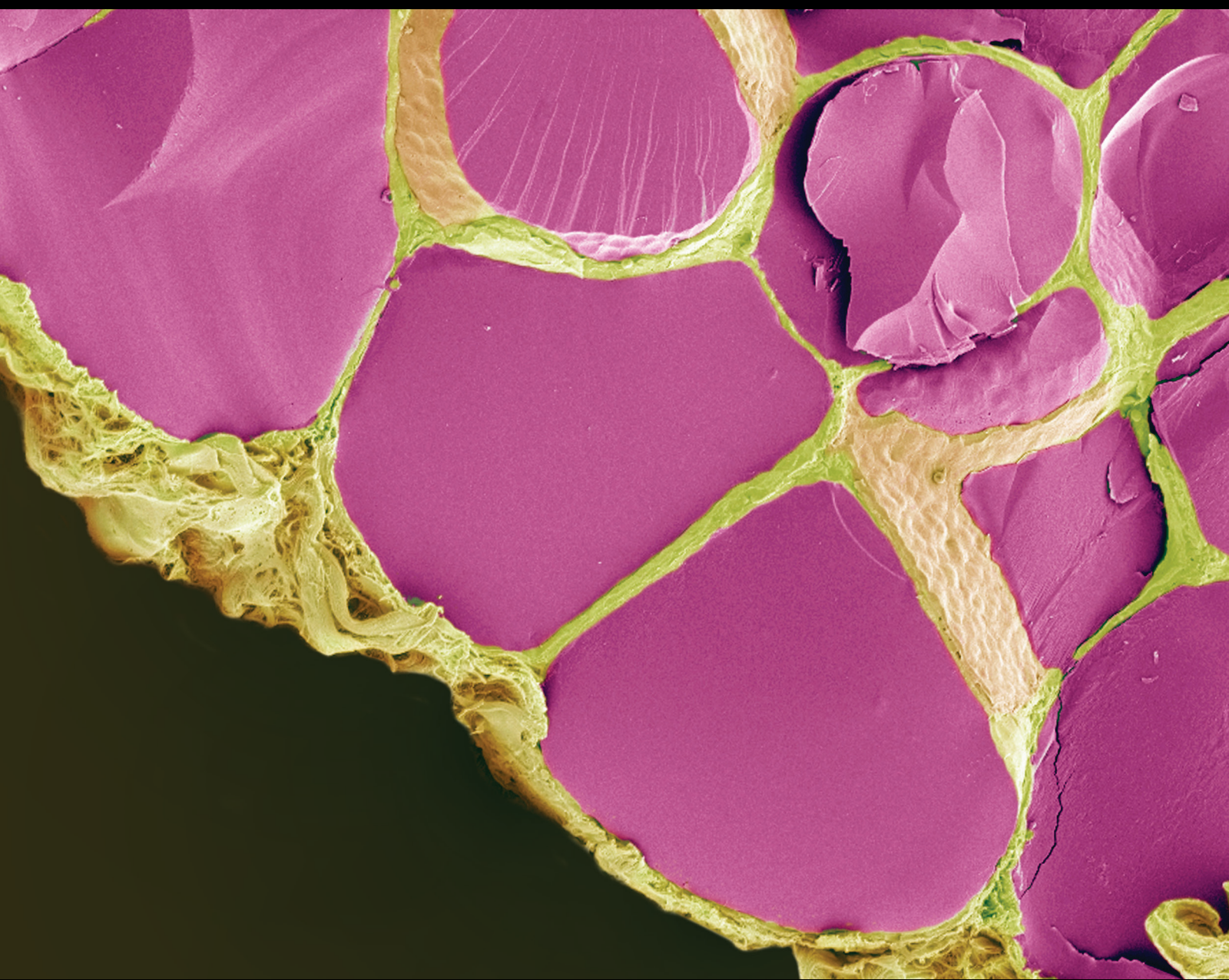

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Editorial Board

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Editorial

Circulating Noncoding RNAs as Candidate Biomarkers of Endocrine and Metabolic Diseases

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Over the last decade, several studies focused on circulating nucleic acids as potential cell-cell communication mediators or as disease biomarkers, thus potentially improving and extending the strategies to understand and diagnose/prognose many different diseases. Particularly, noncoding RNAs (including microRNAs, piRNAs, and long noncoding RNAs) received a bulk of attention due to their role as intracellular regulators of gene expression. Most of the efforts have focused on microRNAs which are single-stranded small noncoding RNAs of 19–24 nucleotides in length that negatively regulate gene expression through their binding to the 3′ untranslated region (UTR) of the messenger RNA (mRNA) target. MicroRNAs have been demonstrated to be part of pivotal cellular processes through their ability to posttranscriptionally modulate expression levels of many target genes; consequently, they were associated to the regulation of important metabolic processes ranging from insulin secretion (pancreatic islet) and insulin sensitivity (muscle, adipose tissue) to bone deposition and absorption.

It is now well known that microRNAs can be secreted by virtually every cell type and deploy important functions in cell-cell and interorgan communication, being found in most of the extracellular fluids through the associations with several circulating components including Argonaute proteins, HDL particles, microvesicles, and exosomes.

In this Special Issue, authors focused on different aspects of circulating microRNAs' biology and function in endocrine and metabolic diseases as well as addressed issues that needs further clarification. M. A. C. Pomatto et al. described the role of extracellular vesicles (EVs) in the transport of circulating noncoding RNAs and their role in several diseases including metabolic syndrome, diabetes, thyroid disorders, preeclampsia, and atherosclerosis. They also discuss the challenges associated with EV isolation from different biological fluids (serum, plasma, and cell culture media) and content analysis which could explain the poor technical and data reproducibility observed between different research groups. The importance of EVs in noncoding RNA transport and interorgan communication has been elegantly shown by Thomou et al. whereby circulating EVs derived from adipose tissue modulate hepatic function/metabolism. Further addressing the role of microRNA networks in adipose tissue may help to clarify the entire mechanism by which adipose tissue regulates the activity of distant organs. To this aim, Y. O. N. Lopez et al. elucidated the regulatory network existing between adipose tissue miR-24, miR-146a, and miR-30d and the antiangiogenic factor secreted frizzled-related protein 4 (SFRP4) in obesity and type 2 diabetes (T2D). These microRNAs were found to be upregulated in type 2 diabetes (T2D) and obese patients compared to healthy controls and correlated with elevated whole body adiposity and with SFRP4 levels in visceral adipose tissue, thus supporting the presence of a regulatory loop between microRNAs and SFRP4 in obesity and T2D pathophysiology.

Deciphering interorgan communication by EVs may also represent a novel pathological component of different diseases as observed in gestational diabetes mellitus (GDM) in which circulating microRNAs derived from placenta EVs may trigger insulin resistance and beta-cell dysfunction. Toward this end, E. Guarino et al. now reports on most recent findings on exosomes and circulating noncoding RNAs in GDM and discuss how technical development may improve the identification and characterization of placenta-derived circulating exosomes.

Additionally, microRNAs have been shown to modulate vascular endothelium homeostasis and bone metabolism. F. Barutta et al. and M. Materozzi et al. reviewed the current knowledge on the pathological effect of noncoding RNAs in microvascular complications in diabetes and bone osteoporotic alterations, respectively. Whereas alteration in microRNA activity may play a crucial role in vascular complications found in diabetics, these tiny regulatory circulating molecules may represent potential biomarkers of bone activity as well.

Finally, several ongoing studies are trying to characterize the potential use of circulating miRNAs as biomarkers of therapeutic response, in order to anticipate the final outcome. In this special issue, G. Catanzaro et al. explored such possibility by analyzing circulating miRNAs in plasma of T2D patients before and after dipeptil dipeptidase IV inhibitor (DPP-IVi) sitagliptin therapy. Analysis of circulating microRNA profiles revealed three microRNAs correlating with responses to DPP-IVi in those patients: miR-378 which was associated with a resistance to DPP-IVi and miR-126-3p and miR-223 which were positively correlated with DPP-IVi response in T2D patients. This study highlights the potential usefulness of circulating microRNAs in predicting the response of patients to glucose-lowering drugs used and may help contribute to improving personalized medicine in the near future.

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Review Article

The Potential Role of miRNAs as New Biomarkers for Osteoporosis

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Osteoporosis is the most common metabolic bone disorder affecting up to 40% of postmenopausal women, characterized by a reduction in bone mass and strength leading to bone fragility and fractures. Despite the available tools for diagnosis and stratification of a fracture risk, bone loss occurs insidiously and osteoporosis is often diagnosed after the first fracture has occurred, with important health-related outcomes. Therefore, the need of markers that could efficiently diagnose bone fragility and osteoporosis is still necessary. Over the past few years, novel studies have focused on miRNAs, small noncoding RNAs that are differentially expressed in many pathological conditions, making them attractive biomarkers. To date, the role of miRNAs in bone disorders remains in great part unclear. In particular, limited and partly conflicting information is available concerning their use as potential biomarkers for osteoporosis, due to differences in patient selection, type of samples, and analytical methods. Despite these limits, concordant information about some specific miRNAs is now arising, making likely their use as additional tools to stratify the risk of osteoporosis and possibly fractures. In this review, we summarize the most relevant studies concerning circulating miRNAs differentially expressed in osteoporotic patients along with their function in bone cells and bone turnover.

1. Introduction

Osteoporosis is the most common metabolic bone disorder in humans and is characterized by a decrease in bone mass and quality leading to decreased bone strength [1]. It is estimated that up to 40% of postmenopausal women and 20% of men over 50 years may be affected worldwide, with millions of fractures registered every year [2]. Considering the aging population, these numbers are expected to increase steadily over the next years, making osteoporosis a major health-economical issue worldwide [3].

Bone is a metabolically active tissue in which the process of remodeling is continuously carried on throughout life. Under normal conditions, osteoblasts (the bone-forming cells) and osteoclasts (the bone-resorbing cells) operate in a well-organized and strictly controlled manner, thus ensuring the renewal of bone tissue in a normal skeletal structure [4, 5]. Remodeling also protects bones from the occurrence of damage by adapting their structure and strength to the circumstantial loading requirements [4]. Aging, as well as the presence of predisposing conditions, may cause either an unbalance in bone remodeling with an increased bone resorption not equally compensated by bone formation, or an increased remodeling velocity, leading to low bone mass, reduced strength, and deterioration of the skeletal microarchitecture [6]. These pathological aspects lead to bone fragility and consequent increased risk of fractures, most commonly involving the forearm, vertebral bodies, and hip [7].

Since many years, the most used method for the diagnosis of osteoporosis and the prediction of a fracture risk consists in the measurement of bone mineral density (BMD), assessed by dual energy X-ray absorptiometry. In fact, the risk of

fragility fractures increases progressively as BMD declines [8]. However, several other components of bone strength affecting either the structural or material properties of bone have been identified that are not necessarily captured with the measurement of BMD. Moreover, the use of markers of bone formation such as serum procollagen type I N-terminal propeptide (s-PINP) and bone resorption such as serum C-terminal telopeptide type I collagen (s-CTX) and urinary N-telopeptide (NTX) is common in the clinical practice [9]. These markers have been developed to provide a noninvasive assessment of bone turnover in different skeletal pathologies and have helped the clinicians to identify patients at a high risk for fractures and to monitor the efficacy of therapies [10]. Nevertheless, to date, s-CTX and s-P1NP show specific limitation such as lack of normative reference population databases, inadequate standardization of quality control, sample handling, and poor association with bone strength and fracture risk [11]. Despite the innovations introduced in the field of diagnostics, bone loss occurs insidiously and it is initially asymptomatic, so that osteoporosis is often diagnosed after the first clinical fracture, with consequent reduction of autonomy and increased mortality [12, 13]. Moreover, these patients often require hospitalization that increases the onset of several complications. In this context, the study of new potential biomarkers which can be used alone or in combination with existing markers, allowing an early and efficient diagnosis before the occurrence of fractures and an evaluation of the patient's response to therapy, would prove to be of great interest, for both clinical practice and translational research [11, 14].

Osteoporosis is a complex and multifactorial disorder with a recognized hereditary component, although genetic variants associated with the disease have limited impact on gene expression and explain only a small fraction of the disease etiology [15]. Thus, the study of new epigenetic factors, connected with this pathology, may increase our knowledge about its pathogenesis and epidemiology.

2. Epigenetics of Osteoporosis

Epigenetic mechanisms include DNA methylation and histone modifications that regulate gene transcription and noncoding RNA (ncRNA) that act at a posttranscriptional level. In fact, while 70–90% of human genome is transcribed into RNA, only 1-2% of these RNAs encode for proteins, suggesting that ncRNA represents most of human transcriptome [16, 17].

Among known epigenetic mechanisms, microRNAs (miRNAs) are one of the most studied regulators of gene expression in both physiological and pathological conditions [14]. miRNAs are noncoding, single-stranded RNA of about 22–24 nucleotides found in both plants and animals and act at a posttranscriptional level, directly modulating their target mRNA through the formation of an RNA-induced silencing complex [18]. They negatively regulate their targets in two ways, depending on the degree of complementarity between the miRNA and the target sequences within 3′ untranslated regions (UTRs) of mRNA. miRNAs that bind with perfect complementarity with the targeted mRNA sequence induce the RNA interference pathway that leads to the degradation of mRNA [19–22]. miRNAs that bind their almost complementary target sequences within 3′ UTRs of mRNA repress gene expression posttranscriptionally at the level of translation, without degradation of mRNA [23, 24].

In the past decade, a lot of evidence showed that also long noncoding RNAs (lncRNAs), ncRNA longer than 200 nucleotides, play important roles in diverse biological process, such as cell growth, transcriptional regulation, tumorigenesis, and stem cell development [25, 26]. Despite the increasing amount of data about the role of lncRNA in the epigenetic regulation of transcription, their role in skeletal basic and clinical biology remains largely unknown [27]. Unfortunately to date, only few studies have investigated the role of some lncRNAs (ANCR, H19, MEG, DANCR, etc.) on bone metabolism and they principally focused on osteoblast differentiation and function, without taking in consideration the role of these ncRNAs as biomarkers for osteoporosis [28–30].

The key role of miRNAs in the regulation of bone homeostasis and metabolism is well established in a lot of experimental observations, showing how these noncoding RNAs may affect osteoblast or osteoclast differentiation, function, apoptosis, and proliferation. This role was first demonstrated *in vivo* using mice knockout for Dicer (protein necessary for the maturation of miRNA) in chondrocytes; these mice showed a reduction in skeletal size and died at weaning [31]. In support of this finding, other two observations demonstrated the fundamental role of miRNAs during normal skeletal development. In the first of these studies, the deletion of Dicer in osteoprogenitor cells impaired bone formation and caused embryonic lethality, while the other study showed that the ablation of Dicer in mature osteoblasts leads to an increased bone mass phenotype [32]. Finally, when we look for new biomarker, it is important to consider that the choice between serum and plasma as starting material and the quality control determination are a crucial point for the success of the experiments [33]. To date, many papers have been published in order to answer the questions on what are the best laboratory practices, the best experimental conditions, and the best starting samples to obtain good biomarkers including miRNAs. In one of these papers, Blondal and colleagues identified 119 miRNAs that are most commonly present in human serum and plasma samples, and for each of them, they developed a normal reference range [34]. Interestingly, some of these miRNAs were found differentially expressed in osteoporotic patients. However, some other miRNAs identified as potential markers of osteoporosis appear to be less common in normal conditions, and, perhaps, they could represent a more specific signature of the disease. A list of such miRNAs is shown in Table 1. Thus, given the great importance of miRNAs as regulators of bone homeostasis and remodeling in both physiological and pathological conditions, the purpose of our review is to summarize what we known so far regarding circulating miRNA in patients with osteoporosis.

3. Diagnostic Biomarkers of Osteoporosis

Accumulated evidence indicated that miRNAs in many cases define the physiology nature of the cell and play significant roles in the regulation of diverse biological processes, such

Table 1: Differentially expressed miRNAs in osteoporotic patients that are commonly or uncommonly expressed in serum and plasma (miRBase V18.0 nomenclature).

Common miRNAs	Uncommon miRNAs
Let-7g	miR-100
miR-122	miR-10b-5p
miR-125	miR-124a
miR-133a	miR-130b
miR-140-5p	miR-151a-3p
miR-142-3p	miR-151b
miR-148a	miR-181
miR-152	miR-188-3p
miR-21	miR-194-5p
miR-22	miR-19a
miR-23a	miR-19b
miR-24	miR-2861
miR-27a	miR-31
miR-29a	miR-330-3p
miR-30b	miR-382
miR-30e	miR-550a-3p
miR-324-3p	miR-590-5p
miR-335	miR-660-5p
miR-423-5p	miR-942
miR-93	miR-96

as development, cell differentiation, proliferation and death, immunity, and metabolism [35, 36]. Furthermore, aberrant miRNA expression should proportionally affect those critical processes and has been implicated in a wide variety of human diseases including cancer, viral infections, nervous system disorders, cardiovascular and muscular disorders, and diabetes [36, 37]. This implies that the use of these aberrantly expressed miRNAs as biomarkers for diseases is not only a valuable diagnostic strategy but it also makes these ncRNAs good candidates for new drug discovery [35, 36].

Furthermore, the presence of several miRNA quantification platforms and the introduction of high-throughput technologies, such as miRNA microarray, Real-Time PCR TaqMan Array microfluid cards, locked nucleic acid-(LNA-)-based high-throughput PCR, and next generation sequencing (NGS), facilitated the analysis of circulating miRNA expression profiles [38]. To date, these methodologies have replaced low-throughput analysis (Northen blotting and cloning) that present a lot of limitation due to their poor quantification output, time-consuming activity, and relatively low sensitivity and are widely used in initial screening of circulating miRNA and for generation of signatures from body fluid in numerous diseases [38, 39].

In this context, the possibility of detecting miRNAs as diagnostic markers of osteoporosis is certainly appealing for the clinical practice, and over the years, it has led to a rapid increase of studies that not only aimed at understanding the function of miRNAs in bone cells, but also understanding their potential as circulating biomarkers and identifying interesting candidates.

Although some of these findings are promising, a unifying method of analysis is missing, outlining a complicated and chaotic picture. Important discrepancies are found between studies, regarding the type of samples (e.g., plasma, serum, or whole blood) or populations used as control groups (e.g., healthy, osteopenic, and osteoarthritic subjects). The analysis is also carried out in different ways considering either a different number of miRNAs evaluated or platforms used, as well as reference genes used to normalize the analysis. Despite these limits, different studies showed partly concordant results, identifying miRNAs that appear to be differentially expressed in osteoporosis, with specific targets and functions on bone turnover, as demonstrated by experimental analyses. Here, we present the most relevant findings regarding circulating miRNAs as diagnostic biomarkers in osteoporosis, trying to describe, when possible, their functions and targets identified at the bone level. A complete list of these miRNAs, along with their biological functions and potential targets, is summarized in Tables 2 and 3 and Figure 1.

3.1. miR-21 and miR-148a. In several studies, a differential expression of miRNAs in the serum of patients can indeed effectively discriminate osteoporotic and nonosteoporotic patients. For example, Seeliger et al. discovered 9 upregulated circulating miRNAs (miR-21, miR-23a, miR-24, miR-93, miR-100, miR-122a, miR-124a, miR-125b, and miR-148a) that could significantly distinguish between serum samples of osteoporotic and nonosteoporotic fractured patients in a cohort of 30 subjects per group [40]. At a cellular level, two of these miRNAs, in particular, miR-21 and miR-148a, are known to play specific roles in bone homeostasis.

miR-21 affects both osteoclasts and osteoblasts. This miRNA is highly expressed during osteoclastogenesis and promotes the differentiation of murine BMMs through the downregulation of PDCD4 (a repressor of OC differentiation) and the survival of mature osteoclasts by the downregulation of FasL (involved in the Fas/FasL pro-apoptotic pathway [41]. It is likely that, at least in part, through this mechanism, estrogen signaling inhibits miR-21 biogenesis and promotes osteoclast apoptosis. This finding further highlights the relevant role of miR-21 in these cells [42]. In osteoblasts as well, miR-21 promotes differentiation and mineralization in MC3T3-E1 cells, targeting the expression of Smad7, a repressor of proliferation, differentiation, and mineralization of osteoblasts [43, 44].

Similarly, miR-148a promotes osteoclast differentiation by directly targeting MAFB, a RANKL-inhibiting protein [45]. In a recent study, it was also shown to inhibit ST2 cell differentiation toward the osteogenic linage by directly targeting lysine-specific demethylase 6b (Kdm6b), a regulator of osteoblast differentiation [46]. Both these miRNAs were also confirmed to be significantly deregulated in the plasma of osteoporotic women in different subsequent studies [47, 48]. However, miR-21 showed opposite modulation and miR-148a is also considered a strong diagnostic marker of osteosarcoma, thus questioning the potential specificity of such miRNAs for osteoporosis [47, 49].

Table 2: miRNA dysregulated in serum of osteoporotic patients and their function.

miRNA	Expression in OP patients	Biological function	Target	Ref.
Let-7g-5p	Downregulated	Promotes osteogenesis and ectopic bone formation and suppresses adipogenesis	HMGA2	[69]
miR-10b-5p	Upregulated	Potent inhibitor of OB differentiation	Bcl6	[80]
miR-19a miR-19b	Downregulated	Part of a cluster of miRNAs that probably maintains OB in undcifferentiated state	Undetected	[64]
miR-21	Upregulated	Promotes OB differentiation and mineralization	Smad7	[43]
		Essential for OC formation and OC bone-resorbing activity, preserves OCs from apoptosis	FasL, PDCD4	[41, 42]
miR-22-3p	Downregulated	Important regulator of the balance between adipogenic and osteogenic differentiation. Promoter of OB differentiation	HDAC6	[68]
miR-23a	Upregulated	Potent inhibitor of OB apoptosis and promoter of osteogenic differentiation	Fas, Runx2	[70, 71]
miR-24	Upregulated	Inhibits OB differentiation and mineralization	Tcf-1	[81]
miR-27	Downregulated	Promotes OB differentiation by inhibition of the suppressor of β-catenin	APC	[56]
miR-29a-3p	Downregulated	Promotes OB differentiation by repressing endogenous levels of Wnt signaling antagonists	DKK1, Kremen2, sFRP2	[82]
miR-30 family	Downregulated	Downregulated during OB differentiation are considered negative regulators of osteogenesis. One component of this family promotes adipogenesis rather than OB genesis	Smad1, Runx2, LRP6	[59]
miR-100	Upregulated	Negative regulator of OB differentiation	BMPR2	[83]
miR-124	Upregulated	Enhances the adipogenic differentiation of BMSCs and inhibits osteogenesis. It is a suppressor of bone formation	Dlx5, Dlx3, Dlx2	[73]
		Negative regulator of RANKL-dependent OC genesis. It inhibits the migration of OC precursors	NFATc1, RhoA, Rac1	[73]
miR-125	Upregulated	Negative regulator of OB precursor proliferation and OB differentiation in the early stages	ErbB2	[63]
miR-140-5p	Downregulated	Downregulated during osteogenesis. It is a suppressor of OB differentiation	BMP2	[65]
miR-142	Downregulated	Positively regulates OB precursor differentiation by activating Wnt signaling	APC	[60]
miR-148a	Upregulated	Negative regulator of adipogenesis and promoter of OB differentiation	Kdm6b	[46]
		Promotes OC genesis and increased the bone resorption area on dentin slices	MAFB	[45]
miR-2861	Upregulated	Promoter of OB differentiation	HDAC5	[75]

Table 3: miRNA dysregulated in plasma of osteoporotic patients and their function.

miRNA	Expression	Function	Target	Reference
miR-21	Downregulated	miR-21 promotes OB differentiation and mineralization	Smad7	[43]
miR-21		Essential for OC formation and OC bone-resorbing activity, preserves OCs from apoptosis	FasL, PDCD4	[42]
miR-31	Upregulated	Promotes OC formation and activity by promoting the formation of the peripheral acting ring	RhoA	[54]
miR-133a	Upregulated	Downregulated during OB differentiation, it is a suppressor of osteogenesis	Runx2	[57]
miR-148a	Upregulated	Promotes OC genesis and increased the bone resorption area on dentin slices	MAFB	[45]

3.2. miR-31 and miR-194. Other miRNAs are also potential candidates (miR-130b-3p, miR-151a-3p, miR-151b, miR-194-5p, miR-590-5p, and miR-660-5p) and display higher levels in the blood of osteoporotic women compared to osteopenic women, and, interestingly, expression levels of miR-151b and miR-194-5p were also negatively correlated

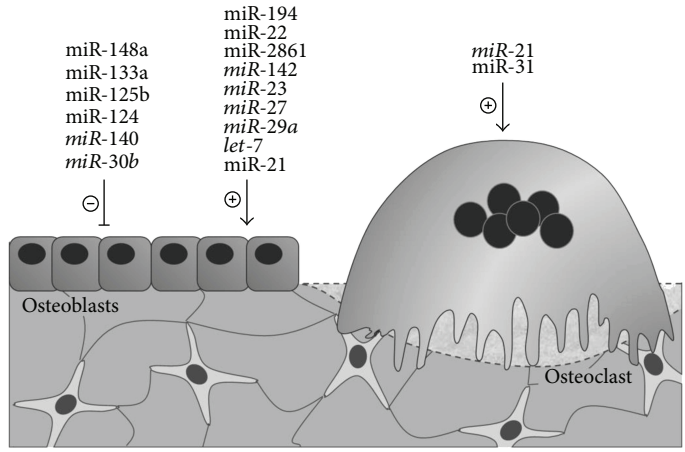

Figure 1: Circulating miRNAs differentially expressed in osteoporotic patients, either upregulated (roman) or downregulated (*italic*), and their functions studied in bone cells.

with femoral neck T-scores [50]. Another miRNA, miR-31, also stands out for being upregulated in both osteoporotic women and men compared to controls. miR-31 has in fact shown higher levels in the plasma of both osteoporotic women (with a fold change increasing with age) and osteoporotic men [51].

While the role of miR-151b on bone homeostasis remains yet unknown, both miR-194 and miR-31 have been shown to play a role in bone biology. miR-194 promotes osteoblast differentiation and activity in studies performed on mouse bone mesenchymal stem cell (BMSCs) cultures, by regulating Runx2 nuclear translocation through STAT1 inhibition and by downregulating COUP-TFII mRNA levels, therefore driving mesenchymal cell differentiation towards osteoblasts instead of adipocytes [52, 53]. miR-31 is highly expressed during RANKL-induced osteoclastogenesis, and its inhibition in murine bone marrow-derived macrophages impairs terminal differentiation and bone resorption activity by targeting RhoA, a GTPase that plays a key role in a crucial step of OC formation, namely the acting ring assembly [54].

3.3. miR-133a and miR-27. In a large study in 120 Chinese postmenopausal women who were divided into three groups (normal, osteopenia, and osteoporosis) according to BMD measurement, miR-133a was found upregulated in the plasma of osteoporotic versus osteopenic patients and negatively correlated with hip and spine BMD [47].

In a more recent analysis, 33 miRNAs were found to be downregulated in the serum of osteoporotic women compared to controls ($n = 5$ each), of which miR-27 showed the strongest reduction, as further validated in a large cohort of 81 women with postmenopausal osteoporosis. However, the controls were younger and thus not age-matched [55].

Indeed, both miRNAs have been also reported to have specific and contrasting functions in osteoblast cell lines. miR-27 directly targets adenomatous polyposis coli (APC) gene expression in hFOB1.19 cells (human fetal osteoblastic cell line), leading to β-catenin accumulation and consequently activating Wnt signaling, the most relevant pathway for osteoblast formation [56]. Conversely, miR-133 is involved in inhibiting osteoblast differentiation, through the downregulation of Runx2 expression, a downstream effector of Wnt signaling pathway [57].

3.4. miR-30b and miR-142. In a more complex analysis involving either animal models of osteoporosis (rats and rhesus monkeys) and postmenopausal women, miR-30b-5p was significantly downregulated in ovariectomized rats and bed-rest rhesus monkeys as well as in the serum of women with low bone mass, together with miR-142-3p and miR-103-3p. Moreover, all these miRNAs positively correlated with the BMD of these patients, making them potential attractive noninvasive biomarkers for osteoporosis [58]. Of interest, miR-30 family miRNAs, including miR-30b, are known *in vitro* negative regulators of BMP-2-induced osteogenesis, inhibiting osteoblast differentiation through targeting of Smad1 and Runx2 expression [59]. Instead, miR-142-3p positively regulates osteoblast differentiation and promotes Wnt signaling by inhibiting APC, similarly to miR-27, as described above [60].

4. miRNAs and Osteoporotic Fractures

By a different approach, Bedene et al. identified a correlation between plasma miR-423-5p levels not only with low BMD values but also with the 10-year probability of major fracture in postmenopausal women, as assessed by the fracture risk assessment tool (FRAX) [48].

Moreover, specific circulating miRNAs have been also correlated with the onset of fractures, the most relevant consequence of osteoporosis, in different studies [2]. Such markers could thus provide some insights on fracture risk, with the potential to specifically identify patients with a history of osteoporotic fractures. In this context, specific miRNA profiles have been found in women with osteoporotic fractures, although, some of these studies were performed comparing osteoporotic women with fractures to healthy controls, or to osteoarthritic women. Thus, the miRNAs identified in such comparisons may actually reflect differences between osteoporotic and nonosteoporotic patients including subjects with osteoarthritis, rather than the actual risk of fracture in osteoporotic patients.

Some of the miRNAs we discuss in this review have been also found to be differentially modulated within the bone tissue of osteoporotic patients, and, although they cannot be considered circulating biomarkers, this aspect further supports their diagnostic value. For example, RNA was extracted from the bone tissue of fractured osteoporotic and nonosteoporotic bone, leading to the identification of 5 miRNAs upregulated in the osteoporotic bone, including miR-21, miR-23a, miR-24, miR-100, and miR-125b, with respect to nonosteoporotic bone [40]. These miRNAs were also differentially expressed in the serum, as previously mentioned, suggesting that the deregulation found in the serum reflects and associates with a similar deregulation within the bone tissue.

4.1. Single miRNAs Modulated in Fractured Osteoporotic Patients. In a small scale study, specifically designed to compare serum miRNA expression profiles between osteoporotic patients with fractures and osteoarthritic controls, 3 miRNAs (miR-122-5p, miR-125b-5p, and miR-21-5p) were positively correlated with fracture prevalence [61]. In the same study, circulating miR-21-5p levels were also positively correlated to those of CTx, a marker of bone resorption. Conversely, in a different study, miR-21-5p levels appeared significantly reduced among osteoporotic/osteopenic women with vertebral fractures (66% sensitivity, 77% specificity in distinguishing women with a vertebral fracture) than in nonfractured controls [62]. While the exact function of both miR-423 and miR-122-5p in bone cells remains unknown, miR-21, as described before, can influence both osteoclast and osteoblast differentiation processes, whereas miR-125b was shown to play a role in osteoblastogenesis, by inhibiting cell proliferation of ST2 cells (murine mesenchymal cells) induced with BMP-4 [63].

A more recent in-depth analysis studied circulating miRNAs in the sera of patients with idiopathic osteoporosis and fractures versus healthy controls. This analysis uncovered 19 miRNAs significantly regulated in premenopausal or postmenopausal women and male idiopathic osteoporosis, compared to age-matched healthy individuals. Eight of these miRNAs (miR-140-5p, miR-152-3p, miR-30e-5p, miR-324-3p, miR-335-3p, miR-19a-3p, miR-19b-3p, and miR-550a-3p) had AUC values of 0.9 for the classification of fracture patients, correctly discriminating between fractured patients and healthy subjects [64].

Some of these miRNAs have been already involved in the regulation of bone metabolism. In vitro, miR-140-5p directly represses the expression of BMP2, inhibiting the differentiation of human mesenchymal cell lines towards the osteoblastic linage [65]. miR-19a and miR-19b are both part of a cluster called miR-17-92, whose haploinsufficiency in mice causes impaired ALP activity and impaired bone calcification [66]. Furthermore, a significant and peculiar regulation in the serum of osteoporotic patients with recent fractures (compared to healthy subjects) was detected concerning miR-10b-5p, miR-133b, miR-22-3p, and let-7 g-5p, but only the last 2 were validated [67].

Indeed, both miR-22 and let-7g-5p were previously demonstrated to either promote or inhibit osteogenic differentiation. miR-22 was found to regulate the fate of MSCs, promoting the differentiation towards osteoblasts and inhibiting adipogenesis in human adipose tissue-derived mesenchymal stem cells (hADMSCs) through the downregulation of HDAC6, a repressor of Runx2, thus underlying an important role in the balance of adipogenesis and osteogenesis [68]. In vitro, let-7 significantly promoted osteogenesis and counteracted adipogenesis of MSCs, by targeting high-mobility group AT-hook 2 (HMGA2) expression [69].

More recently, Yavropoulou et al. described other miRNAs also known to modulate bone turnover (e.g., miR-23a, miR-29a-3p, miR-124-3p, and miR-2861) that were significantly deregulated in the serum of patients with low bone mass and vertebral fractures compared with controls [62]. For example, miR-23a significantly inhibits TNF-α-induced apoptosis in MC3T3-E1 cells (osteoblastic line) by targeting Fas, a proapoptotic protein, and promotes osteogenic differentiation by targeting Runx2 expression in ATDC5 cells [70, 71]. Similarly, miR-29a regulates HDAC4 expression, displaying protective effects from glucocorticoid-induced bone loss by modulating β-catenin accumulation and OB differentiation [72]. In BMSCs, miR-124 inhibits the differentiation toward the osteogenic lineage, favoring adipogenic differentiation, thereby suppressing in vivo bone formation by binding to Dlx5, Dlx3, and Dlx2 [73]. Moreover, miR-124 is a negative regulator of osteoclastogenesis in mouse BMMs. It reduces the expression of NFATc1 protein induced by RANKL stimulation and inhibits the OC precursors by targeting expression of RhoA and Rac1, GTPases involved in cellular motility [74].

Interestingly, a previous study also identified miR-2861 as a promoter of osteoblast differentiation by targeting histone deacetylase 5, resulting in increased Runx2 protein levels in ST2 cells induced with BMP-2 [75]. In this study, two related osteoporotic patients presented a mutation on miR-2861 that impaired its expression, suggesting that the downregulation of this miRNA may contribute to the disease, although, Yavropoulou et al. [62] found it upregulated in osteoporotic patients.

4.2. Combination of miRNAs Identifies Osteoporotic Patients with Fractures. Trying to identify single miRNAs as significant biomarkers for osteoporosis, or any other disease, could be laborious, difficult to apply to various populations, and may not be sufficient to discriminate complex aspects of the disease. As suggested by a recent analysis by Heilmeier et al., a more specific predictive value on fracture risk could be achieved through combinations of different miRNAs [76]. Stronger biomarkers could statistically be identified clustering miRNAs that show differential expression in osteoporosis; a multigene approach could be applied to identify clusters of miRNAs with higher discriminating values [77]. Regarding osteoporosis, few studies have investigated this aspect, but this approach has already been applied to many other disease, including cancer, for example, the miR-183/182/96 cluster expression correlated with metastasis and poor clinical outcome in breast cancer patients [78].

In the study of Heilmeier et al., which also included subjects with type 2 diabetes and fractures, 23 differentially expressed miRNAs were found in osteoporotic fractured

patients and a specific signature of 4 miRNAs, composed of miR-382-3p, miR-188-3p, miR-942, and miR-330-3p, was able to correctly discriminate between postmenopausal women with osteoporotic fractures and postmenopausal women without fractures with the highest AUC value (0.991). Interestingly, a partially different signature was identified in subjects affected by type 2 diabetes and fractures. In fact, only miR-382-3p was downregulated both in diabetic and non diabetic patients with fractures, while 3 additional miRNAs (miRNA-96-5p, miRNA-181-5p and miRNA-550a-5p, all upregulated) were specific for the signature linked to fractures in diabetes. From the experimental point of view, in vitro functional studies demonstrated that in human adipose tissue-derived mesenchymal stem cells, miR-382 is able to significantly enhance osteogenic differentiation [76].

Another cluster worth mentioning is cluster miR-23, composed of miR-23a, miR-27a, miR-24a. Several studies, in vitro and in vivo, have uncovered important functions of this cluster in bone cells, but to date, no clinical studies investigated its potential role as a biomarker for osteoporosis [79]. Given that the single component of miR-23 was indeed found differentially expressed in osteoporotic patients, as described in the previous paragraphs, it would be interesting to explore its potential as a combination.

5. Conclusions and Future Perspectives

It is well established that in the next future miRNAs could become valid biomarkers for several diseases, for diagnostic purposes, prediction of complications, and response to treatment. However, in bone pathologies and more specifically in osteoporosis, we are only at the beginning in this field. To date, the available evidences are indeed promising and have been able to uncover interesting miRNAs that are not only involved in specific functions and roles in the bone biology (as demonstrated in experimental observations), but are also potentially capable of discriminating osteoporotic patients from controls, thus conferring them a relevant value for both medicine and basic research. Of course, we are still far from identifying a strong biomarker for osteoporosis that could be applied to all cohorts of patients, especially because of to the low number of studies on circulating miRNAs and their methodological discrepancies, as mentioned before. In the future, given the potential of such biomarkers, it will be extremely important to perform a further validation of these and other miRNAs in prospective studies of larger population-based samples, taking also into account relevant variables that could influence miRNA expression profile, such as diet or age.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Review Article

Circulating MicroRNAs as Biomarkers of Gestational Diabetes Mellitus: Updates and Perspectives

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Gestational diabetes mellitus (GDM) is defined as any degree of carbohydrate intolerance, with onset or first recognition during second or third trimester of gestation. It is estimated that approximately 7% of all pregnancies are complicated by GDM and that its prevalence is rising all over the world. Thus, the screening for abnormal glucose levels is generally recommended as a routine component of care for pregnant women. However, additional biomarkers are needed in order to predict the onset or accurately monitor the status of gestational diabetes. Recently, microRNAs, a class of small noncoding RNAs demonstrated to modulate gene expression, have been proven to be secreted by cells of origin and can be found in many biological fluids such as serum or plasma. Such feature renders microRNAs as optimal biomarkers and sensors of in situ tissue alterations. Furthermore, secretion of microRNAs via exosomes has been reported to contribute to tissue cross talk, thus potentially represents, if disrupted, a mechanistic cause of tissue/cell dysfunction in a specific disease. In this review, we summarized the recent findings on circulating microRNAs and gestational diabetes mellitus with particular focus on the potential use of microRNAs as putative biomarkers of disease as well as a potential cause of GDM complications and β cell dysfunction.

1. Introduction

Gestational diabetes mellitus (GDM) is defined as any degree of carbohydrate intolerance, with onset or first recognition during second or third trimester of gestation [1].

Insulin resistance physiologically increases during second and third trimester of pregnancy in order to guarantee proper nutrient supply for the fetus [2]: normally, a compensatory increase in insulin secretion maintains glucose homeostasis [3]. The inadequate β cell adaptation to peripheral insulin resistance is likely to be the main pathophysiological mechanism of glucose intolerance and hyperglycemia that characterize GDM [4].

It is estimated that approximately 7% of all pregnancies are complicated by GDM and that its prevalence is rising all over the world [5]. Thus, the screening for abnormal glucose levels is generally recommended as a routine care component for pregnant women [6].

Currently, the screening and diagnosis of GDM is accomplished by a one-step strategy (75 g OGTT at 24th–28th week of gestation) [7]: as a consequence, treatments cannot start before the late third trimester, which already presents a high risk of fetal morbidity and mortality. Therefore, an early screening in the first or second trimester of pregnancy could be important to promptly set up an adequate therapy which normalizes blood glucose levels [8], thereby reducing GDM adverse pregnancy outcomes. Furthermore, a careful evaluation of gestational diabetes risk factors, predisposing to the typical pregnancy alterations of glucose homeostasis, is needed in order to open the path for an earlier diagnosis. Although epidemiological studies on GDM risk factors are limited in number and biased by other potentially

confounding risk factors and study population variables, several of them strongly emerged; indeed, well-established risk factors for GDM include ethnicity, a family history of type 2 diabetes (first-degree relatives affected by T2D), high BMI (obesity or excessive adiposity), advanced maternal age, parity and multiple pregnancies, previous fetal macrosomia (or history of poor obstetric outcomes), and a history of GDM [9, 10]. The association of GDM risk factors to novel potential early biomarkers may help in the prevention of GDM complications during pregnancy and of future metabolic health problem as well. Indeed, GDM not only increases the risk for maternal and fetal complications during pregnancy but also predisposes to long-term complications both in the mother and in the offspring [11]. Once GDM is diagnosed, the risk for type 2 diabetes mellitus (T2DM) and cardiovascular diseases (CVD) increases in the mother. Particularly, the risk of developing T2DM increases by sevenfold, with a cumulative incidence of 60% at 10 years from GDM diagnosis. The rate of T2DM onset increases rapidly after delivery, continuing to increase thereafter without signs of a plateau. Moreover, women with prior GDM have a significantly higher rate of obesity, hypertension, and metabolic syndrome, which, together with altered levels of circulating inflammatory markers, are important risk factors for CVD [12].

Recently, several studies have evaluated the expression of circulating microRNAs (plasma/serum) in diabetes [13]; microRNAs have been associated with the regulation of β cell mass and function and with the immune system homeostasis and certainly represent major players in the pathogenesis of this group of chronic metabolic diseases [14].

Deregulation of microRNA expression has also been associated with GDM; thus, these molecules could represent potential early diagnostic biomarkers, due to their high stability in body fluids and their accessibility from maternal blood throughout gestation [15]. Therefore, a deep understanding of microRNA functions could improve the knowledge on etiology and pathophysiology of GDM and of its complications.

In this review, we aim at providing an overview of recent advances in the characterization of extracellular (plasma/serum) microRNAs in GDM.

2. MicroRNA Biogenesis and Secretion

MicroRNAs are small noncoding ~19–24 nucleotide- (nt-) long RNA molecules that play an important role in the modulation of gene expression [16]. They were discovered in 1993 in *Caenorhabditis elegans* [17] but afterward have been identified in plants, in vertebrates, and in some viruses. The number of discovered microRNAs has progressively increased: each of these molecules can target and regulate multiple genes, whereas a single target gene can be regulated by several different microRNAs [18]. Therefore, it is now clear that microRNAs are involved in many biological processes and that their deregulation or dysfunction can contribute to several diseases, including GDM [19].

MicroRNA biogenesis, function, and secretion are complex events involving a series of molecular mechanisms, which are not yet fully understood. MicroRNA biogenesis starts with microRNA gene transcription by RNA polymerase II (RNA Pol II) or RNA polymerase III (RNA Pol III): the first transcribes intragenic microRNA genes alongside with their host genes [20]; the latter transcribes intergenic microRNAs with their own promoter [21]. The transcription generates a long primary sequence (primicroRNA) that is usually capped and polyadenylated and has one or more long hairpin structure. PrimicroRNAs are then cleaved by the ribonuclease III enzyme *Drosha* and its cofactor DGCR8, generating a long hairpin-structured premicroRNA of ~60–70 nucleotides. The following maturation step is performed outside the nucleus, so the generated premicroRNAs are transported by Exportin-5 into the cytoplasm, where they are further cleaved by RNase III enzyme *Dicer* to generate 22 nucleotide double-stranded RNA with overhangs, consisting in a guide strand and in a passenger strand. While the passenger strand is usually degraded and, therefore, less represented in terms of expression levels, the guide strand, which is normally the most thermodynamically stable, is loaded into Argonaute proteins 1–4 (Ago 1–4) to form RNA-induced silencing complex (RISC) [22]. Functionally, mature microRNAs guide the RISC complex to recognize target mRNAs (messenger RNA), thus inducing a negative regulation. Target recognition is determined by the complementarity between the 3′-untranslated region of mRNA and bases 2–8 of the microRNA (called *seed* sequence) [23].

MicroRNAs can regulate target mRNAs through multiple pathways: the pairing between miRNA and its target site can lead to the degradation of mRNA by endonucleolytic cleavage mediated by Argonaute proteins or by deadenylation of mRNA molecule; the RISC complex can also induce the translational repression of mRNA or stimulate the proteolysis of the nascent peptide; finally, miRNAs have also been shown to upregulate target expression under certain conditions [24]. Translational repression and mRNA decay/degradation are considered the main microRNA mechanisms responsible for the regulation of their target mRNAs: whether these two mechanisms act in parallel or sequentially is still not fully understood. Recent studies have demonstrated that translational repression precedes mRNA degradation but is not always followed by mRNA degradation: indeed, it has been observed that a microRNA-repressed mRNA can be translationally reactivated [23]. It has been hypothesized that approximately 60% of genes are regulated by microRNAs; thus, these molecules are critically involved in the regulation of many biological processes [25].

Despite their function as regulators of gene expression, recent studies have demonstrated that microRNAs are not exclusively intracellular but also extracellular, being present in a cell-free circulating form in many different biological fluids, including serum and plasma [26].

Since the discovery of extracellular microRNAs in 2008 [27], researchers identified multiple mechanisms of microRNA transport, derived from different cell secretion/release pathways [28]:

(i) Passive release after cell death (vesicle-free) of microRNAs associated with AGO proteins

(ii) Passive secretion through apoptotic bodies containing components of apoptotic cells, including microRNAs

(iii) Active secretion of microRNAs packaged into shedding vesicles and exosomes

(iv) Active secretion of microRNAs coupled with high-density lipoproteins (HDL) or low-density lipoproteins (LDL)

The vehicular transport of microRNAs within small lipid vesicles and the association of microRNAs to protein complexes protect them against degradation by RNAses and by other RNA-degradation agents, rendering extracellular microRNAs very stable molecules [29]. Owing to their stability in biofluids, it has been suggested that microRNAs may represent a new form of cell-to-cell communication, both in physiological and in pathological conditions [30]. Furthermore, a putative use of circulating microRNAs as diagnostic, prognostic, and therapeutic biomarkers of many different diseases (including diabetes) has been widely reported, as they can be easily detected and measured in body fluids [31, 32].

3. Placental MicroRNAs in Healthy Pregnancy and GDM

Pregnancy represents an enormous stress for the maternal body and requires many physiological changes in order to guarantee a proper embryo/fetal growth [33]. Surely, a pivotal role in driving the maternal adaptations and/or deregulations is played by placenta-derived molecules, such as placental lactogen, growth hormone and tumor necrosis factor alpha, and by increased cortisol and progesterone levels [2]. Furthermore, additional novel molecules, such as placenta-derived microRNAs, have also been demonstrated to be involved in these regulations, thus suggesting that their alterations could be detrimental for maternal adaptation mechanisms [34].

Several studies reported that human placenta expresses more than 500 microRNAs, and only a part of them are also expressed in other tissues and organs [35]. Due to the importance of the placenta for healthy pregnancy, the characterization of placental microRNAs could be essential to understand the regulatory mechanisms of normal and complicated pregnancies. The group of placental microRNAs has been recently subdivided into (i) placenta-specific, (ii) placenta-associated, and (iii) placenta-derived circulating microRNAs [36].

Placenta-specific microRNAs are specifically or predominantly expressed in the placental tissues and are mainly encoded by three microRNA gene clusters: chromosome 19 microRNA cluster (C19MC), chromosome 14 microRNA cluster (C14MC), and miR-371–373 cluster [37, 38]. C19MC encodes for 58 mature microRNAs, expressed by trophoblasts in the early stages of pregnancy and afterwards by placental differentiated cells. C14MC encodes for 63 mature microRNAs and is highly expressed in trophoblasts. Finally, miR-371–373 cluster is exclusive to mammals and encodes for six mature microRNAs, mostly expressed by placental differentiated cells and embryonic stem cells [36]. All these placenta-specific microRNAs are linked to cell proliferation, apoptosis, migration, and angiogenesis in trophoblasts, that is, the critical processes needed for adequate placentation in early pregnancy [39].

Placenta-associated microRNAs are ubiquitously expressed in the placenta and in other tissues, with different expression profiles across pregnancy. Gu et al. have recently identified 191 differentially expressed microRNAs between placentas at first versus third trimester of gestational age: during the first trimester, oncogenic, angiogenic, and antiapoptotic microRNAs predominated, whereas during the third trimester, microRNAs related to cell differentiation and tumor suppression prevailed [36, 40]. Many studies demonstrated that both placenta-specific and placenta-associated microRNAs can be deregulated in pregnancy complications, such as pregnancy loss, preeclampsia, intrauterine growth restriction/fetal growth restriction, preterm birth, and GDM, suggesting their role in the pathogenesis of these conditions [41]. Several studies analyzed the expression of placenta microRNAs during disease conditions and highlighted GDM-specific deregulations of such molecules. One of the first studies in such direction uncovered placenta-specific microRNA miR-518d alteration in GDM. Indeed, microRNA miR-518d, one of the highest expressed C19MC placenta-specific microRNAs, was found to be hyperexpressed in placenta obtained from GDM patients with respect to nondiabetic women who delivered between the 37th and 40th week of gestation. The same research group uncovered a specific regulation of miR-518d on peroxisome proliferator-activated receptor-α (PPARα) gene (associated with metabolic adaptations during pregnancy), thus modulating its expression; interestingly, placental expression of PPARα is inversely correlated to miR-518d, thus further demonstrating the control of PPARα expression by such microRNA during pregnancy [42].

A subsequent study performed a similar analysis by taking into consideration placental tissues obtained by GDM and non-GDM patients [43]. The microarray analysis revealed a set of deregulated microRNAs (miR-508-3p, miR-27a, miR-9, miR-137, miR-92a, miR-33a, miR-30d, miR-362-5p, and miR-502-5p) which, collectively, targets key genes involved in epidermal growth factor receptor (EGFR) signaling cascade, thus highlighting their potential involvement in the induction of macrosomia, a typical fetal GDM-related complication, strongly associated to the alteration of EGFR signaling [44].

Additional studies uncovered several other microRNAs whose expression is altered in placenta of GDM patients and potentially linked to its function or development. This is the case of miR-221 and miR-222, whose expression was found to be upregulated in human fetoplacental endothelial cells (fpEC) isolated from third-trimester human placentas after pregnancies complicated by GDM versus healthy pregnancies; importantly, miR-221 and miR-222 were found to target ICAM1 protein species (ICAM-1, V-CAM, and E-selectin), whose expression was reduced in GDM placentas. The authors pointed out this mechanism as a

protection against leucocyte transmigration from blood to placenta which may worsen the inflammation due to hyperglycemia during GDM [45].

Finally, it has been reported that placenta-derived circulating microRNAs are released into maternal circulation mostly by syncytiotrophoblasts. They are carried in plasma by exosomes as early as the sixth week of gestation [46], even though other microRNA carriers (e.g., AGO proteins, HDL) cannot be excluded. These microRNAs reflect (at least partially) the expression of placenta-specific and placenta-associated microRNAs, mirroring physiological and pathological conditions during pregnancy. For this reason, many researchers have proposed and investigated their use as diagnostic biomarkers [41], as microRNAs can be easily detected and measured from the blood.

The first study demonstrating a specific case of placenta-blood mirroring in GDM and involving microRNAs has been recently published by Xu and colleagues. The authors identified the microRNA miR-503 which was upregulated both in the placenta and peripheral blood of GDM patients ($n = 25$) versus nondiabetic subjects ($n = 25$). Interestingly, the hyperexpression of miR-503 in rat β cell line INS-1 reduces the proliferation and insulin secretion while promoting apoptosis. Although such study needs specific validations and further experimental evidences, it opens to the possibility of a microRNA-based specific cross talk between placenta and β cells in gestational diabetes [47].

4. Circulating MicroRNAs as Candidate Biomarkers of Gestational Diabetes Mellitus

Recently, several studies have evaluated the expression of circulating microRNAs (plasma/serum) in diabetes, in order to establish whether microRNAs may represent early biomarkers of this group of chronic metabolic diseases and to clarify their eventual involvement in the pathogenetic mechanisms [13]. As a matter of fact, circulating microRNAs have been associated with β cell function and regulation as well as with immune system homeostasis, representing major players in diabetes pathogenesis [14, 48, 49], and deregulation of microRNA expression has been associated with metabolic disorders characterized by impaired insulin secretion and/or action [50].

Previous studies have been mainly focused on the expression of circulating microRNAs in T1D and T2D, while only few have investigated the expression and the diagnostic utility of circulating serum/plasma microRNAs in GDM (Table 1).

The first published study reporting an association between circulating microRNAs and GDM was performed by Zhao et al. in 2011. The authors evaluated microRNAs expression in sera obtained from GDM patients and non-GDM controls between the 16th and 19th week of pregnancy, with the aim to identify a microRNA signature potentially useful for an earlier diagnosis and prediction of GDM. Initially, by using TaqMan MicroRNA Array microfluidic cards, the authors analyzed a pool of 24 sera derived from GDM patients and a pool of 24 sera derived from nondiabetic subjects; differentially expressed microRNAs were further validated in another cohort of 36 GDM patients and 36 nondiabetic controls. They reported that three microRNAs (miR-29a, miR-132, and miR-222) were downregulated in serum of women affected by GDM versus nondiabetic subjects [51]. Despite such differential expression, the receiver operating characteristic (ROC) curves did not retrieve high sensitivity and specificity (combined microRNAs: sensitivity = 66.7% and specificity = 63.3%) to clearly distinguish between GDM and controls. Therefore, although a potential future use of these microRNAs as biomarkers of GDM requires further studies, their involvement in GDM pathogenic mechanisms could be of immediate interest. Indeed, the authors reported that, among the validated target genes of miR-29a, there is *Insig1*, an inhibitor of proteolytic activation of sterol regulatory element-binding proteins (SREBPs). This latter, in turn, activates genes involved in cholesterol and fatty acid metabolism and, probably, in glucose homeostasis, such as PCK2, a key enzyme in hepatic gluconeogenesis [52, 53]. Moreover, recent studies have demonstrated that both miR-29a and miR-222 directly and/or indirectly regulate the glucose transporter member 4 (GLUT4), which plays a key role in glycaemic control and in insulin-induced glucose uptake by muscle and adipose tissues; as a matter of fact, its expression/translocation is reduced in prediabetes and in diabetes [54]. Therefore, the hypothesis of a cross-talk effect mediated by circulating microRNAs and targeting insulin-sensitive tissues is of particular interest in order to understand the molecular cues underlying GDM pathogenesis.

Zhu et al. have evaluated microRNA expression in the plasma of 10 GDM patients and 10 non-GDM controls at 16th–19th week of pregnancy, using next-generation sequencing approach. With respect to the previous study, they identified a different microRNA signature composed of five microRNAs (miR-16-5p, miR-17-5p, miR-19a-3p, miR-19b-3p, and miR-20a-5p) which are upregulated in the plasma of women affected by GDM versus nondiabetic subjects. Such discrepancy could be attributed to the use of different starting sample (serum versus plasma) or differences in the use of the analysis platform. However, the microRNAs identified by Zhu et al. are reported as mainly associated with MAPK signaling pathway, insulin signaling pathway, TGF-β signaling pathway, and mTOR signaling pathway, which are all involved in insulin secretion [55].

In another recent study, Cao et al. tried to confirm the results of the pilot study by Zhu et al. in a larger group of patients (85 GDM women and 72 non-GDM women), by analyzing the same differentially expressed microRNAs (miR-16-5p, miR-17-5p, miR-19a-3p, miR-19b-3p, and miR-20a-5p) found by Zhu and colleagues. They did not find any significant differences in miR-19a-3p and miR-19b-3p expression between GDM and non-GDM patients, whereas miR-16-5p, miR-17-5p, and miR-20a-5p were confirmed as progressively upregulated during pregnancy in the plasma samples of GDM women at 16th–19th week, 20th–24th week, and 24th–28th week of pregnancy [56]. The role of these upregulated microRNAs in the pathogenesis of GDM is not yet fully understood, although some studies reported the involvement of miR-16-5p and of miR-17-5p in T2D and in other metabolic diseases. Interestingly, miR-16-5p

Table 1: The results reported by several studies on circulating microRNAs and GDM.

Study	Study design	Source	Method employed (statistical analysis)	Upregulated microRNA in GDM	Downregulated microRNA in GDM	Limits of the study
Zhao et al. [51]	24 GDM patients and 24 controls at 16th–19th week of pregnancy	Serum	TaqMan low-density array qRT-PCR (Student's *t*-test + risk score analysis)	/	miR-29a, miR-123, miR-222	Normalization using only spiked synthetic exogenous cel-miR-39
Zhu et al. [55]	10 GDM patients and 10 controls at 16th–19th week of pregnancy	Plasma	Ion Torrent qRT-PCR (Student's *t*-test)	miR-16-5p, miR-17-5p, miR-19a-3p, miR-19b-3p, miR-20a-5p	/	Identified microRNAs are enriched in erythrocytes (limitation for their use as biomarkers)
Cao et al. [56]	85 GDM patients and 72 controls at 16th–20th, 20th–24th, and 24th–28th week of pregnancy	Plasma	qRT-PCR (Student's *t*-test, ROC curves)	miR-16-5p, miR-17-5p, miR-20a-5p	/	Identified microRNAs are enriched in erythrocytes (limitation for their use as biomarkers)
Wander et al. [65]	36 GDM patients and 80 controls at 7th–23rd week of pregnancy	Plasma	qRT-PCR (logistic regression adjusted for gestational age at blood draw)	miR-155-5p, miR-21-3p, miR-210-3p, miR-155-5p, miR-146b-5p, miR-223-3p, miR-517-5p, miR-29a-3p	/	Limited number of microRNAs analyzed (associated with pregnancy course and complications)
Lamadrid-Romero et al. [69]	54 GDM patients, 57 non-GDM controls at first, second, and third trimesters of pregnancy, 10 nonpregnant healthy women	Serum	qRT-PCR (Turkey's multiple comparison test + unpaired Student's *t*-test)	miR-183-5p, miR-200b-3p, miR-125-5p, miR-1290	/	Limited number of microRNAs analyzed (neural development associated)
Sebastiani et al. [13]	21 GDM patients, 11 non-GDM control subjects (28th–33rd week of pregnancy)	Plasma	Stem-loop RT PCR (Student's *t*-test + nonparametric Mann–Whitney *U* test)	miR-330-3p	/	To be validated in a larger cohort

target genes (*CUL4A*, *SMAD1*, *EGFR*, *ACTB*, *RRP12*, and *DAB2*) have been reported to be downregulated in T2D [57]; moreover, insulin receptor substrate (IRS) proteins 1 and 2, known as adaptor proteins that mediate insulin-like growth factor-1 (IGF-1) and insulin signaling in insulin-sensitive tissues (e.g., adipose tissue, bone, and liver) [58, 59] are reported as miR-16 target genes. In addition, IRS1 and IRS2 promote Wnt/β-catenin signaling that is critical for cell growth: it has been demonstrated that the dysregulation of this signaling pathway leads to cancer, obesity, and diabetes [60]. Although the confirmation of circulating miR-16-5p alteration in the plasma of GDM patients in two independent studies revealed a potential pivotal role for such microRNA in the pathophysiology of GDM, it is important to note the limited use of miR-16-5p as a potential biomarker; indeed, it has been demonstrated that miR-16-5p is highly expressed and enriched in erythrocytes, thus leading to the possibility of its expression level variation upon hemolysis of the sample; such characteristic renders potentially misleading the measurement of circulating miR-16-5p expression levels in order to monitor the development of GDM and, therefore, cannot be taken into consideration as a reliable biomarker.

Regarding miR-17-miR-20b microRNA family, their involvement in smooth muscle cell proliferation has been previously reported, potentially suggesting a specific role for these microRNAs in vascular complications in diabetic patients [61]. Furthermore, another study previously associated miR-17 and miR-20b to preeclampsia [62], a complication of pregnancy, which (i) affects perinatal outcomes, (ii) is highly correlated to GDM in terms of degree of glucose intolerance [63], and (iii) shares common risk factors with GDM [64]. It is not unlikely that different pregnancy complications (GDM, preeclampsia, etc.) may share similar circulating microRNA alterations due to the observed overlaps of these complications during pregnancy.

More recently, Wander et al. have evaluated the expression of 10 microRNAs (miR-126-3p, miR-155-5p, miR-21-3p, miR-146b-5p, miR-210-3p, miR-222-3p, miR-223-3p, miR-517-5p, miR-518a-3p, and miR-29a-3p), selected for their pivotal roles in pregnancy and its complications and/or previously associated to T2D, in a case-control prospective cohort study of pregnancy complications including plasma samples from 36 GDM patients and from 80 non-GDM controls collected during early–mid pregnancy (7th–23rd week of gestation). They found that high plasma levels of two microRNAs (miR-155-5p and miR-21-3p) were associated with GDM, while levels of other two microRNAs (miR-21-3p and miR-210-3p) were specifically associated with overweight/obese GDM women; finally, levels of six microRNAs (miR-155-5p, miR-21-3p, miR-146b-5p, miR-223-3p, miR-517-5p, and miR-29a-3p) were associated with GDM only among patients carrying male fetuses [65]. Previous studies have found an association of these microRNAs to the pathogenesis of T2D as well. For example, microRNA miR-29a was found upregulated in serum of patients with newly diagnosed or existing T2D [66] and regulates hepatic gluconeogenesis [51], insulin resistance in adipocytes cell lines [67], and GLUT4 expression [54]. Conversely, Zhao et al. have found miR-29a downregulated in serum of GDM patients, in contrast to the results of the previous study. Importantly, microRNAs associated with GDM observed by Wander et al. were limited to overweight/obese prepregnancy patients, probably due to the selection of candidate microRNAs analyzed, all involved in pathways that link obesity to T2D [65].

Finally, our group recently analyzed the expression profiles of plasma microRNAs in GDM patients versus nondiabetic subjects at 28th–33th week of gestation. Due to the high heterogeneity of the resulting differentially expressed microRNAs obtained in previous studies, we hypothesized that the preanalytical factors may strongly affect microRNA stability, leading to differences in their expression mainly due to different sampling methods and nonstandardization of blood collection and processing. Using a highly standardized approach, we identified the hyperexpression of miR-330-3p in the plasma of GDM patients versus nondiabetic subjects; furthermore, such microRNA expression levels were able to distinguish two subpopulations of GDM patients characterized by high and low miR-330-3p expression (miR-330-high, miR-330-low) and by differences in diabetic outcome. Moreover, as miR-330-3p targets key genes involved in β cell function (e.g., E2F1), we hypothesized that its plasma hyperexpression may be detrimental for β cell function and/or proliferation if transferred to them [68].

Although the alterations of circulating microRNAs in the plasma of GDM patients have been reported to be potentially associated to β cell dysfunction and impaired compensation, several authors started also to evaluate whether GDM microRNAs were associated to fetal abnormalities related to maternal diabetes. Fetal and neonatal neural system development alterations, resulting in intellectual and behavioral abnormalities, are strongly associated to GDM; although such association has been clearly established, the underlying mechanisms are less clear. Therefore, in order to verify whether microRNAs could be linked to such aspect of GDM-related complication, specific studies have been performed in such direction. To this aim, Lamadrid-Romero et al. evaluated the expression of 12 fetal neural development-related microRNAs (miR-183-5p, miR-200b-3p, miR-9-5p, miR-17-5p, miR-30b-5p, miR-30c-5p, miR-124-3p, miR-125b-5p, miR-128-3p, has-191-5p, miR-1290, and miR-137) in serum of nonpregnant healthy women and pregnant women (GDM and non-GDM) with the aim to find potential correlations between GDM and alterations of circulating microRNAs involved in fetal neural system development. The results showed that the levels of miR-193-5p, miR-200b-3p, and miR-125-5p were higher in the second trimester versus the first trimester of pregnancy, independently of GDM, while levels of miR-137 were higher in the third trimester in relation to the first trimester, revealing the time-related heterogeneity of these neural development-associated microRNAs. Furthermore, during the first trimester of pregnancy, GDM patients showed higher levels of miR-183-5p, miR-200b-3p, miR-125-5p, and miR-1290 versus controls, suggesting potential alterations of fetal neural differentiation and cell proliferation in this group of patients [69]. Interestingly, previous studies demonstrated that miR-183 and miR-200 gene

families regulate cell proliferation in human glioblastoma cells [70, 71] and are involved in the equipoise between neuroepithelial proliferation and neuroblast emergence in the optic lobe of flies [72]. Furthermore, miR-200 can suppress the expression of Sox2 and reduce proliferation and multipotency of NSCs in the midbrain/hindbrain region in mice, thus strongly suggesting that its hyperexpression during GDM could impair fetal neural system development [73]. Collectively, the increased levels of these microRNAs in GDM patients during the first trimester, as reported by Lamadrid-Romero et al., suggest a decrease in cell proliferation and an increase in neuron differentiation during the development of fetal central nervous system [69], confirming the results of previous studies in mice [74, 75]. Finally, they suggest that neural development-associated microRNAs can be detected in the serum of pregnant women, potentially representing a mirroring of the physiological or pathological growth of the fetus during pregnancy. Indeed, it has been previously well established that fetal DNA and/or RNA may derive from fetal cell debris, and recent studies revealed that these molecules could have biological implications and may act as mediator of cell-to-cell communications between the mother and the fetus, both in physiological and in pathological conditions [36, 76]. However, whether such circulating microRNA alterations during GDM represent a cause of neural impairment or a consequence remains to be established, and further studies are needed in such context.

5. Exosomes and Gestational Diabetes Mellitus

In the last decade, several studies have focused on extracellular vesicles (EVs), classified as exosomes or microvesicles, according to their size, cell or tissue of origin, and functions [77].

Particularly, exosomes are bilayered lipid vesicles of 40–120 nm diameter and originate from the endosomal compartment by the fusion of multivesicular bodies with the plasma membrane of multiple cell types; they contain a wide range of molecules, such as RNA (including microRNAs) and proteins, and are involved in cell-to-cell communications by delivering their cargos into target cells. Therefore, exosomes play a key role in many biological processes and could be useful biomarkers of physiological and pathological conditions, as they can be isolated from body fluids (e.g., plasma, saliva, and urine) [29].

EVs and exosomes play several roles during pregnancy, from regulation of immune responses to maternal metabolic adaptation to gestation [78]. Interestingly, placenta can communicate with other organs/tissues and regulate maternal function via exosomes [79], whose secretion can be modulated by many extracellular stimuli, such as low oxygen tension or high glucose concentration [80, 81].

Importantly, it has been shown that the plasma concentration of specific placenta-derived exosomes is increased significantly with gestational age during first trimester in pregnant women compared to nonpregnant women [82, 83]; more specifically, such placenta-derived exosomes can be detected in maternal plasma at early gestation (~6 weeks) [46], and studies by Sarker et al. [83], Salomon et al. [84], and Nardi et al. [85] independently demonstrated that their concentration progressively increases across pregnancy and correlates with gestational age, while the release of specific trophoblast-derived exosomes decreases during the third trimester.

The first study, demonstrating the association of exosomes to GDM pathophysiology, was performed by Rice et al. [81], who demonstrated a significant increase of exosome concentration in the plasma of GDM pregnant women compared to non-GDM pregnant women and, furthermore, reported that high glucose concentration enhanced exosome release from first-trimester trophoblast cells. Salomon et al. [86] confirmed these results and further showed a differential release of proinflammatory cytokines from endothelial cells treated with placental exosomes from GDM women.

Additionally, Elfeky et al. [87] demonstrated that the total number of exosomes in maternal circulation was strongly correlated with maternal BMI, an important risk factor for GDM. A higher maternal BMI was also correlated with a decreased concentration of placental exosomes with respect to the total exosomal population and with an increased release of IL-6, IL-8, and TNF-α from endothelial cells, showing a possible contribution of exosomes to the maternal systemic inflammation during pregnancy [87].

Surely, further studies are required to elucidate the role of exosomes in GDM pathogenesis. In addition, considering that the deregulation of microRNA expression has been associated with complicated pregnancy, microRNA (or other noncoding RNAs) content within exosomes could be profiled and used as biomarkers for gestational diseases such as GDM.

6. Conclusion and Future Perspectives

Circulating microRNAs have been proposed as potential diagnostic, prognostic, and therapeutic biomarkers of several diseases, being potentially secreted in biological fluids by virtually all cell types [88]. Furthermore, they have been suggested as mediators of tissue cross talk, both in physiological and in pathological conditions [89]. Therefore, the characterization of microRNA expression pattern may also reveal new pathogenic mechanisms, thus improving our understanding of several diseases. Clearly, there is a paucity of data regarding the expression of circulating serum/plasma microRNAs in GDM. The results of the studies reported above highlight a large number of potential candidate circulating biomarkers for GDM. Although informative, the data are discordant, probably due to the different samples analyzed (serum versus plasma) and to the different processing protocols used.

As for the choice between serum or plasma, recent evidence suggests the use of plasma over serum to avoid biases linked to coagulation: as a matter of fact, during this process, microRNAs are released from intact cells or platelets, possibly altering the subsequent results [13, 90]. In other diseases involving different alterations, it may be worthwhile to analyze miRNA profiles in serum over plasma [13, 91, 92].

Another important issue to be considered is the lack of global accepted and standardized operating procedures for sample processing, quality control evaluation, RNA

extraction, microRNA profiling method, and data analysis [93]. Therefore, a strong scientific community effort in standardizing common protocols to definitely analyze circulating noncoding RNAs is undoubtedly needed. A step forward is the advancement of novel analytical methodologies. The recently introduced technologies based on next-generation sequencing (NGS) approaches for the evaluation of RNAs represent a novel tool to render circulating microRNA analysis more precise and powerful in terms of absolute quantification and RNA identification. In addition, several novel cDNA library preparation chemistries for small RNA analysis have been recently developed; such methods allow the preparation of complete and highly representative libraries from very small amount of plasma RNA (from 1 ng), thus opening to the possibility to work with low input volume of starting plasma. Finally, NGS technologies allow also the identification of novel classes of small RNAs (e.g., piRNA, tRNA fragments) which may represent additional disease biomarkers in order to be added to the plethora of potential interesting target.

Additionally, the analysis of noncoding RNA content of circulating exosomes represents another aspect to be carefully taken into consideration for future liquid biopsy approaches. Indeed, it is now possible to isolate circulating exosomes derived from a specific cell source, following to an adequate identification of a unique, distinguishing tissue/cell transmembrane protein marker which characterizes that exosomal population [94]. Specifically, as for placenta-derived exosomes, the transmembrane enzyme PLAP (placental alkaline phosphatase), identified as placenta-specific marker, could be potentially used to immunocapture circulating exosomes derived from placenta thus specifically allowing the analysis of their content [95, 96].

Surely, microRNAs represent potential biomarkers for early GDM diagnosis, and to comprehend its pathogenic mechanisms, however, additional studies are necessary to grasp the physiological and pathological patterns of expression of these molecules in pregnancy. Moreover, the characterization of standard operating procedures (SOPs) to collect serum or plasma, to extract RNA, to measure circulating microRNAs, and to analyze their expression profile is required to achieve this important goal.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Circulating MicroRNAs in Elderly Type 2 Diabetic Patients

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The circulating microRNAs (miRNAs) associated with type 2 diabetes (T2D) in elderly patients are still being defined. To identify novel miRNA biomarker candidates for monitoring responses to sitagliptin in such patients, we prospectively studied 40 T2D patients (age > 65) with HbA1c levels of 7.5–9.0% on metformin. After collection of baseline blood samples (t_0), the dipeptidyl peptidase-IV (DPP-IV) inhibitor (DPP-IVi) sitagliptin was added to the metformin regimen, and patients were followed for 15 months. Patients with HbA1c < 7.5% or HbA1c reduction > 0.5% after 3 and 15 months of therapy were classified as "responders" (group R, $n = 34$); all others were classified as "nonresponders" (group NR, $n = 6$). Circulating miRNA profiling was performed on plasma collected in each group before and after 15 months of therapy (t_0 and t_{15}). Intra- and intergroup comparison of miRNA profiles pinpointed three miRNAs that correlated with responses to sitagliptin: miR-378, which is a candidate biomarker of resistance to this DPP-IVi, and miR-126-3p and miR-223, which are associated with positive responses to the drug. The translational implications are as immediate as evident, with the possibility to develop noninvasive diagnostic tools to predict drug response and development of chronic complications.

1. Introduction

With >400 million patients worldwide, type 2 diabetes (T2D) is among the most frequently diagnosed metabolic disorders. T2D is a multifactorial disease: genetic, lifestyle, and environmental factors combine to render target tissues insensitive to insulin, resulting in increasingly high blood levels of glucose. The disease is associated with serious and frequently disabling long-term complications, including cardiovascular disease, renal failure, neuropathy, and blindness, and it is therefore one of the leading causes of the global increase in morbidity and mortality [1–3]. Outcomes could be improved by earlier diagnosis, while the disease is still in the initial phase, and more rational use of currently available therapies (i.e., targeting drugs to the patients most likely to benefit from them). For this reason, there is an urgent need for new biomarkers with potential applications in the prevention and early diagnosis of T2D and for predicting its response to therapy, especially for fragile elderly patients [1, 2, 4].

Recently, microRNAs (miRNAs)—short (21-22 nucleotides), single-stranded, noncoding RNAs—have been detected in human plasma and other biological fluids, and in some cases, their expression profiles prove to be disease-specific [5–9]. Compared with many more conventional biomarkers, miRNAs offer several advantages, such as high stability, even under drastic conditions. In addition, miRNAs have been identified as major regulators of pancreatic β-cell mass and function, that is, the two key factors in the pathogenesis of T2D [9–11]. For these reasons, they are

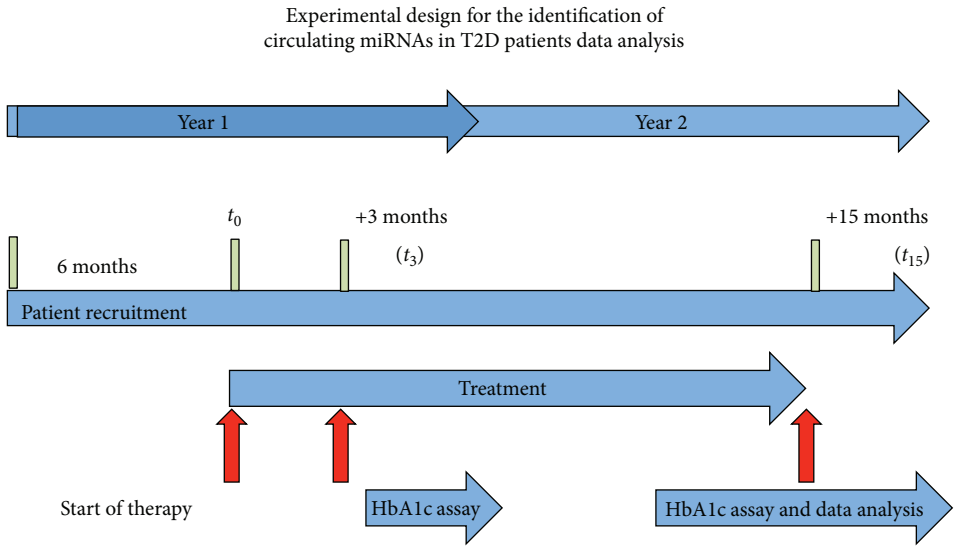

Figure 1: Experimental design for the identification of circulating miRNAs in T2D patients. The duration of the study was 2 years. The blue horizontal arrow indicates the duration of the different phases of the project. The red vertical arrows indicate the main study periods. In year 1, patients were enrolled and started on metformin + sitagliptin (see Materials and Methods for details). After 3 and 15 months of treatment (t_3 and t_{15}), HbA1c values were reassessed and patients were classified as nonresponders (NR) or responders (R). Plasma miRNA levels at baseline (t_0) and t_{15} from groups R and NR were compared as indicated. Comparison of plasma pools: (1) NR-t_0 versus R-t_0, (2) R-t_{15} versus R-t_0, (3) NR-t_{15} versus NR-t_0, and (4) NR-t_{15} versus R-t_{15}.

considered a promising source of biomarkers for diagnosing and staging T2D as well as for predicting their response to therapy [1, 12].

Glucagon-like peptide-1 (GLP-1) is an incretin hormone that stimulates insulin release by β-cells in a glucose-dependent manner and, at the same time, reduces glucagon secretion by alpha-cells. GLP-1-based therapeutic strategies have an increasing role in T2D treatment and consist of GLP-1 agonists and dipeptidyl peptidase-IV (DPP-IV) inhibitors (gliptins) [13]. In detail, gliptins are able to rapidly deactivate GLP-1, resulting in the decrease in insulin production, and are a recent addition to the class of oral glucose-lowering drugs used to treat T2D. Several components of this class, including vildagliptin, sitagliptin, saxagliptin, alogliptin, and linagliptin, have already been approved for this indication by the US Food and Drug Administration or by the European Medicines Agency; others are awaiting for approval or still in development. Gliptins can be used as single-agent therapy or combined with other antidiabetic agents (e.g., metformin) when the latter fail to produce or maintain adequate glycemic control [14, 15]. DPP-IVi would indeed induce insulin secretion in a glucose-dependent manner, with minimal risk of hypoglycemia; accordingly, DPP-IVi can produce a significant reduction in HbA1c. Moreover, they are usually well tolerated, with no weight gain or gastrointestinal side effects [15–18]. Interestingly, they also appear to offer added benefits consisting of the epigenetically mediated restoration of normal gene activity in dysfunctional pancreatic islets [19]. A recent study in diabetic CD1 mice also indicates that gliptin therapy can ameliorate T2D-related kidney fibrosis [20], an effect that was mediated by the drug's induction of the expression of miR-29.

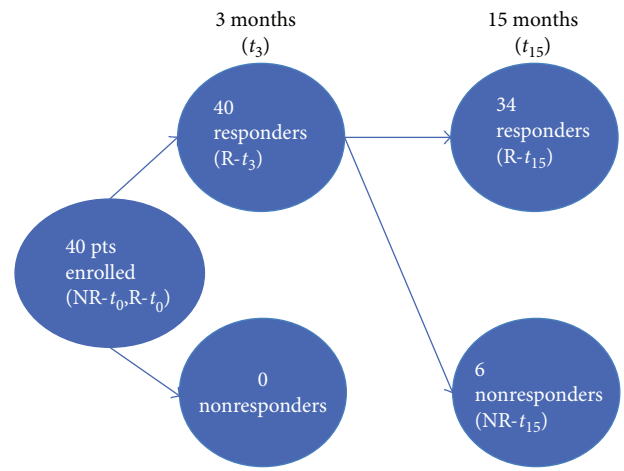

Figure 2: Glycemic control statuses of the patients at baseline and 3 and 15 months after initiation of metformin + sitagliptin. All 40 patients in poor metabolic control were enrolled. HbA1c levels were evaluated after 3 and 15 months from the addition of sitagliptin. On the basis of HbA1c values, patients were divided into responders and nonresponders. All patients showed an initial metabolic response to therapy (t_3), whereas after 15 months (t_{15}), 34/40 were responders. Based on this information, patients were divided into five groups: (1) t_0 responder samples (R-t_0), (2) t_0 nonresponder samples (NR-t_0), (3) t_3 responder samples (R-t_3), (4) t_{15} responder samples (R-t_{15}), and (5) t_{15} nonresponder samples (NR-t_{15}). miRNA profiling was performed at baseline and after 15 months of sitagliptin addition.

These findings prompted us to investigate the circulating miRNA profile of elderly patients with poorly controlled T2D and to identify the changes it undergoes

Table 1: Characteristics of R and NR patients enrolled in the study.

Parameters	Responders (R)			Nonresponders (NR)		
Age (y)	66.62 ± 2.31			67.89 ± 2.24		
Gender, males/females (n)	17/17			4/2		
Time since T2D diagnosis (y)	11 ± 2			10 ± 3		
Time points (in months)	t_0	t_3	t_{15}	t_0	t_3	t_{15}
Body weight (kg)	72.30 ± 4.92	70.36 ± 4.89	70.43 ± 5.23	79.10 ± 12.19	78.57 ± 13.25	79.33 ± 12.99
HbA1c (%)	7.75 ± 0.38	6.38 ± 0.18	7.10 ± 0.28	7.59 ± 0.16	6.73 ± 0.39	7.81 ± 0.51

Data are means ± SD unless indicated otherwise.

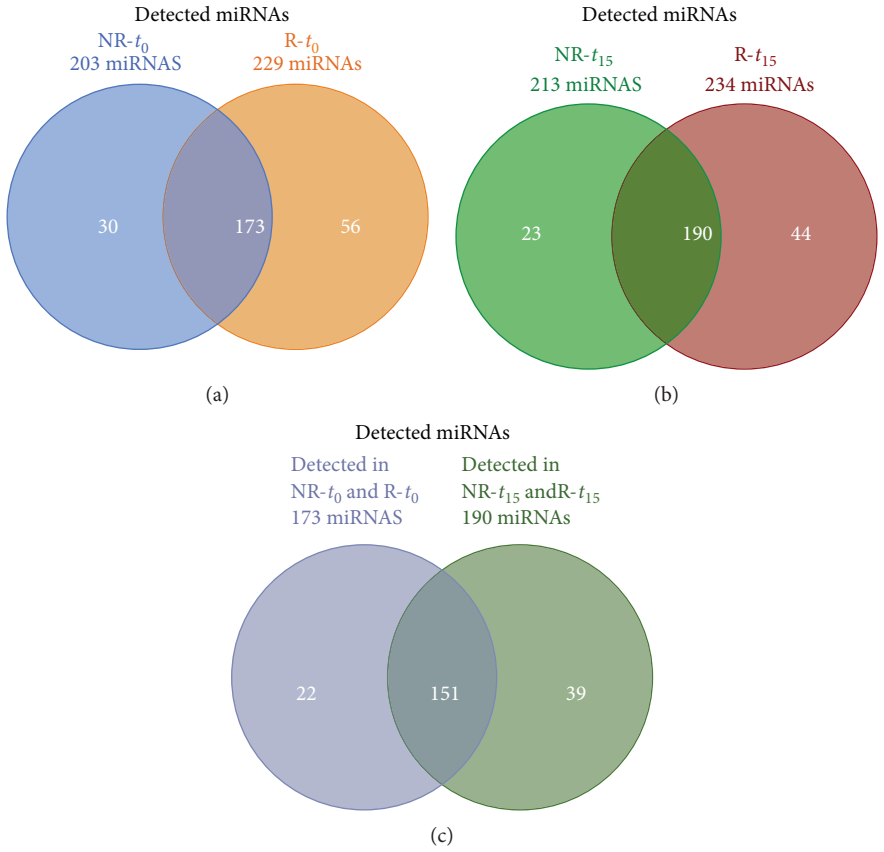

Figure 3: Venn diagram of circulating miRNAs detected in the elderly T2D cohort and in the R and NR subcohorts. Patients were classified as responders (R) or nonresponders (NR) based on their glycemic control status after 15 months of treatment with metformin + sitagliptin (t_{15}). Venn diagrams show the number of miRNAs detected in NR and R plasma samples collected (a) before the start of combined therapy (baseline, t_0) and (b) at t_{15} and (c) at both t_0 and t_{15}.

during DPP-IVi therapy with sitagliptin. The plasma levels of three miRNAs (miR-1208, miR-550a-3p, and miR-30c-5p) displayed directionally similar trends in responders and nonresponders during the 15 months of sitagliptin treatment. These miRNAs thus appear to be modulated by sitagliptin, but not correlated with metabolic response. In contrast, three other miRNAs emerged as promising candidates for use as positive (miR-126-3p and miR-223) and negative (miR-378) biomarkers of responses to sitagliptin therapy in this elderly patient population.

2. Materials and Methods

2.1. Patients. The Ethics Committee of the Tor Vergata University of Rome Medical Center approved this study protocol, and written informed consent was obtained from all patients involved in the study. Patients (males and females) were eligible for enrollment in the study if they met all the following criteria: (1) age > 65 years, (2) a ≥1-year history of T2D, and (3) poor glycemic control (HbA1c levels ranging from 7.5% to 9.0%) on current treatment with maximum-

dose metformin. The following exclusion criteria were applied at baseline and during follow-up: insulin therapy, major organ failure (e.g., congestive heart failure and respiratory and/or hepatic insufficiency), positive history for atrial fibrillation or a coronary or cerebrovascular event during the previous 6 months, known neoplastic disease, and/or acute infections.

Blood samples for HbA1c measurement and circulating miRNA profiling (details below) were drawn upon enrollment (t_0), and the patients were then started on sitagliptin (100 mg once daily) as an adjunct to their metformin therapy. Glycemic control was assessed 3 and 15 months later (t_3 and t_{15}, resp.) (as shown in Figure 1). Patients were classified as "responders" (R) if they exhibited good glycemic control at both time points, reflected by an HbA1c level of <7.5% or an HbA1c reduction of >0.5% relative to the level recorded at t_0. Patients who failed to meet these criteria were classified as "nonresponders" (NR). On the basis of these findings, plasma samples were pooled into the following five groups: (1) baseline samples from patients that emerged as responders (R-t_0 pool), (2) baseline samples from nonresponders (NR-t_0 pool), (3) t_3 samples from responders (R-t_3 pool), (4) t_{15} samples from responders (R-t_{15} pool), and (5) t_{15} samples from nonresponders (NR-t_{15} pool) (Figure 2). Characteristics of the patients enrolled in the study are reported in Table 1.

2.2. Isolation and Profiling of Circulating miRNAs. For miRNA studies, 5 mL of blood was collected from each patient in EDTA-treated tubes. Within 30 minutes of collection, the samples were centrifuged for 10 minutes at 2000 ×g at room temperature (RT), and the plasma thus obtained was divided into 250 μL aliquots and stored at −80°C. To eliminate the risk of bias related to hemolysis [21], all plasma samples were visually assessed and those that were hemolyzed, icteric, or lipemic were excluded from the analysis. We also evaluated the expression of miRNAs susceptible to hemolysis, such as miR-324-3p, miR-454, and miR-652 [21]. Indeed, these miRNAs were not detected in our samples, confirming that none of the samples utilized in the study were hemolyzed.

Plasma samples from all patients in a given group (see above) were thawed on ice and pooled. A miRNA ABC purification kit (Applied Biosystems, Thermo Scientific) was used, according to the manufacturer's instructions. Briefly, 50 μL of each plasma pool was mixed with 100 μL of lysis buffer and centrifuged briefly before the addition of 100 nM of ath-miR-159a (used as a positive external control). Samples were then mixed with freshly prepared magnetic beads (80×10^6) and incubated in a magnetic rack (40 min at 30°C). Bead-hybridized miRNAs were then washed to remove any contaminants. Elution buffer (50 μL) was added, and the sample was incubated for 3 minutes in a ThermoMixer (1200 rpm, 70°C) and placed for 1 minute in a magnetic rack to clear solutions. The supernatants were then transferred into clean tubes and placed on ice.

miRNAs were reverse-transcribed using specific primers according to Applied Biosystems protocols. The cDNAs were preamplified using reagents from Applied Biosystems (Thermo Scientific), and the products were subjected to miRNA expression profiling. The latter was performed by RT-qPCR with Taqman Low-Density Array microfluidic cards (Human miR v3.0, Applied Biosystems), as previously described [22].

Table 2: miRNAs with known links to T2D found in baseline (t_0) plasma pools from groups NR and R.

T2D-linked microRNAs at t_0
hsa-let-7d
hsa-miR-103
hsa-miR-126
hsa-miR-130b
hsa-miR-142-3p
hsa-miR-144
hsa-miR-145
hsa-miR-146a
hsa-miR-155
hsa-miR-17-5p
hsa-miR-186
hsa-miR-191
hsa-miR-192
hsa-miR-195
hsa-miR-197
hsa-miR-20b
hsa-miR-21
hsa-miR-222
hsa-miR-223
hsa-miR-23a
hsa-miR-26a
hsa-miR-27a
hsa-miR-29a
hsa-miR-30d
hsa-miR-30e
hsa-miR-342
hsa-miR-34a
hsa-miR-375
hsa-miR-378
hsa-miR-423-5p
hsa-miR-451a
hsa-miR-483-3p
hsa-miR-486
hsa-miR-571
hsa-miR-572
hsa-miR-593
hsa-miR-661
hsa-miR-770-5p
hsa-miR-9
hsa-miR-92a

2.3. miRNA Expression Analysis. Statistical analysis was performed with StatMiner™ software, v. 5.0 (Integromics™) [23]. miRNA expression levels in plasma pools were subjected

Table 3: miRNAs with known links to T2D found in t_{15} plasma pools from groups NR and R.

T2D-linked miRNAs at t_{15}
hsa-let-7d
hsa-miR-103
hsa-miR-106b
hsa-miR-122
hsa-miR-126
hsa-miR-130b
hsa-miR-132
hsa-miR-140
hsa-miR-140-3p
hsa-miR-142-3p
hsa-miR-144
hsa-miR-145
hsa-miR-146a
hsa-miR-155
hsa-miR-17-5p
hsa-miR-181a
hsa-miR-186
hsa-miR-18a
hsa-miR-191
hsa-miR-192
hsa-miR-195
hsa-miR-197
hsa-miR-20b
hsa-miR-21
hsa-miR-221
hsa-miR-222
hsa-miR-223
hsa-miR-23a
hsa-miR-24
hsa-miR-26a
hsa-miR-27a
hsa-miR-28-3p
hsa-miR-30d
hsa-miR-30e
hsa-miR-320
hsa-miR-342
hsa-miR-34a
hsa-miR-375
hsa-miR-378
hsa-miR-423-5p
hsa-miR-451a
hsa-miR-483-3p
hsa-miR-486
hsa-miR-571
hsa-miR-572
hsa-miR-593
hsa-miR-661
hsa-miR-770-5p
hsa-miR-92a
hsa-miR-96

to global expression normalization, and relative levels were calculated with the comparative threshold cycle (Ct) method. miRNAs with Ct values > 33 were excluded. Differential expression between groups was assessed with the limma test and considered statistically significant when P values were <0.05. Heat maps were generated in the R environment (http://www.r-project.org/), using differentially expressed miRNAs as input. The Bray-Curtis and average linkage methods were used to cluster samples (hclust) and generate heat maps (heatmap.2).

3. Results and Discussion

All 40 enrolled patients completed 15 months of treatment with metformin + sitagliptin without occurrence of adverse events. After three months of the study treatment, all patients met the predefined criteria for good metabolic control. In contrast, at t_{15}, only 34/40 (85%) were still in good metabolic control; the remaining six (15%) had HbA1c levels that exceeded 7.5% ($n = 6$). On the basis of these findings, patients were divided into responders (group R), which included the 34 patients in good glycemic control at t_3 and t_{15}, and nonresponders (group NR, 6/40), whose initial response to the addition of sitagliptin at t_3 was not maintained at t_{15}.

3.1. Circulating miRNA Profiles of Groups R and NR. We then compared groups R and NR in terms of miRNA expression profiles in their t_0 plasma sample pools. Of the 754 miRNAs analyzed, 203 (27%) were detected in group NR and 229 (30%) in group R (Supplementary Tables 1 and 2, resp.). The reliability of our findings is supported by the fact that 173 miRNAs found in both groups of plasma samples (Figure 3(a)) included several miRNAs previously known to be associated with T2D; specifically, 36% of those were found in the BioM2MetDisease database (http://www.bio-bigdata.com/BioM2MetDisease/browse) and identified in a recent systematic review by He et al. [24] (Table 2). Similar findings emerged when we compared circulating miRNA profiles after 15 months of combined metformin + sitagliptin therapy. Indeed, the number of detectable miRNAs in the NR group (213/754, 28%) was comparable to that in the R group (234/754, 31%) (Supplementary Tables 3 and 4). In addition, the subset of miRNAs found in both t_{15} pools (Figure 3(b)) comprised many of those with known links to T2D (39% in BioM2MetDisease and He et al. [24]) (Table 3). Finally, 151 miRNAs were detected in the plasma pools for both groups, before (t_0) and after the addition of sitagliptin (t_{15}) (Figure 3(c), Supplementary Table 6). Notably, miRNAs described to be linked to T2D in previous studies were detected also in our cohort of elderly T2D patients, further strengthening their potential role as T2D biomarkers.

Table 4: miRNAs that were differentially expressed in t_0 plasma pools from groups NR and R.

Regulation	miRNA	Linear fold change	P value
Upregulated in NR-t_0	hsa-miR-1208	2.05	$1.84E-02$
	hsa-miR-1225-3p	2.27	$1.20E-02$
	hsa-miR-1252-5p	901.88	$4.52E-02$
	hsa-miR-338-5p	7.56	$4.22E-02$
	hsa-miR-375	68.83	$4.35E-02$
	hsa-miR-378*	15.26	$3.81E-02$
	hsa-miR-571	2.39	$1.32E-02$
	hsa-miR-595	540.84	$4.66E-02$
	hsa-miR-601*	14179.56	$4.97E-02$
	hsa-miR-885-5p	4.58	$3.00E-02$
Downregulated in NR-t_0	hsa-let-7b-5p*	0.18	$1.53E-03$
	hsa-let-7d-5p	0.19	$1.11E-02$
	hsa-miR-1247-5p	0.20	$9.91E-03$
	hsa-miR-16-5p*	0.21	$1.51E-03$
	hsa-miR-223-5p	0.32	$1.83E-02$
	hsa-miR-23a-5p*	0.20	$8.79E-03$
	hsa-miR-30b-5p*	0.36	$2.74E-02$
	hsa-miR-320a	0.23	$5.22E-03$
	hsa-miR-451a*	0.31	$3.88E-03$
	hsa-miR-93-5p*	0.09	$2.03E-02$

*Hemolysis-susceptible miRNAs as reported in Kirschner et al. [21].

3.2. Circulating miRNAs in Elderly T2D Patients: Differential Expression between Responders and Nonresponders at Baseline. Our next goal was to identify circulating miRNAs that might be correlated with a positive metabolic response to therapy. To this end, we first compared the plasma levels of the 173 miRNAs found in the t_0 plasma pools from groups R and NR. This analysis revealed 20 miRNAs that were differentially expressed in the two groups (Table 4, Figure 4(a)) and might thus be potentially useful for predicting the response to therapy. As noted in Table 4, plasma levels of eight of these 20 miRNAs are known to be potentially influenced by blood cell contamination [21]. However, this factor is unlikely to have played a role in this analysis, since occurrence of hemolysis was excluded in all plasma samples analyzed.

Expression of 10 miRNAs was significantly lower in plasma from NR-t_0 versus R-t_0 (Table 4, Figure 4(a)); these included three miRNAs of particular interest: let-7d, miR-223, and miR-23a, whose expression was reported to be downregulated in T2D patients when compared to healthy individuals [6, 12, 25, 26]. Reduced expression of let-7d, miR-223, and miR-23a in NR patients suggests that these miRNAs may represent potential biomarkers of response to sitagliptin therapy.

Other miRNAs resulted to be significantly more expressed in NR-t_0 plasma samples. These included miR-375, whose high expression has been associated with β-cell dysfunction [27, 28]; miR-571, previously reported to be hyperexpressed in plasma samples from T2D patients compared to healthy controls [29]; and miR-378, which was associated with obesity-related insulin resistance [30, 31].

Increasing evidence has shown the involvement of miRNAs in the development of the endocrine pancreas, as well as in the regulation of insulin secretion, insulin signaling, and insulin gene transcription. Indeed, in Dicer-1 conditional knockout mice, loss of miRNAs in β-cells causes major defects in glucose homeostasis and in insulin secretion, with a marked reduction in insulin content. Moreover, studies in rodent models of T2D have revealed changes in miRNA expression in β-cells [32].

In this context, our data provide evidence that some miRNAs previously reported as regulators of glucose homeostasis in critical tissues (e.g., endocrine pancreas) may also represent circulating biomarkers for disease staging and/or for predicting response to glucose-lowering therapy.

3.3. Circulating miRNAs in the Elderly T2D Patients Who Responded to Sitagliptin Treatment. To identify miRNAs potentially modulated by sitagliptin therapy, we compared miRNA expression levels at t_0 and at t_{15} in plasma samples from the R group of patients. Twenty-one miRNAs were differentially expressed between the two plasma pools (Table 5, Figure 4(b)). Of note, expression levels of miR-222, previously reported to be hyperexpressed in plasma samples from T2D patients versus healthy controls [33], were reduced at t_{15} versus t_0.

Conversely, three miRNAs were found significantly upregulated in the R-t_{15} plasma pool (Table 5). These included miR-126-3p, which was reported to be decreased in T2D patients (with or without complications) versus healthy controls [34] and in T2D patients with major cardiovascular events [35]. Moreover, this miRNA has been proposed as a biomarker for the detection of prediabetes and diabetes. miR-30c, another miRNA significantly upregulated in the R-t_{15} plasma pool, was recently shown to exert protective effects on diabetic nephropathy [36] and cardiomyopathy [37]. Interestingly, gliptins inhibit the degradation of several peptides and chemokines and reduce tissue inflammation by suppressing macrophage activation and M2 macrophage response. These findings suggest that glucagon-like peptide-1- (GLP-1-) based treatments provide additional benefits beyond glycemic control, including vascular protection and improved bone health [38].

3.4. Circulating miRNAs in Elderly T2D Patients Who Did Not Respond to Sitagliptin Treatment. We then compared miRNA expression levels at t_0 and at t_{15} in plasma samples from the NR group of patients. As shown in Table 6 and in Figure 4(c), 21 miRNAs were differentially expressed between the two time points, with 5/21 (miR-1208, miR-550a-3p, miR-30c-5p, miR-1260a, and miR-1291) showing similar posttreatment changes to group R (Tables 4 and 5). Consequently, this observation suggests the exclusion of these five miRNAs as possible biomarkers of response to sitagliptin therapy. Nevertheless, the protective effects of

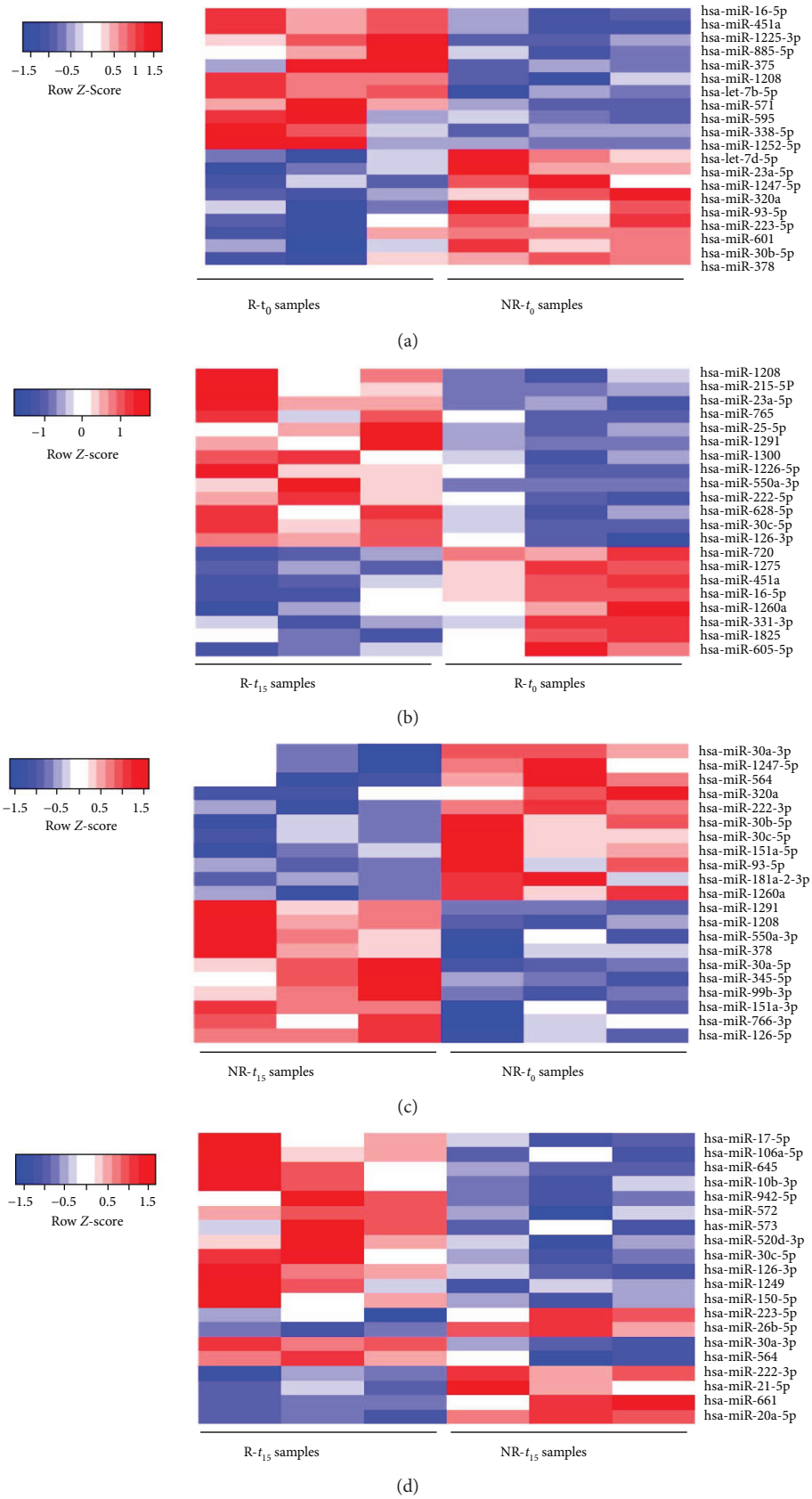

FIGURE 4: Heat maps showing circulating microRNAs that were differentially expressed in plasma samples from (a) R and NR patients at baseline (t_0), prior to the addition of sitagliptin to the maximum-dose metformin regimen; (b) R at t_0 and after 15 months of sitagliptin (t_{15}); (c) NR patients at t_{15} and t_0; and (d) R and NR patients at t_{15}.

Table 5: miRNAs that were differentially expressed in t_0 and t_{15} plasma pools from group R.

Regulation	miRNA	Linear fold change	P value
Upregulated in R-t_{15}	hsa-miR-126-3p	3.30	$2.05E-02$
	hsa-miR-30c-5p	3.44	$4.71E-03$
	hsa-miR-331-3p*	163.08	$3.12E-02$
Downregulated in R-t_{15}	hsa-miR-1208	0.53	$3.55E-02$
	hsa-miR-1226-5p	0.42	$2.78E-02$
	hsa-miR-1260a	0.35	$4.07E-02$
	hsa-miR-1275	0.24	$1.93E-03$
	hsa-miR-1291	0.15	$3.98E-02$
	hsa-miR-1300	0.29	$2.08E-02$
	hsa-miR-16-5p*	0.27	$2.23E-02$
	hsa-miR-1825	0.50	$4.13E-02$
	hsa-miR-215-5p	0.05	$3.76E-02$
	hsa-miR-222-5p	0.26	$3.66E-02$
	hsa-miR-23a-5p*	0.18	$4.28E-03$
	hsa-miR-25-5p*	0.03	$3.26E-02$
	hsa-miR-451a*	0.35	$9.49E-03$
	hsa-miR-550a-3p	0.34	$1.27E-02$
	hsa-miR-605-5p	0.19	$1.34E-02$
	hsa-miR-628-5p	0.00	$3.07E-02$
	hsa-miR-720	0.41	$5.80E-03$
	hsa-miR-765	0.01	$4.16E-02$

*Hemolysis-susceptible miRNAs as reported in Kirschner et al. [21].

Table 6: miRNAs that were differentially expressed in t_0 and t_{15} plasma pools from group NR.

Regulation	miRNA	Linear fold change	P value
Upregulated in NR-t_{15}	hsa-miR-1247-5p	4.57	$2.50E-02$
	hsa-miR-126-5p	1.62	$4.72E-02$
	hsa-miR-151a-3p	2.50	$1.87E-02$
	hsa-miR-151a-5p	3.73	$9.29E-03$
	hsa-miR-181a-2-3p	134.20	$1.75E-02$
	hsa-miR-222-3p	1.85	$1.94E-02$
	hsa-miR-30a-3p*	1.70	$4.95E-02$
	hsa-miR-30a-5p	1.59	$4.80E-02$
	hsa-miR-30b-5p	2.18	$2.63E-02$
	hsa-miR-30c-5p	2.08	$3.87E-02$
	hsa-miR-320a	2.95	$3.40E-02$
	hsa-miR-378*	2.05	$4.62E-02$
	hsa-miR-564	3.39	$1.41E-02$
	hsa-miR-766-3p	3.46	$4.62E-02$
	hsa-miR-93-5p*	6.81	$2.63E-02$
Downregulated in NR-t_{15}	hsa-miR-1208	0.23	$3.70E-03$
	hsa-miR-1260a	0.37	$8.75E-03$
	hsa-miR-1291	0.28	$1.11E-02$
	hsa-miR-345-5p	0.15	$9.84E-03$
	hsa-miR-550a-3p	0.48	$4.08E-02$
	hsa-miR-99b-3p*	0.29	$7.73E-03$

*Hemolysis-susceptible miRNAs as reported in Kirschner et al. [21].

miR-30c-5p on diabetic nephropathy and cardiomyopathy [36, 37] underline the need for further investigation on the functional role of miR-30c in elderly T2D patients.

A comparison between the differentially expressed miRNAs of NR versus R at baseline (Table 4) and NR at t_{15} versus baseline (Table 6) allowed us to observe that one of the miRNAs that were more abundant in NR plasma than in R plasma at baseline, miR-378, increased during the treatment period, reaching levels in the NR-t_{15} plasma pool that were significantly higher than those in the NR-t_0 pool. This result allowed us to propose miR-378 as a negative biomarker candidate for response to sitagliptin therapy.

In this context, our results could open new perspectives providing the basis for further investigations of the reported dysregulated miRNAs as markers of response to gliptins.

3.5. Circulating miRNAs in Elderly T2D Patients after 15 Months of Sitagliptin Treatment. To identify miRNAs that might be affected by sitagliptin treatment, we compared circulating miRNA levels in plasma samples at t_{15} from the two subgroups. Twenty miRNAs were differentially expressed in plasma from NR versus R patients (Table 7, Figure 4(d)). Ten of the 20 miRNAs were significantly more expressed in the plasma samples from the NR group. Among the upregulated miRNAs, we observed an increased expression of miR-661 and miR-572 in NR-$t_{15,}$ in accordance with previous studies performed in T2D patients [26, 39].

miRNAs that were hypoexpressed in the NR-t_{15} pool included two of particular interest: miR-126-3p and miR-223. In the previous analysis of group R, miR-126-3p levels at t_{15} were higher than those in the t_0 plasma pool, suggesting that this miRNA is a good candidate biomarker of successful metabolic response to therapy. Additionally, our previous comparison of t_0 plasma pools from the two groups revealed substantially lower levels of miR-223 in NR. This miRNA has already been associated with pancreatic islet β-cell function and glycemic control, and its expression is reportedly higher in individuals with pre-T2D and normal controls than in T2D patients [40, 41]. In light of these results, miR-223 appears to be a possible positive biomarker for monitoring patients' responsiveness to sitagliptin therapy.

Finally, we checked for a possible correlation between differentially expressed miRNAs, miR-126-3p, miR-223, and miR-378, and the patients' clinical features. We did not observe any statistically significant correlation between miR-126-3p, miR-223, and miR-378 and age, time from T2D diagnosis, body weight, and HbA1c levels.

An increasing body of evidence has linked diabetes to cardiovascular disease, renal failure, neuropathy, and osteoporosis, especially in elderly individuals, with a consequent

Table 7: miRNAs that were differentially expressed in the t_{15} plasma pools from groups NR and R.

Regulation	miRNA	Linear fold change	P value
Upregulated in NR-t_{15}	hsa-miR-10b-3p	2.49	$3.39E-02$
	hsa-miR-1249	4.17	$4.27E-02$
	hsa-miR-30a-3p*	3.84	$1.62E-03$
	hsa-miR-520d-3p	6.78	$1.79E-02$
	hsa-miR-564	2.86	$2.19E-02$
	hsa-miR-572	2.15	$1.81E-02$
	hsa-miR-573	7.99	$4.10E-02$
	hsa-miR-645	2.85	$1.90E-02$
	hsa-miR-661	6.57	$3.68E-03$
	hsa-miR-942-5p	2.63	$1.70E-02$
Downregulated in NR-t_{15}	hsa-miR-106a-5p*	0.41	$2.68E-02$
	hsa-miR-126-3p	0.42	$1.13E-02$
	hsa-miR-150-5p	0.49	$4.21E-02$
	hsa-miR-17-5p*	0.43	$3.28E-02$
	hsa-miR-20a-5p*	0.18	$3.61E-04$
	hsa-miR-21-5p*	0.17	$1.59E-02$
	hsa-miR-222-3p	0.35	$5.14E-03$
	hsa-miR-223-5p	0.54	$4.95E-02$
	hsa-miR-26b-5p*	0.41	$4.04E-03$
	hsa-miR-30c-5p	0.45	$2.62E-02$

*Hemolysis-susceptible miRNAs as reported in Kirschner et al. [21].

increase in mortality, morbidity, and socioeconomic costs. Reliable biomarkers to predict drug response in these patients are urgently needed but still lacking. Glucose-lowering drugs, such as DPP-IVi, are known to differentially impact metabolic control and disease-related complications.

Our study is the first to provide a description of the circulating miRNAs that can be used as novel biomarkers for monitoring response to therapy in elderly T2D patients.

4. Conclusion

The results we obtained suggest that miR-378, miR-126-3p, and miR-223 represent candidate plasma biomarkers for disease staging and for predicting response to therapy in T2D elderly patients. High circulating levels of miR-378 appear to be a negative predictor of response to sitagliptin in elderly T2D patients. Indeed, miR-378 was more expressed in the NR-t_0 plasma pool than in the R-t_0 pool. In addition, its levels in the NR-t_{15} plasma were even higher than those found in the NR-t_0 pool, and this result highlights its possible role as a biomarker of resistance to sitagliptin. In contrast, miR-126-3p and miR-223 seem to be markers of response to the drug. miR-126-3p levels have been reported to be lower in T2D patients than in healthy individuals [34]. Consistently, this miRNA was not differentially expressed in the plasma of R and NR patients at baseline. After 15 months of sitagliptin therapy, however, plasma levels in responders were significantly higher than those found in the NR group, suggesting that the addition of the DPP-IVi may have restored miR-126-3p levels to the range found in healthy subjects. As for miR-223, its expression at baseline was already significantly higher in the R group, and this difference persisted after 15 months of sitagliptin addition. This behavior is consistent with its potential role as a positive predictor of response to the drug.

Further work is needed to validate the role of these miRNAs as biomarkers in T2D patients, since this was a discovery study and the number of NR patients was limited. Interestingly, circulating miRNAs may reflect phenomena occurring at the level of those organs involved in T2D pathophysiology (e.g., endocrine pancreas, liver, and adipose tissue). Therefore, our data provide a snapshot of the circulating miRNAs that deserve further studies.

The translational relevance of our findings is immediate; in fact, data regarding miRNA profiles can produce results of potential impact not only predicting drug response of T2D elderly patients but also helping in selecting patients that could be suitable candidates to this therapy.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Giuseppina Catanzaro, Zein Mersini Besharat, Agnese Po, and Elisabetta Ferretti contributed equally to this work.

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Supplementary Materials

Supplementary Tables 1–5 list the detected microRNAs in the plasma samples of interest (NR-t_0, R-t_0, NR-t_{15}, and R-t_{15}). *(Supplementary Materials)*

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Review Article

Noncoding RNAs Carried by Extracellular Vesicles in Endocrine Diseases

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RNA molecules are essential and fine regulators of important biological processes. Their role is well documented also in the endocrine system, both in physiological and pathological conditions. Increasing interest is arising about the function and the importance of noncoding RNAs shuttled by extracellular vesicles (EVs). In fact, EV membrane protects nucleic acids from enzyme degradation. Nowadays, the research on EVs and their cargoes, as well as their biological functions, faces the lack of standardization in EV purification. Here, the main techniques for EV isolation are discussed and compared for their advantages and vulnerabilities. Despite the possible discrepancy due to methodological variability, EVs and their RNA content are reported to be key mediators of intercellular communication in pathologies of main endocrine organs, including the pancreas, thyroid, and reproductive system. In particular, the present work describes the role of RNAs contained in EVs in pathogenesis and progression of several metabolic dysfunctions, including obesity and diabetes, and their related manifestations. Their importance in the establishment and progression of thyroid autoimmunity disorders and complicated pregnancy is also discussed. Preliminary studies highlight the attractive possibility to use RNAs contained in EVs as biomarkers suggesting their exploitation for new diagnostic approaches in endocrinology.

1. Introduction

The larger fraction of transcribed RNAs is composed by noncoding RNAs, instead of mRNAs coding for proteins [1]. Noncoding RNAs include a wide variety of RNA molecules, such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), circular RNAs (cRNAs), and small nucleolar RNAs (snoRNAs) with several different regulatory and structural functions. They are involved in mRNA translation and splicing, transcription initiation, but also cell cycle regulation, chromosome maintenance and segregation, chromatin remodeling, and epigenetic memory regulation [1]. Noncoding RNAs also include cytoplasmic Y RNAs (yRNAs) and vault RNAs (vtRNAs). YRNAs are involved in chromosomal DNA replication and in RNA stability when complexed with Ro60 ribonucleoprotein particle. YRNAs also modulate cell death and inflammation [2, 3]. VtRNAs are a part of large ribonucleoprotein particles present in the cytoplasm of many eukaryotic cells, which are suggested to be involved in several processes, including multidrug resistance of cancer cells, DNA damage repair, innate immune response, apoptosis resistance, nuclear pore complex formation, and nucleocytoplasmic transport [4, 5]. However, their functions are still not completely elucidated. Two other well-studied classes of noncoding RNAs are microRNAs (miRNAs) and small interference RNA (siRNAs), short single-strand RNA molecules (20–22 nucleotides) derived from hairpin or double-stranded RNA precursors. These RNAs are loaded by the Dicer complex into a member of the Argonaute protein subfamily to form the RNA-induced silencing (RISC) complex, which recognizes a complementary sequence in the

target mRNA and mediates degradation or inhibits translation into protein [6]. miRNAs regulate posttranscriptional gene silencing of up to 60% of protein-coding genes targeting one or several mRNAs. They have been associated to quite all biological processes, including development, proliferation, differentiation, metabolism, apoptosis, and cancer [7]. Finally, a large part of the mammalian noncoding transcripts is composed by long noncoding RNAs (lncRNAs), RNA molecules of approximately 200 nucleotides or more. LncRNAs take part in several biological processes: they regulate transcription by affecting the activity of specific transcription factors and polymerases. They mediate posttranscriptional regulation, by influencing splicing, transport, translation, and degradation of mRNAs, and they take part in epigenetic modifications, by regulating chromatin remodeling and X chromosome inactivation in mammals [8, 9]. Interestingly, lncRNAs can also modulate the biological activity of other RNA species. LncRNAs can interact with miRNAs and inhibit their effect by acting as "competing endogenous RNAs" (ceRNAs). LncRNAs containing miRNA-binding sequence regions can impound miRNA molecules and reduce their availability for target mRNAs [10]. This mechanism of interaction has been recently demonstrated to play a critical role in several pathological processes, including cancers [11–13], fat deposition [14], diabetic retinopathy [10], and osteoarthritis [15], and in biological processes such as cellular apoptosis [16–18] and stem cell differentiation [19].

During the last few years, extracellular vesicles (EVs) have been recognized as carriers of RNA molecules from their cell of origin to recipient cells all over the organism. Indeed, EVs are a heterogeneous class of vesicles ranging from 20 to 1000 nm, delimited by plasma membrane (PM) and containing proteins and nucleic acids. In this review, we will collectively refer to EVs that include several subpopulations, such as microvesicles, microparticles, and exosomes, basing on their size, biogenesis, molecular markers, and isolation techniques [20, 21].

2. EVs and Communication

It is now clear that EVs play an important role in cell to cell communication between neighboring and distant cells. In fact, EVs are released by quite all cell types and have been detected in several biological fluids [22]. Recipient cells uptake EVs by receptor-mediated interactions or by direct fusion with the PM [23]. In this way, EVs transfer lipids, membrane receptors, proteins, or nucleic acids to recipient cells. First evidence that EVs mediate the horizontal transfer of proteins and mRNAs and reprogram recipient cells was provided by Ratajczak et al. [24]. Recently, some interesting *in vivo* studies have used Cre recombinase technique [25–27] or a combination of fluorescent and bioluminescent reporters tagging EV membrane and RNA molecules [28] to demonstrate that mRNAs are transferred from cell to cell by EVs. Being mediators of cell to cell interaction, EVs play a complex role in pathophysiology of several organs and diseases. The biological effects of EVs depend on their origin, on the status, and on the environment. Stem cell-derived EVs elicit, at least in part, the regenerative properties of their cell of origin. Particularly, EVs released by mesenchymal stromal cells (MSCs) have been widely studied and showed immunomodulatory properties and a protective role in cardiovascular disease, kidney disease, lung disease, and liver disease [29]. MSC-derived EVs exploit both antitumor and protumorigenic activity based on the context [30]. On the other hand, EVs released by cancer stem cells, such as renal cancer stem cells [31] or prostate cancer stem cells [32], can promote tumor metastasis. In general, EVs act influencing different biological processes and tumor-derived EVs can support tumor in several ways, by promoting cell migration, invasiveness, angiogenesis, and premetastatic niche formation in distant sites [33]. EVs from different other cells, adipose mesenchymal stem cells (ASCs), endothelial cells, proangiogenic progenitors, and tumor cells, show proangiogenic properties *in vitro* and *in vivo*, in ischemic injury or in tumors [34].

3. EV Biogenesis and Cargo

EVs can be classified into two different groups based on their biogenesis: microvesicles and exosomes. Microvesicles originate by shedding from the cell surface. Changes in the composition of lipids, proteins, and other components of the PM modify the curvature of the membrane and facilitate the microvesicle budding. This process relies, in part, on the interaction of proteins such as arrestin domain-containing protein-1 (ARRDC1) with the late endosomal protein TSG101. Microvesicle fission is due to myosin and actin cytoskeletal rearrangements regulated by the Ras-related GTPase ADP-ribosylation factor 6 (ARF6) and its signaling cascade [35]. Exosomes are formed by budding of the membrane into the lumen of multivesicular bodies (MVB), which are part of the endocytic pathway and fuse with the PM releasing exosomes outside the cell. Several proteins are involved with the formation of exosomes, such as the components of the endosomal sorting complex required for transport (ESCRT) machinery and the accessory proteins TSG101, ALIX, and VPS4. However, other ESCRT-independent mechanisms have been described [36]. The docking of MVB with the PM is mediated by several components of the RAB family of small GTPase proteins (RAB2B, RAB5A, RAB7, RAB9A, RAB11, RAB27A, RAB27B, RAB35 [37], and RLP-1 [38]). Some members of the tetraspanin family, such as CD63, CD81, and CD9, are enriched in exosomes and have been recognized as exosome markers [37]. On the other hand, specific markers for microvesicles are lacking and it is difficult to discriminate EVs based on biomarkers. Moreover, even if the biogenesis of microvesicles and exosomes is based on different processes, some mechanisms are shared [36].

EV content varies depending on the originating cell and the biogenesis mechanism. However, the compartmentalization of proteins and RNAs is, at least in part, a regulated process. Comparative lipidomic, proteomic, and transcriptomic analysis usually finds an enrichment of subsets of lipids, proteins, or RNAs in EVs compared with their cells of origin [39–43]. Several proteins involved in EV biogenesis regulate compartmentalization into EVs. For example, ESCRT complex recruits proteins into both exosomes and

microvesicles [44]. In human liver stem cells, ALIX is associated with Ago2, a member of the Argonaute protein family, which binds miRNAs. The complex ALIX-Ago2-miRNA was found in EVs [45]. In breast cancer, EVs contain functional Ago2-associated miRNAs, which are mature and induce transcriptome alterations in target cells [46]. The heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) recognizes specific motifs and regulates miRNA loading into EVs [47]. The RNA-binding protein Y-box protein I (YBX1) binds to miR-223 and is necessary for its packaging into EVs [48]. In colorectal cancer cells, KRAS seems to mediate miRNA sorting into EVs [49] by regulating Ago2 secretion [50]. Besides miRNAs, increasing reports show that EVs are particularly enriched in other types of small RNAs, such as tRNA fragments, yRNAs, vtRNAs, and miRNA fragments [51–53]. The presence of a regulated mechanism of small RNA sorting into EVs has been observed also in Leishmania, suggesting that it is conserved throughout evolution [54]. However, the biological functions of small RNAs found in EVs are not completely clear.

4. Techniques for EV Isolation

Before addressing the main topic of this review relative to the role of noncoding RNA shuttled by EVs in endocrine pathophysiology, it is useful to discuss the technical challenges in EV purification as these may influence the analysis of RNAs contained in EVs [55]. In fact, EVs are heterogeneous in size and molecular composition (membrane lipids, surface proteins, and cargo), and thus in density and charge. The heterogeneity is increased as EVs derive from different cell of origin (e.g., biological fluids such as blood or saliva). This is a relevant problem since the purity, the integrity, the yield, and the biological activity of EVs are influenced by the isolation technique [56, 57]. Each isolation technique presents advantages and disadvantages, and the choice of the methods should be based on the starting material and volume, on the grade of purity desired, and on the purpose of the isolation (research, therapeutic, or diagnostic use).

The standard technique is differential ultracentrifugation. This protocol is based on several consecutive centrifugation steps. First steps are necessary to discard cells and debris and consist in brief centrifugation at low centrifugal forces (g). The consecutive steps isolate different populations of EVs based on density (i.e., 10,000 g for microvesicles and 100,000 g for exosomes). The protocol was firstly described by Raposo et al. [58] and then widely adopted with slight variations depending on the source of EVs (cell supernatant or biological fluids) [59, 60]. It is often combined with other techniques, mostly the density gradient with or without cushion, to stratify EVs based on their density and obtain a pure population, free of contaminant proteins, especially to perform proteomic or other molecular analysis [61–64]. Iodixanol (OptiPrep), rather than sucrose, is preferred for gradient because it reduces contaminants such as HDL [65, 66], small apoptotic bodies, and virus [67]. Recently, an upward floating method into iodixanol gradient has been proposed by Kowal et al. [68] to better separate and to characterize subtypes of EVs. Some concerns exist over the possibility that high-speed centrifugation may damage EVs and create aggregates [69]. Another issue is related to the low reproducibility of the technique. Adopting different rotors, speeds, and times, yield, protein, and RNA content of the pellet might vary [70]. Nevertheless, differential ultracentrifugation is still the most widely used method, especially for conditioned cell culture media [64].

Another technique is size-exclusion chromatography (SEC). EVs flow into a filtration column that elutes EVs of different sizes in different fractions. This method is preceded by a preconcentration step and followed by ultracentrifugation that can damage EVs [71]. In other cases, SEC is used as a stand-alone technique [72, 73]. It seems that SEC is preferentially used to isolate EVs from complex biological fluids, such as plasma [74, 75], urine [76], and milk [73]. Two studies comparing ultracentrifugation and SEC suggest that SEC provides a higher yield of EVs [77] and contaminant reduction [78]. Other size-based isolation methods are ultrafiltration, flow field-flow fractionation, hydrostatic filtration dialysis [79], an integrated double-filtration microfluidic device [80], and lab-on-a-disc integrated with two nanofilter devices [81].

Moreover, EVs can be isolated with immunoaffinity capture-based techniques, which use microbeads coated with antibodies for EV membrane receptors to recognize and isolate EVs. This approach selects specific subpopulations of EVs without considering their size or density [71]. Therefore, this method may be unsuitable for some application, but it is very useful for others, such as the detection of diagnostic or prognostic markers expressed on EV surfaces. Moreover, this method can be coupled to flow cytometry, Western blotting, and real-time PCR to furtherly characterize EVs [71]. The same method has been used to develop an ELISA microplate for the capture and quantification of EVs from urine, serum, and plasma [82]. Besides, immunoaffinity isolation is more used for complex biological fluids than for cell supernatants, or with small starting volumes [64].

Another method of EV isolation is based on precipitation. Polymers such as polyethylene glycol (PEG), which separate water from solutes, are incubated overnight at 4°C with cell culture media or biological fluids, and EVs are collected by low-speed centrifugation or filtration [83]. Moreover, EV negative charge has been exploited to develop a charge-based precipitation method, which uses protamine, a positively charged molecule, coupled with PEG to separate EVs [84]. Precipitation techniques recover EVs more efficiently than ultracentrifugation [84, 85]. Moreover, precipitation techniques require very small volumes and are easier to perform. For this reason, this technique is suitable for diagnostic use. On the other hand, it is possible to coprecipitate contaminants, such as lipoproteins and the polymer itself [86]. However, these particles can be removed by a preisolation centrifugation and a post isolation filtration through Sephadex G-25 spin columns [57, 84].

5. Metabolic Syndrome

In the last years, it has been suggested that miRNAs, both intracellular and extracellular, can control the metabolic

Table 1: EV-miRNA molecules in endocrine diseases.

Pathology	EV source	miRNA alteration	Target	Recipient cell	Activity	Reference
Metabolic syndrome	Macrophages of adipose tissue of obese mice	↑ miR-155	PPARγ	Liver and muscle cells	Glucose metabolism, insulin sensitivity	[103]
Obesity	Obese visceral adipocytes	↓ miR-148b ↓ miR-4269 ↑ miR-23b ↑ miR-4429	TGF-β Wnt/β-catenin	—	Involvement in liver diseases	[107]
	White and mostly brown adipocytes	miR-99b miR-325 miR-743b miR-98	FGF21 UCP1 PGCIα	Liver cells	Metabolic injury (glucose metabolism injury)	[109]
Liver	ASCs of obese subjects	↓ miR-126	↑ Spred1 ↑ E2K1/2 MAPK	—	Reduced EC migration and angiogenesis	[111]
	NAFLD mouse model	↑ miR-122 ↑ miR-192	—	—	EVs as potential biomarkers of hepatic injury	[125]
Pancreas	Human pancreatic islets	miR-27b miR-126 miR-130 miR-296	—	Human islet ECs	Beta cell function, insulin secretion, angiogenesis	[131]
	EPCs	miR-126 miR-296	—	Human islet ECs	Angiogenesis promotion *in vitro* and *in vivo*	[132]
	Skeletal muscle	miR-16	Ptch1	Pancreatic beta cells	Development of insulin resistance	[142]
Diabetes	Urine of patients with diabetic nephropathy	↑ miR-130a ↑ miR-145 ↓ miR-155 ↓ miR-424	—	—	Involvement in diabetes progression	[152]
	ECs	miR-143/ miR-145 cluster	—	Vascular smooth muscle cells	Endothelium stabilization	[164]
Thyroid disorders	AITD patients	↑ miR-146a ↑ miR-155	SMAD4	—	Function and development of Treg and Th17 cells	[176]
	Intractable Graves' disease	miR-92a-3p miR-23b-5p miR-339-5p let7g-3p	—	—	Potential role in upregulation of cytokine production	[181]
Preeclampsia	STB in *in vitro* model of PE	↓ miR-517a ↓ miR-517b ↓ miR-141	—	—	—	[213]
	PE patients	↑ miR-486-1-5p ↑ miR-486-2-5p	—	—	Potential PE biomarkers	[214]

EV: extracellular vesicle; PPARγ: peroxisome proliferator-activated receptor gamma; TGF-β: transforming growth factor beta; FGF21: fibroblast growth factor-21; UCP1: uncoupling protein 1; PGCIα: peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ASCs: adipose mesenchymal stem cells; Spred1: sprouty-related EVH1 domain-containing 1; E2K1/2 MAPK: extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase; EC: endothelial cell; NAFLD: nonalcoholic fatty liver disease; EPCs: endothelial progenitor cells; Ptch1: protein patched homolog 1; AITD: autoimmune thyroid disease; SMAD4: SMAD family member 4; Treg: T regulatory cells; Th17: T helper 17 cells; STB: syncytiotrophoblast; PE: preeclampsia. Expression trend: ↑: miRNA increased expression; ↓: miRNA reduced expression.

homeostasis and may impact the main tissues involved in the development of the metabolic syndrome [87]. In effect, miRNAs represent important modulators of glucose and lipid metabolism in the liver [88], insulin production in the pancreas [89], and leptin signaling in the hypothalamus [90]. In addition, miRNAs have been involved in many processes associated to metabolic disorders such as oxidative stress, inflammation, insulin signaling, adipogenesis, and angiogenesis. In this part, we describe the recent knowledge on the role of RNAs shuttled by EVs in metabolic syndrome and correlated disorders. Most relevant EV-cargoes involved in endocrine diseases discussed here and in the following paragraphs are summarized in Table 1.

5.1. Obesity. Obesity is a chronic condition characterized by an excessive accumulation of body fat potentially associated to other metabolic disorders, hypertension, insulin resistance, and cardiovascular diseases. Human obesity associates with a chronic state of low-grade inflammation with augmentation of inflammatory protein production, increased infiltration of macrophages, lipolytic activity of adipocytes with increased systemic liberation of fatty acids, and potential insulin resistance. The increased production of proinflammatory cytokines and the decrease of anti-inflammatory cytokines may take part in the metabolic complications linked with obesity and leading to metabolic syndrome. This condition is modulated by signal transduction networks, and miRNAs may regulate the expression and production of inflammatory proteins with amplification of the inflammatory effect [91]. Adipose tissue is a heterogeneous tissue composed by several cell types. Adipocytes and ASCs for instance may release EVs that regulate adipocyte metabolism. In effect, when the adipose-specific knockout of Dicer is performed, a depletion of most miRNAs is observed [92–94]. miRNA deficit prejudices adipocyte basic functions like differentiation, metabolism, and signaling [94]. It has been shown that several miRNAs may contribute to the infiltration of macrophages in the adipose tissue [95–97] and to their transition to a proinflammatory phenotype [98–101] favoring the low-grade chronic inflammation characteristic of obese people and the induction of insulin resistance [102].

A recent work by Ying et al. [103] showed that adipose tissue macrophages may regulate systemic insulin responses via EVs. The authors reported that macrophages from adipose tissue of obese mice released EVs carrying miRNAs able to induce insulin resistance and glucose intolerance in lean mice. At variance, EVs from adipose tissue macrophages of lean mice were able to ameliorate glucose metabolism and insulin sensitivity of obese mice. MiR-155, targeting the peroxisome proliferator-activated receptor gamma (PPARγ) gene, was upregulated in EVs of obese adipose tissue macrophages. EV-mediated transfer of miR-155 to insulin target cells of the liver and muscle may have an impact on metabolism [103]. In humans, PPARγ exerts several important functions in adipocyte differentiation, lipid metabolism, and glucose homeostasis [104]. As a matter of fact, some studies have outlined that EVs released from adipose tissue may take part in the metabolic features associated with obesity [105–107] and differ from those of lean individuals [107, 108].

The potential benefits of EVs for biomarker identification are related to their easy purification, their stability when stored at −80°C, the protection of nucleic acids from enzyme degradation, and the possibility of EV subset discrimination based on the expression of membrane markers of the cell of origin. Karolina et al. [108] analyzed the profile of miRNAs in EVs of patients with metabolic syndrome, hypercholesterolemia, hypertension, or type 2 diabetes mellitus alone, showing a distinct miRNA profile for each single disease. Ferrante et al. [107] demonstrated a different expression of several miRNAs in obese in respect to lean visceral adipocyte EVs. In particular, downregulation of miR-148b and miR-4269 and upregulation of miR-23b and miR-4429 were reported in obese visceral adipocyte EVs. These altered miRNAs targeted the TGF-β and Wnt/β-catenin signaling linked to the modulation of chronic inflammation and adipogenic differentiation of ASCs [107]. It has been shown that EVs from adipose tissue intravenously injected in an obese mouse model (B6 ob/ob mice) or in mice fed with a high-fat diet were incorporated in peripheral blood monocytes following macrophage activation in a TLR4-dependent way. This led to increased secretion of TNF-α and interleukin-6 and development of insulin resistance [105]. Recently, Thomou et al. [109] demonstrated that the adipose tissue is a great source of circulating vesicular miRNAs capable of modulating gene expression in other tissues. The authors showed that circulating vesicular miRNAs are decreased in mice with a fat-specific knockout of Dicer, and in humans with lipodystrophy. The transplantation of white adipose tissue and mostly of brown adipose tissue into Dicer knockout mice was followed by restoration of circulating vesicular miRNAs, amelioration of glucose tolerance, and reduction of fibroblast growth factor-21 (FGF21) mRNA in the liver and of protein in blood [109]. FGF-21 is a hormone that is induced in the liver by fasting [110] and was repressed when liver cells were incubated with normal serum EVs but not with Dicer knockout EVs [108]. In EVs from Dicer knockout mice, the effect was restored by introduction of miR-99b, a predicted regulator of mouse FGF21. Moreover, the authors demonstrated that transplantation of brown adipose tissue reestablished other miRNAs such as miR-325 and miR-743b, predicted to target the uncoupling protein 1 (UCP-1, an uncoupling protein found in the mitochondria of brown adipose tissue), and miR-98 predicted to target the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGCIα), the master regulator of mitochondrial biogenesis. Taken together, these data suggest that miRNAs carried by EVs could be transferred to other tissues where they are able to modulate gene expression [109]. It has been shown that ASCs release EVs capable of promoting endothelial cell migration, proliferation, and neoangiogenesis [111]. In obese subjects, the ASC-EV angiogenic potential resulted weakened in comparison with normal individuals with reduced endothelial cell migration and vessel-like structure formation [112]. This effect was mainly due to a reduced miR-126 content in EVs leading to Spred1 upregulation and inhibition of the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase (Erk1/2 MAPK) pathway in endothelial cells. Moreover, the treatment of nonobese ASCs with palmitic acid induced the release of EVs similar to those from obese, whereas the treatment of obese ASCs with high glucose decreased the miR-126 content in EVs and the *in vitro* angiogenesis [112].

Beside miRNAs, EVs were shown to contain lncRNAs [113]. Little is known on the role of lncRNAs carried by EVs in endocrine physiology and pathology. However, it has been reported that lncRNAs exert critical roles in homeostasis and differentiation of metabolic tissues interacting with target mRNAs or miRNAs [114, 115].

To date, accumulating evidence supports an association of diabetes and metabolic syndrome to liver disease

[116, 117]. Like EVs released by adipose tissue, EVs produced by hepatocytes are sensitive to metabolic signals, but their importance in the interplay between metabolically active tissues and the liver is still uncertain [118]. It has been suggested that EVs carrying miRNAs released from different hepatic cells are involved in liver functions and play a role in liver diseases [119, 120].

Obesity and metabolic syndrome may enable the accumulation of fat in the hepatic tissues with the development of nonalcoholic fatty liver disease (NAFLD) [121]. It can initiate as simple steatosis, then advance to nonalcoholic steatohepatitis (NASH) in which inflammation and fibrosis associate to steatosis, eventually leading to cirrhosis [122]. It has been shown that macrophages and natural killer T cells of subjects suffering NAFLD or NASH release a higher amount of EVs in the circulation, in respect to healthy individuals [123]. Since their release correlated with alanine aminotransferase (ALT) levels and the degree of histologic damage, EVs have been suggested as a new noninvasive and quantitative diagnostic tool [123]. Povero et al. [124], in a NASH *in vitro* model of lipotoxicity, demonstrated that hepatocytes released EVs with proangiogenic activity on endothelial cells. This process was dependent on the EV membrane expression of the protein vanin-1, which mediated EV internalization in target cells and subsequent caspase 3 activation. Therefore, hepatocyte-derived vanin-1-positive EVs have been suggested as potential biomarker or target for therapy of NASH [124]. Moreover, the same authors showed that the quantity of liver and blood EVs was markedly increased in a choline-deficient L-amino acid (CDAA) diet experimental model of NAFLD in comparison with control mice [125]. The number of EVs increased over time and correlated with the degree of histopathological lesions. Interestingly, these circulating EVs showed a different pattern of proteins and an enrichment of two well-known miRNAs highly expressed in hepatocytes, miR-122 and miR-192. These results suggest a potential use of these EVs as biomarkers of hepatic damage [125]. Hirsova et al. [126] showed that toxic lipids, such as palmitate and its active metabolite lysophosphatidylcholine, induced an increased release of EVs from human hepatocytes. This process was mediated by the activation of the death receptor 5 signaling pathway in hepatocytes with downstream activation of caspases and of Rho-associated coiled-coil containing protein kinase 1 (ROCK1) [126]. These EVs carried the protein tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and could represent a possible connection between liver lipotoxicity and macrophage-mediated inflammation in NASH. In fact, the treatment with the ROCK1 inhibitor fasudil decreased the number of total and hepatocyte-derived circulating EVs in an *in vivo* model of NASH. Importantly, it induced a reduction of inflammation, hepatic damage, and fibrosis, suggesting the role of EVs in these mechanisms [126]. Moreover, Koeck et al. [127] isolated EVs from visceral adipose tissue of obese and lean patients and investigated their effects on cultured hepatic cell lines. Interestingly, incubation of liver cells with EVs from obese subjects dysregulated the TGF-β pathway, a process correlated with the pathogenesis of NAFLD [127]. As mentioned above, Ferrante et al. [107] demonstrated that EVs derived from obese subjects carry miRNAs which interfere with TGF-β pathway, suggesting their involvement in liver diseases. A recent study of Thomou and colleagues demonstrates that EVs derived from adipose tissue can guide metabolic insults in the liver via the transfer of miRNAs [109].

5.2. Diabetes

5.2.1. EVs in Endocrine Pancreas Physiology. Pancreatic beta cells, situated within the islets of Langerhans, are crucial for the regulation of blood glucose homeostasis by secreting insulin. Several evidences demonstrated that noncoding RNAs control beta cell function and viability in health and disease [128]. Recently, it has been demonstrated that these cells release EVs involved in signal interactions influencing the activity of adjacent pancreatic beta cells. In fact, EVs isolated from beta cell lines and from rat, mice, or human islets contained miRNAs that can be transferred to neighboring beta cells, influencing their behavior. Importantly, EV-miRNA content varies based on cell conditions and EVs released by beta cells treated with proinflammatory cytokines were shown to lead to apoptosis of naive beta cells [129]. Besides the direct interaction between beta cells, the physiopathology of pancreatic islets has been demonstrated to depend on a crosstalk between beta cells and endothelial cells [130]. EVs seem to contribute to cell communication, and EVs isolated from human pancreatic islets were shown to be biologically active, inducing insulin mRNA expression, enhancement of angiogenesis, and protection from apoptosis. In fact, these EVs shuttle specific mRNAs and miRNAs into target human islet endothelial cells, including several mRNAs (VEGFa, eNOS) and microRNAs (miR-27b, miR-126, miR-130, and miR-296) involved in beta cell function, insulin secretion, and angiogenesis [131]. Moreover, EVs derived from endothelial progenitor cells (EPCs) induce *in vitro* islet endothelial cell migration, proliferation, organization in vessel-like structures, and resistance to apoptosis. *In vivo*, EVs were shown to favor survival, insulin secretion, and revascularization of islets transplanted in SCID mice. Of note, their effect is due to the transfer of proangiogenic miRNAs, miR-126 and miR-296 [132].

5.2.2. EVs in Diabetes. Insufficient and/or ineffective insulin secretion or both of them can lead to diabetes mellitus (DM). The autoimmune beta cell destruction in the pancreas is the cause of insulin-dependent type 1 diabetes (T1D), whereas insulin resistance (IR) or inadequate insulin release is the cause of non-insulin-dependent diabetes (T2D). A potential involvement of miRNAs in the dialogue between immune system and pancreatic islets has been suggested [133]. A recent study [134] has suggested that EVs may take part in the beginning and acceleration of autoimmune pancreatic islet reactions in T1D demonstrating that rat and human pancreatic beta cells secrete EVs carrying autoantigens GAD65, IA-2, and insulin/proinsulin which target and activate dendritic cells promoting autoimmunity in particular under proinflammatory conditions. Evidence indicates the crucial role of miRNAs in the beta cell differentiation,

acquirement of a mature phenotype, and dysfunction. In effect, the pancreatic-specific deletion in mouse embryos of Dicer was followed by a dramatic deficiency of insulin producing beta cells linked to upregulation of the notch-signaling target Hes1 and decrease in the formation of endocrine progenitor cells expressing the neurogenin3 gene [135].

In DM, miRNAs can regulate various molecular and cellular pathways like insulin synthesis and secretion in pancreatic beta cells, exocytosis of insulin granules, beta cell fate, and formation of islets [136]. Many miRNAs contribute to the adaptive features of beta cells to increase requirements of insulin such as miR-132 [137, 138], miR-184 [139], miR-338-3p [140], and miR-375 [141, 142].

EVs regulate intercellular signaling in diabetes mediating the exchange of RNAs between cells. Recently, miRNAs delivered by EVs have been demonstrated to mediate the crosstalk between skeletal muscle and beta cells in diabetes [143]. This study used mice fed with a high palmitate diet inducing hyperglycemia, glucose intolerance, hyperinsulinaemia, and insulin resistance. Interestingly, EVs isolated from skeletal muscle can be internalized by pancreatic beta cells with delivering of proteins and miRNAs, suggesting that they contribute to modification of the beta cell mass arising during insulin resistance. In particular, lipid-induced insulin-resistant muscle EVs transferred miR-16 to beta cells regulating Ptch1, a receptor involved in the sonic hedgehog pathway and suggested to exert a significant role in insulin resistance development [143].

In addition to miRNAs, EVs can shuttle other noncoding RNAs, which regulate cell gene expression also interacting with miRNAs. In fact, recent studies have shown that EVs may carry and transfer cRNAs that may have functional and diagnostic relevance [144, 145]. In particular, the human ciRS-7 (also termed Cdr1as) was detected in EVs [146] and is the first miRNA sponge identified, negatively regulating miR-7 [147]. Of note, a recent study underlined a critical relationship between ciRS-7/miR-7 and diabetes [148]. The authors demonstrated how the overexpression of Cdr1as augmented the insulin mRNA concentration and the granule secretion in beta cells via the inhibition of miR-7 in islet MIN6 cells and mouse islets [148]. In fact, miR-7 has been extensively related to diabetes impairing insulin secretion and beta cell dedifferentiation. Importantly, the knockdown of miR-7 in obese mice has been shown to ameliorate beta cell failure and glycemia [149]. Then, Cdr1as has been shown to improve beta cell function by preventing miR-7 interaction with its target genes. Among these, the authors demonstrated the importance of the paired box protein Pax-6 (Pax-6), which enhances insulin gene 1 (Ins1) and gene 2 (Ins2) transcripts by directly binding to their promoters increasing insulin content and secretion, and of Myrip (myosin VIIA and Rab-interacting protein), which forms a complex with Rab27a and MyosinVa and mediates insulin granule transportation and secretion [148]. These data suggest the presence of multiple types of vesicular RNAs involved in diabetes and highlight the necessity of further investigations to understand their complex role in this pathology. Taken together, these results suggest the therapeutic potential of EV-RNAs to induce the rescue of beta cell function.

Finally, miRNAs have been suggested as potential biomarkers for both type 1 and type 2 diabetes [150]. Recently, Garcia-Contreras et al. [151] have shown that plasma-derived EVs express a distinct signature in long lasting diabetes. Several deregulated miRNAs have been shown to be involved in diabetes progression. Barutta et al. [152] studied urinary EVs in incipient diabetic nephropathy showing an enrichment of miR-130a and miR-145 and a reduction of miR-155 and miR-424 in patients with microalbuminuria. In an experimental model of diabetic nephropathy, a similar upregulation of miR-145 in urinary EVs as well as in glomeruli was observed [152]. *In vitro* experiments showed that high glucose exposure of mesangial cells upregulated miR-145 with consequent increased expression of this miRNA in secreted EVs [152]. Since tissue-specific EVs may allow monitoring of transplanted tissue rejection, EVs could be also used as biomarkers to monitor the outcome of islet transplantation [153]. Moreover, it has been recently suggested that EVs carrying small RNAs released from MSCs may act as immune modulators to improve islet transplantation [154]. In fact, MSC-derived EVs have been already demonstrated to modulate immunity in type 1 diabetes inducing the formation of regulatory dendritic cells [155, 156].

5.3. Atherosclerosis. Dysfunctional adipose tissue and proinflammatory signals of metabolic syndrome are associated to the development of atherosclerosis, a condition characterized by a low-grade chronic inflammation of arterial wall [157, 158]. The process is initiated by endothelial damage and followed by deposition of lipoproteins in the subendothelial place. Beside signaling and molecular regulatory pathways critical for the formation and evolution of the atherosclerotic plaques, miRNAs are considered crucial modulators at a fine tune level of the different players implicated in the pathophysiological processes of atherosclerosis. In response to different stimuli, miRNAs such as the miR-181 family are suggested to be capable of modifying the balance of pro- and anti-inflammatory molecules implicated in the pathogenesis of atherosclerosis. The miR-181 family has a crucial role in vascular inflammation by regulation of signaling pathways and targets known to be critical for endothelium activation and immunity, thus contributing to the onset and development of vascular inflammatory diseases [159]. Several miRNAs involved in lipid metabolism have emerged as contributors of the pathogenic process of atherosclerosis such as miR-33 [160, 161], miR-27a/b [162, 163], and miR-122 [164]. miRNAs mediating the endothelial damage induced by disturbed flow have been identified and extensively studied (see [165] for review). In situations of shear stress, it has been shown that the increasing of the Kruppel-like factor (KLF2) transcription factor induced the expression of functional miR-143/miR-145 cluster which was loaded in endothelial cell EVs and taken up by vascular smooth muscle cells in coculture [166], with acquisition of a contractile phenotype and stabilization of endothelium. Noteworthy, EVs from endothelial cells were able to decrease

atherosclerotic lesion formation *in vivo* in the aorta of ApoE$^{-/-}$ mice in a miR-143/miR-145-dependent manner exerting an atheroprotective effect [166]. In another study, the exchange of miR-143/miR-145 through intercellular tunneling nanotubes has been shown to occur between vascular smooth muscle cells and endothelial cells resulting also in this case in vessel stabilization [167]. EVs derived from vascular smooth muscle cells overexpressing Kruppel-like factor 5 (KLF5) contained miR-155 and were able to promote atherosclerotic progression *in vitro* and *in vivo*. Thus, EVs could either promote or prevent atherosclerosis based on their RNA content [168]. In a recent study, de Gonzalo-Calvo et al. [169] investigated the miRNA content in EVs released from human coronary artery smooth muscle cells (CASMC) and its modification after exposure to atherogenic lipoproteins. Interestingly, these miRNA modifications were reproduced in the plasma of patients with familial hypercholesterolemia (FH). Among miRNAs deregulated by atherogenic lipoproteins, plasma miR-130a was suggested as potential biomarker of significant coronary atherosclerosis, and miR-24-3p and miR-130a present in circulating EVs as potential biomarkers for FH [169].

6. Thyroid Disorders

The thyroid gland is one of the largest endocrine glands in the body [170], and EVs containing undegraded thyroglobulin (Tg) have been suggested as an alternative way to conventional exocytosis of Tg [170, 171]. The presence of Tg-containing EVs in bovine serum [172] suggests the physiological involvement of this mechanism in the hormone release into circulation. In pathophysiological conditions, such as subclinical hypothyroidism, an increased number of preapoptotic vesicles might promote endothelial dysfunction and cardiovascular risk [173]. Although the precise mechanisms of autoimmunity are still under investigation [174], it is well known that EVs can modulate immune response [175]. Recent studies highlight the importance of an equilibrium between T effector and T regulatory cells to preserve the immune tolerance in thyroid, and of its alterations in the development of the autoimmune thyroid disease (AITD) [176]. Increasing evidence suggests a role of EVs also in thyroid autoimmune disorders. Graves' disease (GD) patients have higher levels of EVs in blood that are significantly reduced after antithyroid therapy with thiamazole [177]. The increase of circulating EVs most likely reflects the activation of immune and inflammatory processes and the resulting cell apoptosis. In addition, patients with GD have higher number of both E-selectin and VE-cadherin-positive EVs, suggesting endothelial dysfunction. The increase in monocyte-EVs indicates an activation and an increased turnover of monocytes. Based on these findings, EVs have been candidate biomarkers for diagnosis and prognosis of GD [177]. In human AITD, EVs seem to have a relevant role in the modulation of the inflammatory response since circulating EVs regulate Tregs and Th17 differentiation [178]. In particular, an increase in platelet-derived EVs and a decrease in leukocyte and endothelial cell-derived EVs have been detected in AITD patients compared to healthy controls. Of note, patient-derived EVs inhibited the *in vitro* differentiation of T regulatory cells and induced the differentiation of proinflammatory Th17 cells. RNA dysregulation, including both miRNAs and lncRNAs, has been associated to the pathogenesis of AITD [176, 179–181]. EVs derived from AITD patients showed a higher expression of miR-146a and miR-155, involved in the differentiation and function of innate and adaptive immunity [178]. Both of these miRNAs are instrumental for function and development of Treg cells. miR-155 also favors the development of inflammatory T cells including Th17 cells. miR-146a and miR-155 can induce immune cell unbalance characteristic of AITD patients by targeting the SMAD family member 4 (SMAD4) [178]. Circulating EVs may mediate the crosstalk between immune cells, leading to the promotion of cytokine expression in peripheral blood mononuclear cells (PBMCs) and contributing to GD pathogenesis [182]. In particular, four miRNAs (miR-92a-3p, miR-23b-5p, miR-339-5p, and let7g-3p) have been detected in EVs [183], suggesting their role in the upregulated cytokine production in intractable GD. The coincubation of EVs isolated from GD patients' sera can stimulate the mRNA expression of IL-1β and TNF-α in PBMCs, compared with EVs from GD patients in remission or from healthy controls [182]. GD patients in remission show an increase in circulating miR-23b-5p and miR-92a-3p and a decrease of let-7g-3p and miR-339-5p compared to intractable GD patients [182]. The higher expression of these two miRNAs suggests their role as regulators of immune suppression in proinflammatory mechanisms of autoimmune disorders and their association to disease's remission. In addition, miR-339-5p is decreased in GD patients in remission versus intractable GD patients and regulates the expression of sodium-iodine symporter. This protein is involved in the response to radioactive iodine (RAI) therapy [184], a treatment for hyperactive thyroid states including thyroid cancer and Graves' disease [185]. Together, these studies candidate EVs as accessible biomarkers for monitoring or predicting disease activity as well as therapy outcomes in thyroid diseases.

7. The Role of EVs in Preeclampsia

EVs and their RNA cargo have been intensively studied for their role in physiology and pathophysiology in the context of reproduction such as sperm maturation, ovarian follicle and oocyte maturation, as well as fertilization, and pregnancy (as previously reviewed [186, 187]). During pregnancy, placental EVs physiologically increase their concentration into circulation and mediate several biological processes, including endometrium remodeling and immunological communication between the mother and the fetus [188–192]. For instance, hypoxia during early pregnancy induces the release from cytotrophoblast cells and placental MSCs of a higher number of EVs with different contents and activities [193, 194]. EVs have been reported to contribute to spiral artery remodeling essential to provide a satisfactory nutrient exchange at the maternal-fetal interface [195]. One example of EV immunological properties is represented by their capacity to confer viral resistance. In fact, both

placenta and trophoblast-derived EVs are able to induce resistance of recipient cells against viral infections [196, 197]. Delorme-Axford et al. [196] demonstrated that this mechanism is mediated by the transfer of specific placental miRNAs. Here, we focus on preeclampsia (PE) and the purported role of EVs. Preeclampsia is a multisystem pregnancy disorder that is associated with major maternal and neonatal morbidities [198, 199]. The cellular mechanisms triggering the development and the progression of PE are still not completely elucidated [200]. PE is characterized by hypertension and a decreased utero-placental blood flow linked with impaired trophoblast invasion. Moreover, hypoxia of the placenta promotes the release of harmful substances into the maternal and feto-placental circulation leading to endothelial dysfunction [201]. Increasing evidence suggests that EVs contribute to the initiation and progression of PE by mediating the complex interactions at the maternal-fetal interface [192, 200–202]. In normal pregnancy, EVs released from the syncytiotrophoblast (STB) (STB-EVs) contribute to communication between the maternal endometrium and the embryo [203]. In PE, STB-EVs are released in significantly increased number and show proinflammatory, procoagulant, and antiangiogenic activities, implicating them in the maternal systemic inflammation and endothelial dysfunction [204, 205]. EVs promote vasculogenesis and angiogenesis possibly by transferring miRNAs [201]. Placental EVs contain placental-specific molecules, including proteins (e.g., placental alkaline phosphatase (PLAP)) and miRNAs (e.g., chromosome 19 miRNA cluster) [206, 207]. EVs can also transfer placental-specific miRNA (miR-571a-3p) as it has been shown into human Treg cells and Jurkat leukemic T-cell line, inducing the repression of a gene target (PRKG1) [208]. Increasing studies have highlighted the potential use of EVs and placenta-derived miRNAs as PE diagnostic tools [192, 205, 209, 210]. In fact, EVs are increased in PE maternal blood [191] and early onset PE [211]. Oxygen tension and hypoxia can modulate the release and the content of EVs from placental and trophoblast cells [193, 194, 212–214]. Using an *in vitro* model of PE, it has been demonstrated an alteration of miRNA cargo in a specific subpopulation of STB-EVs, with a downregulation of miR-517a, miR-517b, and miR-141 [215]. In a recent study, Salomon et al. [216] demonstrated an increase of total and placenta-EVs in plasma of PE pregnancies compared with healthy subjects. Importantly, EVs in PE patients showed a different content of miRNAs, mainly related to biological processes dysregulated in PE such as migration, placenta development, and angiogenesis. Among miRNAs, the authors identified the upregulation of miR-486-1-5p and miR-486-2-5p as candidate biomarkers to distinguish PE and normal pregnancies for the early detection of women at risk [216]. Therefore, it is plausible that EVs and their miRNA cargo could be useful as early biomarkers of PE thus improving pregnancy outcomes through the prevention and the reduction of PE severity. In the future, more information about the role of EVs and their associated RNAs will provide a better understanding as to their capacity to modulate gene targets, endothelial dysfunction, and angiogenesis.

8. Conclusion

In conclusion, this review summarizes the current knowledge on the role of noncoding RNAs shuttled by EVs in endocrine diseases. EVs may act locally as paracrine mediators and/or at distant sites being released in biological fluids. RNAs contained in EVs are described to be essential mediators of cell communication in endocrine physiopathology. These molecules carried by EVs can modulate the gene expression and the biological function of target cells. The majority of studies have focused on the role of miRNAs carried by EVs since small RNAs are the RNA species more abundant in EVs [205]. Although investigation on EV-lncRNAs and other noncoding RNAs is relatively at the beginning, the evidence suggests their key role in association to miRNAs in regulation of the endocrine system.

EVs and their associated noncoding RNAs are involved in the physiological cellular communication in endocrine organs. In the pancreas, EVs mediate the exchange of information between beta cells with endothelial cells and adjacent beta cells [129, 130]. Through their RNA cargo, EVs regulate beta cell function, insulin secretion, and angiogenesis [131]. In the liver, they can control the metabolic homeostasis and modulate glucose and lipid metabolism [119, 120]. EVs are also essential mediators of normal communication in endocrine glands, such as thyroid, and in the immune system [171, 172, 175, 217]. EV modulation of immune response and angiogenesis suggests also their involvement in reproductive processes. In particular, during pregnancy, EVs and their cargo mediate the immunological interaction at the maternal-fetus interface and control endothelial remodeling [188–192, 195]. Moreover, RNAs carried by EVs have been involved also in the establishment and progression of several endocrine diseases, such as thyroid autoimmunity disorders, complicated pregnancy, and in metabolic dysfunctions, including obesity and diabetes, and their related manifestations. In metabolic syndrome, EV-associated noncoding RNAs can modulate biological processes (oxidative stress, inflammation, insulin signaling, adipogenesis, and angiogenesis) which can promote disease progression. EVs released by macrophages of adipose tissue can modulate liver and muscle glucose and insulin metabolism, favoring diabetes [103]. Skeletal muscle can also modulate the fate of pancreatic beta cells through EV-RNAs leading to insulin resistance [143]. In addition, adipocytes can release EVs that can activate macrophages and lead to inflammation and insulin resistance in obese subjects [105]. EVs from adipose tissue can mediate liver insults and promote chronic inflammation [109, 127] and thus favor the atherosclerosis progression [157, 158]. The modulatory role of EVs in immune response and inflammation has been reported in autoimmune endocrine disorders [182] and in preeclampsia [204, 205]. A better understanding of the role of EVs and their molecular content in pathological and physiological conditions may provide insight for new therapeutic strategies. EVs and their associated noncoding RNAs could be targeted as therapeutic approach. When EV-associated RNAs positively correlate with the progression of endocrine diseases, interventions aimed to reduce their release or their bioactive cargo could

Table 2: Most relevant EV-miRNAs for biomarker application in endocrine diseases.

miRNA	Change	Source	Disease		Reference
miR-23b	↑	Adipocyte-derived EVs	Obesity	*Metabolic syndrome*	[107]
miR-122	↑	Circulating EVs	NAFLD	*Metabolic syndrome*	[125]
miR-130a	↑	Urinary EVs	Diabetic nephropathy	*Metabolic syndrome*	[152]
	↓	Circulating EVs	Coronary atherosclerosis	*Metabolic syndrome*	[169]
miR-141	↓	STB-derived EVs	Preeclampsia		[215]
miR-145	↑	Urinary EVs	Diabetic nephropathy	*Metabolic syndrome*	[152]
miR-146a	↑	Circulating EVs	AITD	*Thyroid disorders*	[178]
miR-148b	↓	Adipocyte-derived EVs	Obesity	*Metabolic syndrome*	[107]
miR-155	↓	Urinary EVs	Diabetic nephropathy	*Metabolic syndrome*	[152]
	↑	Circulating EVs	AITD	*Thyroid disorders*	[178]
miR-192	↑	Circulating EVs	NAFLD	*Metabolic syndrome*	[125]
miR-424	↓	Urinary EVs	Diabetic nephropathy	*Metabolic syndrome*	[152]
miR-486-1-5p	↑	Circulating EVs	Preeclampsia		[216]
miR-486-2-5p	↑	Circulating EVs	Preeclampsia		[216]
miR-517a	↓	STB-derived EVs	Preeclampsia		[215]
miR-517b	↓	STB-derived EVs	Preeclampsia		[215]
miR-4269	↓	Adipocyte-derived EVs	Obesity	*Metabolic syndrome*	[107]
miR-4429	↑	Adipocyte-derived EVs	Obesity	*Metabolic syndrome*	[107]

EV: extracellular vesicle; NAFLD: nonalcoholic fatty liver disease; STB: syncytiotrophoblast; AITD: autoimmune thyroid disease. miRNA expression change: ↑: increased expression; ↓: reduced expression.

inhibit disease. For instance, miR-320 contained in EVs from diabetic rat can inhibit angiogenesis, but a decreased miR-320 content in EVs can restore and have a therapeutic impact [218]. Otherwise, the loading of EVs with therapeutic RNAs can have a beneficial relevance and the enrichment of endothelial cell-derived EVs with miR-146a demonstrated to attenuate dementia-like pathology following administration in diabetic db/db mice [219].

On the other hand, since EVs carry the molecular signature of the cell of origin, they can be exploited as diagnostic tools. In fact, EVs present in the biological fluids vary in number and molecular content depending on the physiological or pathological conditions. In particular, noncoding RNAs present in EVs may provide a picture of ongoing biological processes in the organism. In fact, vesicle-included RNA species are protected from enzyme degradation and are an accessible source of RNAs released in the biological fluid by different cell types and organs. To date, EVs represent a potential diagnostic instrument and increasing studies are highlighting their utility to identify endocrine diseases. In fact, a recent clinical trial has begun to investigate EVs released by beta cells isolated from plasma of diabetes mellitus patients to identify islet-specific antigens (ClinicalTrials.gov identifier: NCT03106246). Likewise, EV-associated noncoding RNAs are arising interest as disease's biomarkers and they are currently investigated in a clinical trial focused on cholangiocarcinoma (ClinicalTrials.gov identifier: NCT03102268). In endocrine diseases, current results on EV-associated noncoding RNAs here presented and summarized in Table 2 give hope to exploit them as an easy and noninvasive diagnostic instrument. Moreover, RNA cargo of EVs could provide clinically useful information and be exploited to staging and in predicting response to therapy. In that context, the optimal definition of the EV isolation procedures is an essential challenge to improve the purity of starting material for downstream analysis. In particular, standardization of techniques will better define the presence of coisolated free circulating RNAs. These non-EV-associated molecules are connected to protein (e.g., Ago2) or lipoprotein complexes, and they can be coisolated with EV-associated RNAs during isolation procedures [55]. Different EV isolation techniques allow different grades of non-EV RNA contamination and can lead to discordant results in the literature. In future, the constant progress and consensus in technology platforms may solve the presence of discrepancies due to technical methodologies which still exist.

Abbreviations

AITD: Autoimmune thyroid disease
ALIX: ALG-2-interacting protein X or programmed cell death 6 interacting protein
ALT: Alanine aminotransferase
ARF6: ADP-ribosylation factor 6
ARRDC1: Arrestin domain-containing protein-1
ASCs: Adipose mesenchymal stem cells
CASMC: Coronary artery smooth muscle cells
CDAA: Choline deficient L-amino acid
ceRNAs: Competing endogenous RNAs
cRNAs: Circular RNAs
DM: Diabetes mellitus
EPCs: Endothelial progenitor cells
Erk1/2 MAPK: Extracellular signal-regulated kinase 1/2 mitogen-activated protein kinase
ESCRT: Endosomal sorting complex required for transport

EVs: Extracellular vesicles
FGF21: Fibroblast growth factor-21
FH: Familial hypercholesterolemia
g: Centrifugal forces
GD: Graves' disease
hnRNPA2B1: Heterogeneous nuclear ribonucleoprotein A2B1
Ins1: Insulin gene 1
Ins2: Insulin gene 2
IR: Insulin resistance
KLF2: Kruppel-like factor
KLF5: Kruppel-like factor 5
lncRNAs: Long noncoding RNAs
miRNAs: MicroRNAs
Myrip: Myosin VIIA and Rab-interacting protein
MSCs: Mesenchymal stromal cells
MVB: Multivesicular bodies
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
Pax-6: Paired box protein Pax-6
PBMCs: Peripheral blood mononuclear cells
PE: Preeclampsia
PEG: Polyethylene glycol
PGCIα: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PM: Plasma membrane
PPARγ: Peroxisome proliferator-activated receptor gamma
RAI: Radioactive iodine
RISC: RNA-induced silencing complex
ROCK1: Rho-associated coiled-coil containing protein kinase 1
rRNAs: Ribosomal RNAs
SEC: Size-exclusion chromatography
siRNAs: Small interference RNAs
SMAD4: SMAD family member 4
snoRNAs: Small nucleolar RNAs
snRNAs: Small nuclear RNAs
STB: Syncytiotrophoblast
T1D: Type 1 diabetes
T2D: Type 2, non-insulin-dependent diabetes
Tg: Thyroglobulin
TGFβ: Transforming growth factor β
TRAIL: Tumor necrosis factor-related apoptosis-inducing ligand
tRNAs: Transfer RNAs
TSG101: Tumour susceptibility gene 101 protein
UCP-1: Uncoupling protein 1
vtRNAs: Vault RNAs
YBX1: RNA-binding protein Y-box protein I
yRNAs: Cytoplasmic Y RNAs.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article. The affiliation of Ciro Tetta to Unicyte AG does not lead to any conflict of interest on the present review article.

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Research Article

Elevated and Correlated Expressions of miR-24, miR-30d, miR-146a, and SFRP-4 in Human Abdominal Adipose Tissue Play a Role in Adiposity and Insulin Resistance

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Objective. We explored the relationships among microRNAs (miRNAs) and SFRP4, as they relate to adipose tissue functions including lipolysis, glucose and glycerol turnover, and insulin sensitivity. *Methods*. Abdominal adipose tissue (AbdAT) levels of thirteen microRNAs (miRNAs), SFRP4, and VEGF in lean nondiabetic subjects ($n = 7$), subjects with obesity ($n = 5$), and subjects with obesity and type 2 diabetes (T2DM) ($n = 5$) were measured by qPCR. Insulin sensitivity was measured by the euglycemic-hyperinsulinemic clamp. Osmium fixation and Coulter counting were used for adipocyte sizing. Data were analyzed using generalized linear models that adjusted for age, gender, and ethnicity. *Results*. AbdAT miR-24, miR-30d, and miR-146a were elevated in subjects with obesity ($P < 0.05$) and T2DM ($P < 0.1$) and positively correlated with measures of percent body fat by DXA ($r_{miR.24} = 0.894$, $r_{miR.146a} = 0.883$, $P < 0.05$), and AbdAT SFRP4 ($r_{miR.30} = 0.93$, $r_{miR.146a} = 0.88$, $P < 0.05$). These three miRNAs additionally correlated among themselves ($r_{miR.24\sim miR.146a} = 0.90$, $r_{miR.30\sim miR.146a} = 0.85$, $P < 0.01$). *Conclusions*. This study suggests a novel association between the elevated levels of miRNAs miR-24, miR-30d, and miR-146a (apparently coregulated) and the level of SFRP4 transcript in AbdAT of subjects with obesity and T2DM. These molecules might be part of a regulatory loop involved in AbdAT remodeling/adiposity and systemic insulin resistance. This trial is registered with NCT00704197.

1. Introduction

Obesity increases the risk of developing serious health complications including hypertension, steatohepatitis, fatty liver, dyslipidemia, and type 2 diabetes (T2DM) [1]. For a condition as prevalent as obesity, with eventual life-threatening complications, our knowledge of mechanisms leading to obesity-associated pathophysiological alterations is limited. Pathological adipose tissue expansion leads to massive enlargement of adipocytes, limited angiogenesis, and ensuing hypoxia [2]. In addition, obesity associates with insulin resistance (including insulin suppression of lipolysis) and increased fat cell size (FCS). However, enlarged subcutaneous abdominal adipocyte size, but not obesity itself, was shown to predict T2DM, independent of insulin resistance [3].

Earlier, we found that subcutaneous adipose tissue from humans with obesity has inadequate vascularization, hypoxia, inflammation [4, 5], and fibrosis [6]. These alterations were found to be correlated with insulin resistance [4–10]. We have also demonstrated that the antiangiogenic factor secreted frizzled-related protein 4 (SFRP4) is associated with adipose tissue rarefaction (capillary drop out) and may lead to inflammation and ultimately insulin resistance in people with obesity [11]. Other groups demonstrated the clinical

utility of circulating SFRP4 protein [12] by showing it could predict the development of T2DM up to five years before the onset of T2DM [13]. Mechanistically, SFRP4 appears to act by decreasing insulin secretion from the pancreatic beta cells [13], by preventing transcription of angiogenesis-associated genes (including vascular endothelial growth factor, VEGF) [12], and by modulating Wnt signaling (pathway involved in glucose metabolism) [14]. Concordantly, our group has shown that the dysregulation of Wnt signaling, driven by changes in the expression of a related family member (SFRP3), is also associated with inflammation, glucose metabolism, and insulin resistance in the skeletal muscle of insulin-resistant humans [15]. We have also shown the potential biomarker utility of the circulating SFRP4 protein in an independent cohort by demonstrating its differential abundance in plasma from subjects with obesity, prediabetes, and T2DM [16].

MicroRNAs (miRNAs) are endogenous, small noncoding RNAs that are abundant in many cell types and tissues including the adipose tissue [17]. miRNAs play important functions in the regulation of a broad spectrum of physiological and metabolic processes including obesity, metabolic dysfunction, diabetes, and aging, among others [18–21]. miRNAs are reported to stimulate or inhibit the differentiation of adipocytes and to regulate specific metabolic and endocrine functions [22]. Like adipokines, miRNAs can also be secreted from fat cells into the blood circulation and function in inter tissue or organ communication in an endocrine fashion and thus may serve as markers of dysregulated adipose tissue function [23–25]. Given their role in regulating transcriptional networks, miRNAs in adipose tissue might offer attractive biomarkers of adiposity as well as potential therapeutic targets for treating metabolic disorders.

The goal of this study was to explore relationships among miRNAs and SFRP4, as they relate to adipose tissue functions including lipolysis, glucose and glycerol turnover, and insulin sensitivity. We hypothesize that miRNAs might be involved in the regulation of SFRP4 causing adipose tissue rarefaction and inflammation and ultimately leading to insulin resistance in patients with obesity. To test this hypothesis, we conducted a pilot retrospective cross-sectional study to evaluate the relationship between AbdAT miRNAs, SFRP4, and related adipose tissue phenotypes and functions.

2. Methods

The parent clinical trial was conducted at Pennington Biomedical Research Center (PBRC) and the main study results previously described by Pasarica et al. [4–6]. Subjects were excluded for previous use of thiazolidinediones or drugs known to affect lipid metabolism or body weight. Subjects with T2DM were treated with lifestyle modifications, metformin, or glipizide. The protocol was approved by the PBRC Institutional Review Board and all subjects gave written informed consent. In this secondary study, 7 lean [ND mass index (BMI) $< 25\,kg/m^2$] and 10 subjects with obesity [BMI $> 30\,kg/m^2$] with ($N = 5$) or without T2DM ($N = 5$) were included. One sample from each original group had been depleted; therefore, three subjects were not included in this study.

2.1. Clinical Measurements. Clinical measurements were conducted as described [4, 5]. Briefly, body composition was measured by dual-energy X-ray absorptiometry (DXA) on a Hologic dual-energy X-ray absorptiometer (Hologic, MA). Maximal aerobic capacity (VO2 max) was assessed using a graded treadmill test. Insulin sensitivity was measured during a euglycemic-hyperinsulinemic clamp as the mean rate of exogenous glucose infusion during the 30 minutes steady-state, corrected for changes in glycemia and divided by fat-free mass. Insulin suppression of lipolysis was assessed as the percent change in the rate of appearance of glycerol from the basal to insulin-stimulated state. Subcutaneous adipose tissue biopsies were obtained from the abdominal areas using a blunt-ended needle.

2.2. Laboratory Assays. Analysis of adipose tissue SFRP4 and VEGF mRNA and serum SFRP4 protein levels was previously described [5, 11]. VEGF and SFRP4 gene expressions were measured by qRT-PCR using TaqMan® (Applied Biosystem) and normalized to the housekeeping gene beta-actin. Mean adipocyte size was measured by osmium fixation and counting on a Coulter counter. Adipose tissue capillary density was measured on a paraffin-embedded adipose tissue by using tetramethylrhodamine isothiocyanate-conjugated lectin from *Ulex europaeus* (Sigma-Aldrich) for labeling capillaries and was previously presented [5].

2.3. miRNA Profiling. A panel of ten miRNAs (hsa-miR-21, hsa-miR-24, hsa-miR-29a, hsa-miR-30d, hsa-miR-34a, hsa-miR-126, hsa-miR-146a, hsa-miR-148a, hsa-miR-375, and hsa-miR-376) associated with diabetes and/or obesity and three potential endogenous control miRNAs (hsa-miR-191, hsa-miR-423, and hsa-miR-451) was profiled by qRT-PCR using TaqMan microRNA assays (Thermo Fisher, CA). These miRNAs are part of a metabolically involved miRNA panel we have designed and tested for biomarker discovery efforts at the Translational Research Institute for Metabolism and Diabetes, Florida Hospital [16, 26–28]. Briefly, total RNA was extracted using the miRNeasy mini kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. Complementary cDNA was reverse transcribed from 10 ng of total RNA using a primer pool of 1 : 100 diluted TaqMan microRNA assays 5xRT and the TaqMan microRNA reverse transcription kit (Thermo Fisher), following the manufacturer's recommendations. The cDNA was then quantitatively amplified using 20x TaqMan microRNA assays and 2x TaqMan Universal Master Mix II (Thermo Fisher), on a ViiA™ 7 real-time PCR system, following manufacturer's recommendations. A panel of miRNAs associated with obesity and metabolic diseases. The qRT-PCR reactions were performed in triplicate. The $-\Delta Ct$ method was used for the data analysis. The NormFinder algorithm [29] was implemented in the R programing environment and used to identify hsa-miR-191 as the best endogenous miRNA for data normalization.

2.4. Statistical Analysis. The data were analyzed using generalized linear models (GLMs) with gamma family and log link

Table 1: Demographic and clinical characteristics of the study cohorts.

	Level	ND lean ($N = 7$)	ND with obesity ($N = 5$)	T2DM with obesity ($N = 5$)	P
Age (median [IQR])		21 [20, 23]	26 [22, 27]	52 [51, 56]	0.004
Gender (%)	F	2 (28.6)	1 (20.0)	4 (80.0)	0.106
	M	5 (71.4)	4 (80.0)	1 (20.0)	
Ethnicity (%)	AA	2 (28.6)	3 (60.0)	3 (60.0)	0.636
	Asian	1 (14.3)	0 (0.0)	0 (0.0)	
	Caucasian	4 (57.1)	2 (40.0)	2 (40.0)	
BMI (median [IQR])		21.70 [21.30, 22.70]	31.50 [31.15, 31.70]	32.40 [29.30, 32.90]	0.002
Waist-to-hip ratio (median [IQR])		0.80 [0.75, 0.80]	0.90 [0.90, 0.90]	0.90 [0.90, 0.90]	0.027
% fat (median [IQR])		18.45 [14.80, 22.77]	31.56 [22.51, 32.04]	34.04 [33.45, 45.30]	0.005
VO2 max (ml kg min) (median [IQR])		41.51 [34.46, 41.58]	21.73 [21.18, 22.29]	20.09 [18.03, 22.37]	0.023
GDR FFM (median [IQR])		10.84 [10.12, 12.66]	5.41 [5.09, 7.12]	6.02 [2.84, 6.76]	0.022
Glucose (median [IQR])		88.25 [87.75, 89.38]	87.00 [84.50, 92.00]	130.50 [126.00, 156.50]	0.006
Insulin (median [IQR])		4.28 [3.73, 5.24]	10.60 [10.30, 11.10]	16.10 [12.75, 31.80]	0.007
HOMA-IR (median [IQR])		0.93 [0.80, 1.11]	2.13 [2.07, 2.36]	5.14 [3.40, 11.15]	0.003

Comparison between groups for continuous variables was done using the Wilcoxon test. Comparison for categorical variables was done using the Fisher exact test. Data is presented as median with [interquartile range (IQR)] or as percent (%). ND: subjects without diabetes; T2DM: subjects with type 2 diabetes mellitus; BMI: body mass index; GDR: glucose disposal rate. Percent body fat was measured by DXA. Glucose disposal rate was measured by hyperinsulinemic-euglycemic clamp to calculate insulin resistance. VO2 max was a measure of aerobic exercise capacity. The clinical data and tissue angiogenesis were previously described [5].

implemented in the R environment, using the *glm* function from the *stats* package. Each GLM modeled the $-\Delta$Ct expression levels of a specific miRNA (the outcome variable) as a function of two explanatory variables: a BMI-related variable with two levels (lean and obesity) and a diabetes-related variable with two levels (ND and T2DM) (ND: subjects without diabetes). The models additionally adjusted for potential confounding effects of age, gender, and ethnicity (also included in the GLM models as explanatory variables). General linear hypothesis testing and multiple comparisons (post hoc Tukey tests) for each variable were implemented using the *glht* function from the *multcomp* package. Plots for the visualization of the relationships between the outcome and the explanatory variable of interest (as the other explanatory variables are held constant) were generated using the *visreg* package. The *visreg* package offers the advantage of superimposing partial residuals (the adjusted values) on the visualization plots. Correlation plots were generated using the *ggplot2* package and the miRNA levels expressed as $-\Delta$Ct data. The reported partial correlations and corresponding P values were calculated using the *pcor.test* function from the *ppcor* package, also controlling for the potential confounding effects of age, gender, and ethnicity. Statistical significance was defined relative to a nominal two-sided 5% type 1 error rate.

3. Results

3.1. Anthropometric Characteristics. A total of 17 subjects participating in this study had a median BMI of 21.7 [interquartile range (IQR): 21.3, 22.7] kg/m^2 in the ND lean group, 31.50 [31.15, 31.70] kg/m^2 in the ND with obesity group, and 32.4 [IQR: 29.3, 32.9] kg/m^2 in the T2DM with obesity group (Table 1, median [IQR], $P < 0.05$). Other clinical characteristics and adipose tissue parameters are shown in Table 1.

3.2. Abdominal Adipose Tissue miRNAs Are Elevated in People with Obesity and Diabetes. To assess the role of AbdAT miRNAs in the context of obesity independently from T2DM and vice versa, we performed miRNA profiling in the specific tissue and implemented generalized linear modeling controlling for the potential confounding effects of age, gender, and ethnicity. We found that AbdAT miR-30d is significantly elevated in people with obesity (as compared to ND lean controls, $P = 0.0083$—the "obesity context" comparison, Figure 1(a)) and in T2DM subjects (as compared to ND lean and ND with obesity controls, $P = 4.3 \times 10^{-6}$—the "T2DM context" comparison Figure 1(b)). Similarly, two other miRNAs, namely, miR-24 and miR-146a, were found significantly elevated in the AbdAT in the "obesity context" ($P = 0.0114$ and $P = 0.0072$, respectively, Figures 1(c) and 1(e)) and marginally significantly ($P < 0.1$) elevated in the same tissue in the "T2DM context" (Figures 1(d) and 1(f)). Notably, these three AbdAT miRNAs were found highly correlated among themselves ($r_{miR.30d\sim miR.146a} = 0.85$ and $r_{miR.24\sim miR.146a} = 0.90$, both with $P < 0.01$, Figures 1(g) and 1(h)).

3.3. Abdominal Adipose Tissue Levels of miR-30d and miR-146a Positively Correlate with the Antiangiogenic Factor SFRP4 in the Same Tissue. AbdAT miR-30d and miR-146a positively and significantly correlated with AbdAT expression levels of SFRP4 ($r_{miR.30d\sim SFRP4} = 0.93$ and $r_{miR.146a\sim SFRP4} = 0.88$, both with $P < 0.05$, Figures 1(i) and 1(k)). Consistent with its reported antiangiogenic functions, transcript levels of SFRP4 in the fat tissue negatively and significantly correlated with the respective tissue

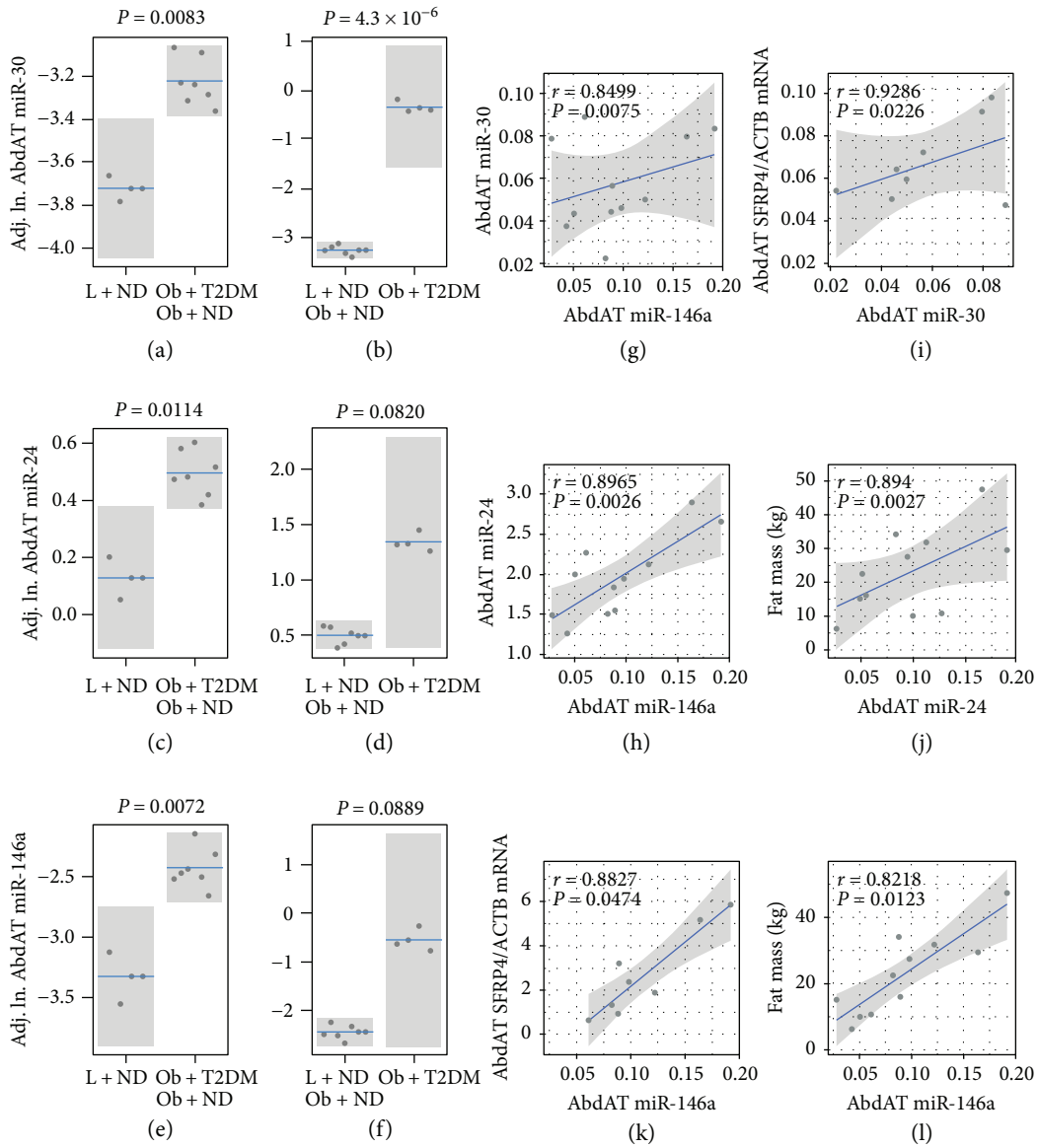

Figure 1: Differential expression and correlation analysis identify key coexpressed abdominal adipose tissue (AbdAT) miRNAs that appear to interact with AbdAT SFRP4. AbdAT miRNAs measured by qRT-PCR are elevated in subjects with obesity and diabetes (a–f). Levels of select miRNAs, mRNAs, and proteins in AbdAT were modeled using generalized linear models (GLMs with a gamma family and log link) as a function of a T2DM-related variable with two levels (ND, T2DM) and a BMI-related variable with two levels (lean, obesity). GLMs controlled for potential confounders including age, gender, and ethnicity. Visualization of the regression models was rendered with the R package *visreg*. Boxplots with overlapped dot plots display the adjusted values (partial residuals). Mean represented by the blue line and 95% confidence interval of the mean as a grey band. ND: subjects without type 2 diabetes; T2DM: subjects with type 2 diabetes; Adj. ln: natural logarithm value of the measurement adjusted for confounders including age, gender, and ethnicity. Partial correlation analysis identified seemingly coregulated miRNAs and significant associations between specific AbdAT miRNAs and AbdAT SFRP4 and measures of adiposity (g–l). Correlation scatterplots including miRNA variables display the $-\Delta Ct$ data from differentially expressed AbdAT miRNAs and were generated with the R package *ggplot2*. The reported partial correlation that controlled for age, gender, and ethnicity was calculated with the *ppcor* package. The blue line and gray band represent the linear fit of the plotted values and the 95% confidence interval of the fit, respectively. The secondary analysis of miRNA expression in abdominal adipose tissue was performed in seventeen subjects (lean $N = 7$ and subjects with obesity $N = 10$), as per sample availability.

capillary density [11]. However, we did not detect significant correlations between AbdAT SFRP4 [11] or the differentially expressed AbdAT miRNAs (this study) with VEGF transcript levels in the fat tissue.

3.4. Abdominal Adipose Tissue Levels of miR-24 and miR-146a Positively Correlate with Measures of Adiposity. AbdAT miR-24 and miR-146a significantly correlated with measures of adiposity including fat mass ($r_{miR.24\sim fat.mass} = 0.894$,

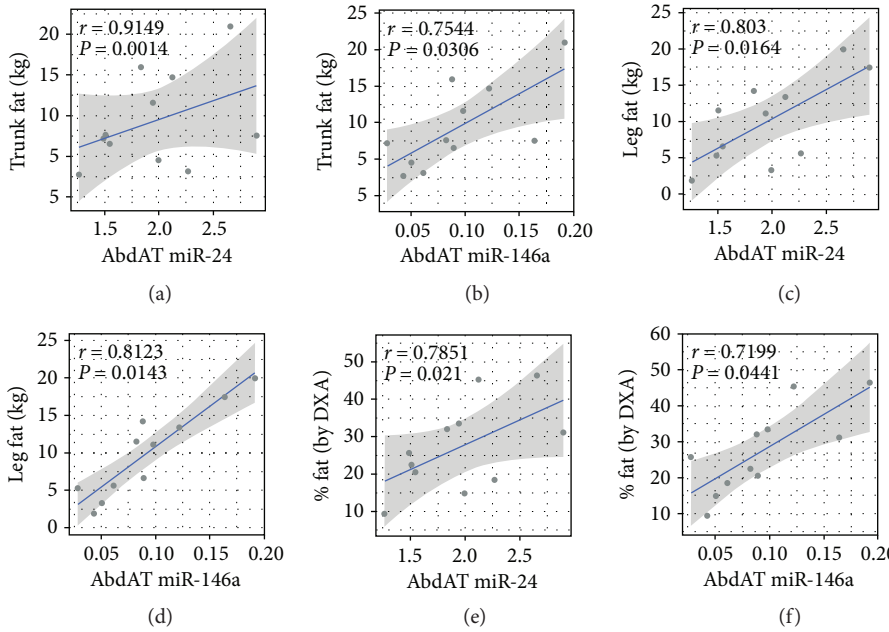

Figure 2: Additional measures of adiposity correlate with abdominal adipose tissue (AbdAT) miR-24 and miR-146a. Partial correlation analysis identified significant associations between specific AbdAT miRNAs and measures of adiposity (a–f). Correlation scatterplots display the −ΔCt data from differentially expressed AbdAT miRNAs and were generated with the R package *ggplot2*. The reported partial correlation that controlled for age, gender, and ethnicity was calculated with the *ppcor* package. The blue line and gray band represent the linear fit of the plotted values and the 95% confidence interval of the fit, respectively.

$r_{miR.146a\sim fat.mass} = 0.822$, both $P < 0.05$, Figures 1(j) and 1(l)), trunk fat ($r_{miR.24\sim trunk.fat} = 0.91$ and $r_{miR.146\sim trunk.fat} = 0.75$, both $P < 0.05$, Figures 2(a) and 2(b)), leg fat ($r_{miR.24\sim leg.fat} = 0.80$ and $r_{miR.146a\sim leg.fat} = 0.81$, both $P < 0.02$, Figures 2(c) and 2(d)), and % fat by DXA ($r_{miR.24\sim\%.fat} = 0.78$ and $r_{miR.146a\sim\%.fat} = 0.72$, both $P < 0.05$, Figures 2(e) and 2(f)).

4. Discussion

miRNAs influence the expression of target genes in multiple ways: directly, by binding to target gene transcripts to alter (usually reduce) mRNA transcript levels; indirectly, for example, by altering the expression of transcription factors, which, in turn, regulates the expression of target genes; and by acting on cofactors of transcription factors or on genes in signaling networks that control the expression of specific target genes/proteins. Target proteins, in turn, can function to regulate feedback loops that control the expression of miRNAs [22].

This study uncovers a potential regulatory link between AbdAT miRNAs and SFRP4 and supports our previous finding that AbdAT is a major contributor of circulating SFRP4 and that SFRP4 plays important roles in adipose tissue and T2DM pathophysiology [11, 16]. In the study by Garufi et al. [11], we reported that circulating SFRP4 protein abundance significantly correlated with the levels of AbdAT SFRP4 mRNA ($r = 0.60$, $P = 0.029$) and negatively correlated with the AbdAT vascularization as measured by capillary density ($r = -0.65$, $P < 0.05$). We also reported that circulating SFRP4 protein levels correlated with AbdAT VEGF mRNA ($r = -0.67$, $P < 0.05$) and with AbdAT local inflammation as measured by secreted MIP1α ($r = 0.74$, $P < 0.05$) [11]. Similarly, we found that AbdAT-SFRP4 was also negatively associated with the AbdAT capillary density ($r = -0.71$, $P < 0.05$) [11]. Importantly, it was previously reported that the levels of circulating SFRP4 negatively correlated with whole body insulin sensitivity and the insulin capacity to suppress lipolysis [11]. Altogether, these findings suggested that SFRP4 might work in a paracrine and endocrine manner to modulate AbdAT capillarity and systemic insulin sensitivity, respectively.

Now, using samples from the same parent study cohort, we have uncovered new evidence that supports the existence of obesity- and T2DM-related mechanisms that upregulate the production of the secreted antiangiogenic factor SFRP4 in the AbdAT, in close association with the expression of fat tissue-derived miRNAs (specifically miR-30d, miR-146a). Interestingly, the differentially abundant levels of miR-146a in the fat tissue of subjects with obesity and diabetes, and its positive correlation with the level of SFRP4 mRNA in the same tissue suggest the existence of a negative feedback loop between these two molecules, as they are reported to impact opposite effects on angiogenesis [11, 30]. Supporting our reasoning, the miRNA target prediction tool miRanda identified SFRP4 as a target of miR-146a. This implies that in AbdAT of subjects with obesity and T2DM, the elevation of SFRP4 levels may induce the expression of miR-146a as a feedback regulatory loop to self-limit the potentially detrimental overexpression of

SFRP4. Consequently, this loop may contribute to fine-tuning the remodeling of the adipose tissue landscape (e.g., capillary microvasculature).

On the other hand, fat cell enlargement was previously reported as an independent marker of insulin resistance and hyperleptinaemia [31], and we previously found that circulating SFRP4 protein correlated with measures of adiposity such as body fat and BMI [11]. We now report that adipose tissue-expressed miRNAs miR-24, miR-30d, and miR-146a also significantly associate with measures of adiposity such as whole body, trunk, and leg fat mass as well as percent body fat (measured by DXA). Remarkably, our published studies profiling circulating miRNAs in independent cohorts covering the full diabetes spectrum [including prediabetes, T2DM, latent autoimmune diabetes of adults (LADA), and type 1 diabetes] have identified these three miRNAs among characteristic signatures that differentially correlate with clinical measures and indices of glycemic control, insulin resistance, and beta cell function and allow for fairly sensitive and specific classification of diabetes subtypes [16, 28].

Taken together, our current and previously published work discussed in this manuscript suggests an important role for the apparently coregulated (in AbdAT) miRNA trio miR-24/miR-30d/miR-146a and SFRP4 in obesity-related pathological events leading to insulin resistance and T2DM. The mechanism through which these molecules exert their effects on obesity is still unclear, but these studies suggest that miRNA-regulated adipose tissue-secreted SFRP4 is related to adiposity and contributes to global insulin resistance in a paracrine and endocrine manner. However, the cross-sectional nature of the study and the limited number of subjects in each cohort limit the generalizability of our conclusions. Because the study is based, at least in part, on the analysis of correlative associations, causal relationships cannot be determined with certainty. Further mechanistic studies are warranted.

5. Conclusion

We show here, for the first time to our knowledge, that correlated levels of miR-24, miR-30d, and miR-146a are elevated in AbdAT from people with obesity and diabetes. The positive correlations between the levels of miR-24, miR-146a, and miR-30d and the levels of SFRP4 transcripts in adipose tissue suggest regulatory loops between these molecules. The correlations between the levels of specific miRNAs and SFRP4 in AbdAT and measures of whole body adiposity (detected in this study) together with previous findings in the same cohort demonstrating associations among AbdAT SFRP4, circulating SFRP4, AbdAT capillarity, and whole body insulin sensitivity [11] suggest a role for abdominal fat and fat-derived miRNA-regulated SFRP4 in the modulation of systemic insulin sensitivity/resistance. Collectively, our findings suggest novel roles for miRNAs in the regulation of mechanisms that impact adiposity and remodeling of the adipose tissue landscape, as well as mechanisms that impact insulin resistance in obesity and T2DM.

Additional Points

Study Importance. What is already known about this subject? (a) There is a strong link between adiposity and insulin resistance and type 2 diabetes (T2DM). (b) Abdominal adipose tissue (AbdAT) is a major contributor to circulating SFRP4. SFRP4 plays important roles (e.g., may inhibit vascularization and promote tissue inflammation) in obesity and T2DM pathophysiology. (c) Circulating levels of miR-24, miR-30d, and miR-146a have been previously involved with adipogenesis, obesity, and T2DM. What does this study add? (a) AbdAT levels of miR-24, miR-30d, and miR-146a are elevated in subjects with obesity and T2DM and positively correlate with the level of SFRP4 transcripts in the same tissue. Notably, SFRP4 and miR-146a display opposite antiangiogenic and angiogenic activities, respectively. This suggests a negative feedback loop between miR-146a and SFRP4 in human AbdAT. (b) These results emphasize the roles of AbdAT miRNAs (i.e., miR-24, miR-30d, and miR-146a) and SFRP4 in regulating mechanisms that govern AbdAT remodeling and systemic insulin sensitivity in obesity and T2DM.

Disclosure

Attila A. Seyhan's current affiliation is Fox Chase Cancer Center, Temple Health, Temple University, Philadelphia, PA, USA.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Yury O. Nunez Lopez and Gabriella Garufi both performed experiments and data analyses and contributed to data interpretation and writing of the manuscript. Magdalena Pasarica provided the study samples and contributed to the study design, study implementation, data interpretation, data analyses, writing, and critical revision of the manuscript. Attila A. Seyhan covered the cost of the research through his startup funds from TRI/Florida Hospital, conceived the research, and contributed to the research design, supervision of the analysis, study implementation, data acquisition, data interpretation, writing of the manuscript, and critical revision/final approval of the manuscript. Attila A. Seyhan is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Yury O. Nunez Lopez and Gabriella Garufi contributed equally to this work.

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Review Article

MicroRNA and Microvascular Complications of Diabetes

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In the last decade, miRNAs have received substantial attention as potential players of diabetes microvascular complications, affecting the kidney, the retina, and the peripheral neurons. Compelling evidence indicates that abnormally expressed miRNAs have pivotal roles in key pathogenic processes of microvascular complications, such as fibrosis, apoptosis, inflammation, and angiogenesis. Moreover, clinical research into innovative both diagnostic and prognostic tools suggests circulating miRNAs as possible novel noninvasive markers of diabetes microvascular complications. In this review, we summarize current knowledge and understanding of the role of miRNAs in the injury to the microvascular bed in diabetes and discuss the potential of miRNAs as clinical biomarkers of diabetes microvascular complications.

1. Introduction

Microvascular complications of diabetes (DMC) have a significant impact on quality of life, morbidity, and mortality, posing a huge burden on the health care system. Diabetic nephropathy is a leading cause of end-stage renal disease (ESRD) and augments the risk of cardiovascular diseases (CVD). Diabetic retinopathy is the major cause of new blindness in adults, and diabetic neuropathy contributes to the development of foot ulcerations that are the predominant cause of nontraumatic lower limb amputation among adults. Therefore, it is urgent to identify novel targets for treatment and to discover innovative noninvasive biomarkers to improve risk prediction, early diagnosis, and prognosis assessment.

MicroRNAs (miRNAs) have been recently implicated in a growing number of pathophysiological conditions. Moreover, miRNAs are also present in biological fluids in a stable form and have been proposed as noninvasive biomarkers. Recently, a growing number of basic science studies have demonstrated the key role of miRNAs in the pathogenesis of DMC. In addition, clinical studies have provided preliminary evidence that miRNAs present in body fluids may be exploited as biomarkers of DMC.

This review summarizes current knowledge on miRNAs as both pathogenic mechanisms and biomarkers of DMC. Furthermore, we will discuss potential future perspectives and limits of this novel line of research.

2. MicroRNAs

MiRNAs are small (~22 nucleotides) noncoding RNA sequences that inhibit gene expression of specific mRNA targets. MiRNAs are present in mammalians, plants, and some viruses. Over 2500 mature miRNAs have been identified in the human genome. Each of them regulates the expression of multiple genes and single mRNAs are targeted by multiple miRNAs; therefore, miRNAs are involved in a vast array of pathophysiological processes [1].

2.1. MicroRNA Synthesis. The first step in miRNA synthesis is the transcription of miRNA genes by RNA polymerase II into large RNA precursors called pri-miRNAs, containing a 5′ cap and a poly-A tail [2]. As multiple miRNAs can be transcribed from the same gene, pri-miRNAs can give rise to various mature miRNAs. Pri-miRNAs are processed in the nucleus by the microprocessor complex, containing the RNase III Drosha that cuts pri-miRNAs and releases stem-

loop sequences of 60–70 nucleotides called pre-miRNAs [3]. The pre-miRNA is transferred from the nuclei to the cytosol by the exportin-5 and Ran-GTP complex [4] In the cytosol, the RNase III Dicer processes pre-miRNAs with formation of duplex miRNAs (dsmiRNA), containing a guide and a passenger strand. dsmiRNAs form an intermediate complex, named miR-RISC (RNA-induced silencing complex), including Dicer and both Argonaute (Ago) and TRBP proteins. During this step, the passenger strand is removed and the mature single-strand miRNA binds to Ago within a functional RISC complex [5–8].

2.2. MicroRNA Function. Mature miRNAs act as a guide allowing the RISC to recognize complementary sequences at the 3′ untranslated region (3′ UTR) of their target mRNAs. In most cases, the recognition occurs between the target mRNA and a sequence of 2–8 bases of the miRNA [9]. The predominant mechanism whereby miRNAs negatively control the expression of their targets is translational repression and subsequent degradation of target mRNAs. Translational repression is usually followed by mRNA degradation; however, this is not always the case and a repressed mRNA may undergo translational reactivation. Degradation may occur through different mechanisms. Ago proteins possess nuclease activity and may directly degrade mRNAs. Alternatively, Ago can function as a binding site for TNRC6-A/B/C proteins that promote mRNA deadenylation. Following deadenylation, mRNAs are rapidly degraded by 3′–5′ exonucleases [10].

3. Diabetes Microvascular Complications

3.1. Diabetic Retinopathy. In the developed countries, diabetic retinopathy (DR) is the leading cause of new blindness in people aged 25–74 years. DR prevalence is strongly related to both level of glycemic control and diabetes duration, and intervention studies have convincingly demonstrated the importance of hyperglycemia in the rate of both development and progression of DR. There are two major DR stages: nonproliferative (NPDR) and proliferative (PDR) retinopathy. In NPDR, thickening of the basement membrane and pericytes/endothelial cell loss result in increased vascular permeability and development of microvascular abnormalities, such as dilated vessels, capillary microaneurysms, shunts, and vascular occlusion. In the severe NPDR stage, vessel obliteration deprives blood supply to areas of the retina that secrete factors to promote formation of new blood vessels. In PDR, both leaking and breaking of immature and fragile new vessels can cause vitreous hemorrhages, macula edema, fibrosis, retinal detachment, and possibly sight loss. A low-grade inflammation is believed to contribute to both DR stages by amplifying hyperglycemia-induced injury and partially mediating neovascularization [11].

3.2. Diabetic Neuropathy. Diabetic neuropathies are the most common complications of DM, affecting as many as 50% of patients. Distal symmetric polyneuropathy (DSPN), the most prevalent form of diabetic neuropathy, is a chronic, symmetrical, length-dependent sensorimotor polyneuropathy. DSPN develops after long-standing hyperglycemia and can be stabilized by rigorous glycemic control. The complication is due to both metabolic derangement, directly damaging the neurons, and injury of the small blood vessels supplying the nerves. Axonal degeneration is the primary structural abnormality; however, demyelination resulting from Schwann cell dysfunction also occurs. DSPN affects predominantly sensory neurons and symptoms vary according to the class of sensory fibers involved. Early symptoms due to the involvement of small fibers include pain and dysesthesias, while anesthesia and poor balance develop at later stages [12].

3.3. Diabetic Nephropathy. Diabetic nephropathy (DN) develops in almost a third of patients with diabetes and accounts for 44% of incident ESRD in the United States. Intervention studies in humans have demonstrated the key pathogenic role of both hyperglycemia and hypertension in DN both onset and progression. DN is characterized by increased glomerular permeability to proteins and progressive renal function decline. Injury and loss of glomerular podocytes, thickening of the glomerular basement membrane, mesangial expansion, and tubule-interstitial fibrosis are the predominant structural abnormalities. Podocyte dysfunction/damage is the major cause of albuminuria development, while excessive extracellular matrix (ECM) deposition leading to sclerosis is a key determinant of progressive renal function loss [13, 14].

4. MicroRNAs as Cellular Mediator of DMC

Several studies have assessed the expression of miRNAs in the tissues/cells from target organs of DMC, providing evidence that miRNA expression profile is altered in both human and experimental DMC (Table 1). However, these studies have used nondiabetic controls as the comparator group, and thus diabetes itself may account for part of the observed changes.

miRNA expression profiling can facilitate the discovery of miRNAs with a key role in DMC and also identify "molecular miRNA signatures" associated with DMC. However, as shown in Table 1, the reproducibility of the results obtained in miRNA profiling studies is scarce even when only highly differentially expressed and PCR-validated miRNAs are considered. The presence of a myriad of potential confounders, including preanalytic and analytic variables, may partially account for this. Regardless of the cause, only part of miRNAs that were differentially expressed in profiling studies was found to be important in subsequent dedicated analyses, and most of the available data come from hypothesis-driven studies, assessing selected candidate miRNAs.

In the next sections, we will review evidence for an involvement of selected miRNAs in common pathophysiological processes across DMC: angiogenesis, inflammation, cell injury/apoptosis, and fibrosis. A list and description of the most relevant miRNAs by complication are reported in Tables 2–4.

4.1. MicroRNAs and Neovascularization in DMC. As mentioned above, hypoxia-induced abnormal neovascularization is a characteristic feature of DR. The cytokine vascular

Table 1: miRNA profiling studies in diabetic microvascular complications.

DMC	Study design	Source	Number of ΔmiRNAs	Selected differentially expressed miRNA	Ref
DR	STZ-DM rats versus controls	Retinas	Δ86	↑ 31, 31*, 34b-3p, 34c, 184,199a, 200a, 200b, 205, 223, 335-3p, 378*, 488, 574-3p ↓ 20b, 499, 690 (*17 PCR-validated miRNAs*)	[22]
		REC	Δ120	↑ *15b*, *19b*, *21*, *31*, *132*, *142-3p*, *146a*, *155*, *339-5p*, *342-3p*, *450a* ↓ *20b-5p*, *29c*, *181c*, *136**, *376c* *(16 PCR-validated miRNAs)*	
	db/db mice versus controls	Retinas	Δ6	↑ 21, 31, 132, 184, 379, 322 ↓ 29b, 294*, 376b, 690, 712 *(>2-fold increase)*	[41]
	DM2 patients versus controls	REC	↑ 60, ↓ 16	↑ 133 ↓ 10a, 10b, 15a *(4 PCR-validated miRNAs)*	[21]
DSPN	STZ-DM mice versus controls	DRG	↑ 74 ↓ 68	↓ let-7i, 103, 16, 107, 130a, 30a, 29a, 138, 27a, 27b, let7g, 30b, let7f, 34a, 338-3p ↑ 191, 466-5p, 149*, 341* *(>1.5 fold-change)*	[51]
	STZ-DM mice versus controls	Spinal dorsal horn	↑ 21 ↓ 21	↑ 184-5p ↓190a-5p *(2 PCR-validated miRNAs)*	[137]
DN	DN FSGS IgAN MPGN Controls	Human glomeruli	DN: Δ18 FSGS: Δ12 IgAN: Δ2 MPGN: Δ17 versus controls	DN versus controls: ↑ 29a, 23a, 214, 21, 5585, 589, 150, 4286 ↓ 486-3p, 486-5p, 1180, 4301, 30a-3p, 30c, 148a, 30a-5p, 3184, 423 DN versus FSGS: ↑ 24, 146a DN versus MPGN:↑ 146a, 146b; ↓ 671	[37]
	DM2 patients versus healthy controls	Kidney	↑ 32 ↓ 39	↑ 146a, 155 *(2 PCR-validated miRNAs)*	[28]
	db/db mice versus controls	Glomeruli	↓ 45		
	HG versus NG	Podocytes	↓ 32	↓ 5	↓ 92a, 92b, 93, 140, 191	[55]
		GEC	↓ 86		

DMC: diabetic microvascular complications; DM: diabetes; DM2: type 2 diabetes; STZ-DM: streptozotocin-induced diabetes; DR: diabetic retinopathy; DN: diabetic nephropathy; DSPN: diabetic peripheral symmetric polyneuropathy; HG: high glucose; NG: normal glucose; FSGS: focal segmental glomerulosclerosis; MPGN: membranoproliferative glomerulonephritis; REC: retinal endothelial cells; GEC: glomerular endothelial cells; DRG: dorsal root ganglia; Δ: differentially expressed miRNAs. *miRNA nomenclature.

endothelial growth factor (VEGF) plays a key role in this process and anti-VEGF therapies have been proven effective for the treatment of both diabetic macular edema and PDR. Therefore, miRNAs that control VEGF signaling have been extensively studied in the context of DR. Among them, miR-126, miR-106, miR-15, and miR-200b directly target VEGF, while miR-150, miR-184, and miR-155 indirectly modulate VEGF either expression or signaling.

miR-126 is one of most studied miRNA in both diabetes and diabetes complications because of its key role in endothelial protection and angiogenesis. Levels of miR-126 are reduced in the retina in experimental diabetes and other hypoxic conditions [15], and there is evidence from studies in animal models of oxygen-induced retinopathy (OIR) of a causal link between hypoxia-induced miR-126 downregulation and the rise in retinal VEGF levels. For instance, intravitreous injection of a plasmid vector expressing miR-126 reduced both VEGF and neovascularization [16]. Of interest, a recent study has shown that treatment with Niaspan, which normalized retinal miR-126 levels, prevented overexpression of VEGF as well as edema, hemorrhages, and apoptosis [17]. Moreover, a small case-control study also reported an association between a miR-126 polymorphism and both severe NPDR and PDR in patients with type 2 diabetes [18]. There is interdependence between hypoxia-induced factor 1α (HIF1-α) and VEGF expression as silencing of HIF1-α resulted in a significant reduction in VEGF protein levels and vice versa in both *in vitro* and *in vivo* models of DR. This interdependence is mediated by shared miRNAs, such as *miR-106*, and overexpression of miR-106a significantly

Table 2: MicroRNAs Involved in Diabetic Retinopathy.

miRNA	Source	Model	miRNA levels	Putative targets	Pathogenic role	Reference
126	Retinas	STZ-DM rats, OIR mice	↓	*VCAM-1* *VEGF*	↑ angiogenesis	[15–17]
	Retinas	STZ-DM rats and DM patients	↓	*VEGF*	↑ angiogenesis	[20]
200b	Vitreous	Patients with PDR, Akita-DR mice, STZ-DM mice	↑	*Oxr1* *Snail1* *Smad2* *p300*	↑ EMT	[98–100]
15a	Retinas RPE cells	STZ-DM mice and STZ-DM rats, HRPE cells	↓	*VEGF-A* *ASM*	↑ angiogenesis, ↑ inflammation, ↑ lipotoxicity	[21]
150	Retinas	STZ-DM rats HFD-DM mice (WT and miR-150$^{-/-}$)	↓	*VEGFR2*	↑ angiogenesis	[22, 23]
184	Retinas	OIR mice	↓	*Frizzled-7*	↑ angiogenesis	[24]
155	Retinas	OIR mice	↑	*SHIP1*	↑ angiogenesis	[26]
146a	Retinas	STZ-DM rats	↓	*CARD10* *IRAK1/2* *TRAF6*	↑ inflammation	[22, 34]
	Retinas	STZ-DM rats and db/db mice	↓	*Fibronectin*	↑ fibrosis	[30]
21	Retinas	STZ-DM rats and db/db mice	↑	*PPARα*	↑ inflammation	[22, 41]
195	Retinas	STZ-DM rats	↑	*SIRT1*	↑ apoptosis	[62]
29b	Retinas	STZ-DM mice, STZ-DM rats	↓	*Sp1*	↑ apoptosis	[39, 52]

RPE: retinal pigment epithelial cells; STZ: streptozotocin; OIR: oxygen-induced retinopathy; DM: diabetes; PDR: proliferative diabetic retinopathy; HFD: high-fat diet; WT: wild type; EMT: epithelial mesenchymal transition.

Table 3: MicroRNAs involved in diabetic neuropathy.

miRNA	Source	Model	miRNA levels	Putative targets	Pathogenic role	Reference
146	Sciatic nerve	db/db mice	↓	*IRAK1/2* *TRAF6*	↑ inflammation, apoptosis	[31, 32]
		STZ-DM rats	↑	*IRAK1/2* *TRAF6*	Dysfunctional NF-κB-miR-146a negative feedback loop	[27]
let-7i	DRG neurons	STZ-DM mice	↓	—	↓ neurotrophism regeneration	[51]
29b	DRG neurons	STZ-DM rats	↓	*Sp1 (?)*	↑ apoptosis, ↓ regeneration	[40]
29c	DRG neurons, sciatic nerve, and foot pad tissues	db/db mice	↑	*PRKCI*	↓ axonal growth	[53]
341	DRG neurons	STZ-DM mice	↑	—	Unknown	[51]

DRG: dorsal root ganglia; STZ: streptozotocin; DM: diabetic.

reduced the expression of both HIF1-*α* and VEGF and prevented high glucose-induced increased permeability [19].

McArthur et al. showed a downregulation of *miR-200b* in both experimental and human DR, and manipulation of miR-200b levels confirmed that retinal VEGF expression was under miR-200b control. Most importantly, they demonstrated that a miR-200b mimic normalizes retinal VEGF levels and reduces both neovascularization and enhanced permeability in DM mice, providing evidence that miR-200b is a key mediator of VEGF rise in DR and a potential target for miRNA-based treatment [20]. Similarly, retinal *miR-15* was reduced in both experimental and human DR, and overexpression of miR-15 reduced VEGF and ameliorated vascular leakage in DM animals. However, miR-15 has also anti-inflammatory properties and controls the expression of acid sphingomyelinase, the central enzyme in the sphingolipid metabolism; therefore, amelioration of inflammation and lipotoxicity is a possible additional protective mechanism [21].

MicroRNA-150, miR-184, and miR-155 can indirectly modulate VEGF signaling in DR. *miR-150* affects VEGF by inhibiting the expression of the type 2 VEGF receptor. This

Table 4: MicroRNA involved in diabetic nephropathy.

miRNAs	Source	Model	miRNA levels	Putative targets	Pathogenic role	Reference
21	Glomeruli Kidney	DM2 patients with albuminuria, STZ-DM mice	↑	*SMAD7*, *TGF-βR2* *PDCD4*, *Col4a1*, *TIMP3*	↓ fibrosis, ↓ podocyte damage	[42]
	Kidney	db/db mice KK/Ay mice	↑	*SMAD7*	↑ fibrosis, ↑ inflammation	[44, 45]
	Kidney	OVE26 mice	↑	*PTEN*	↑ fibrosis	[43]
	Glomeruli	db/db mice	↓	*PTEN*	↑ fibrosis	[72]
25	Kidney	DM2 patients with DN STZ-DM rats STZ-DM mice and db/db mice	↓	*PTEN* *NOX4* *CDC42*	↑ oxidative stress, ↑ apoptosis, ↑ fibrosis, ↑ podocyte injury	[66–68]
26a	Glomeruli	DM2 patients with DN STZ-DM mice	↓	*CTGF*	↑ fibrosis	[102]
27a	Glomeruli Kidney	DM2 patients with DN STZ-DM rats	↑	*PPAR-γ*	↑ podocyte damage, ↑ fibrosis	[58, 101]
29a	Glomeruli	STZ-DM mice	↓	*HDAC4*	↑ podocyte injury, ↑ fibrosis	[54]
29b	Kidney	db/db mice	↓	*SP1*	↑ fibrosis, ↑ inflammation	[38]
29c	Glomeruli	db/db mice	↑	*SPRY1*	↑ fibrosis, ↑ podocyte injury	[59]
34a	Glomeruli	db/db mice	↑	*GAS1*	Glomerular hypertrophy	[69]
93	Glomeruli Kidney	db/db mice DM patients with DN	↓	*MSK2*/*VEGF*	↑ VEGF, podocyte damage	[55, 56]
130b	Glomeruli	STZ-DM mice	↓	*TGF-βR1*	↑ fibrosis	[89]
	Kidney	DM2 patients and STZ-DM rats	↓	*SNAIL*	↑ EMT	[105]
135a	Kidney	DM patients with DN and db/db mice	↑	*TRPC1*	↑ fibrosis	[103]
146a	Kidney	DM2 patients with DN, STZ-DM rats and DM2 rats STZ-DM mice and db/db mice	↑	*IRAK1*, *TRAF6*	↓ inflammation, ↓ fibrosis	[28]
		DM2 patients and ob/ob mice	↓	*ERB-B4*, *NOTCH-1*	↑ podocyte injury	[33]
155	Kidney	DM2 patients with DN, STZ-DM rats and DM2 rats STZ-DM mice	↑	*SOCS1*	↓ inflammation, ↑ podocyte damage	[28, 57]
192	Kidney	Patients with advanced DN ApoE-KO DM mice	↓	*ZEB1/2*	↑ EMT, ↑ fibrosis (tubule-interstitial)	[91, 92]
	Glomeruli Kidney	STZ-DM mice and db/db mice DM2 patients with early DN	↑	*ZEB1/2*	↑ fibrosis (glomeruli)	[77, 78, 80]
195	Glomeruli Kidney	STZ-DM mice (proteinuric)	↑	*BCL2*	↑ podocyte damage/apoptosis	[63]
	Kidney	Early DN mice	↓	*BCL2*	↑ mesangial cell proliferation	[64]
215	Glomeruli Kidney	db/db mice	↑	*CTNNBIP1*	↑ EMT	[104]
	Kidney	ApoE-KO STZ mice	↓	*ZEB2*	↑ EMT, ↑ fibrosis	[87]
216a	Glomeruli	STZ-DM mice and db/db mice	↑	*YBX1*	↑ fibrosis	[81]
200b	Kidney	ApoE KO STZ mice	↓	*ZEB1/2*, *FN*	↑ EMT	[93–95]
200b/c	Glomeruli	STZ-DM mice and db/db mice	↑	*FOG2*, *ZEB1*	Glomerular hypertrophy	[73, 79]
377	Kidney	STZ-DM mice and NOD mice	↑	*PAK1*, *SOD2*	↑ fibrosis	[90]
let-7a	Kidney	DM2 patients with early DN STZ-DM rats	↓	*UHRF1* *TGF-βR1*	↑ fibrosis	[88, 96]
let-7/7b	Glomeruli Kidney	ApoE-KO STZ mice	↓	*Col1a2*, *Col4a1* *TGF-βR1*	↑ fibrosis	[85, 86]

STZ: streptozotocin; DM: diabetes; DN: diabetic nephropathy; KO: knockout; EMT: epithelial mesenchymal transition.

miRNA is of relevance in DR as miR-150 is downregulated in the diabetic retina [22] and miR-150 deletion exacerbated retinal neovascularization in a model of high-fat diet-induced diabetes [23], likely by further enhancing VEGF signaling. *miR-184* was downregulated in a model of ischemia-induced retinal neovascularization [24]. Because miR-184 controls the expression of frizzled-7, downregulation of miR-184 can activate the canonical Wnt/frizzled-7 pathway that plays a central role in DR by enhancing neovascularization through increased VEGF transcription [25]. Expression of *miR-155* is induced by VEGF, and this is a mechanism whereby VEGF enhances its downstream signaling. In fact, miR-155 inhibits SHIP1, a counterregulator of the VEGF-induced phosphoinositide 3-kinase (PI3K)/Akt pathway. Moreover, studies in DR have shown that retinal levels of miR-155 are enhanced in a model of OIR and that treatment with a miR-155 antagomir can reduce neovessel formation in a model of OIR via a SHIP1/PI3K/Akt-dependent pathway [26].

Collectively, these data highlight the importance of miRNAs in modulating VEGF activity and thus retinal neovascularization in DR and suggest that miRNAs may represent novel targets for treatment. However, miRNAs affecting VEGF in the diabetic retina have many other target genes, increasing the likelihood of off-target effects.

4.2. MicroRNAs and Inflammation in DMC. A low-grade chronic inflammation is believed to contribute to the pathogenesis of DMC. Hyperglycemia and both hemodynamic and oxidative stress are the main inducers of the inflammatory response in DMC through activation of the transcription factor NF-κB. Monocyte chemoattractant protein 1 (MCP-1) and other chemokines locally recruit monocyte/macrophages that are predominantly of the M1 proinflammatory phenotype. Both resident cells and infiltrating macrophages release inflammatory cytokines that contribute to cell damage and promote inflammation-driven fibrosis. Among the multitude of miRNAs implicated in the regulation of inflammatory processes, miR-146, miR21, and miR-29 appear of particular relevance in DMC.

miR-146a is a well-known modulator of both the innate and adaptive immune response. In particular, miR-146 induction is a mechanism whereby NF-κB limits its proinflammatory activity. In fact, NF-κB activates miR-146a, which in turn inhibits NF-κB by downregulating interleukin-1 receptor-associated kinase 1/2 (IRAK1/2) and TNF receptor-associated factor 6 (TRAF6). Therefore, miR-146a is crucial to allow the timely resolution of ongoing inflammatory processes.

miR-146a expression has been studied in the kidney, retina, and sciatic nerve from both patients with DMC and experimental diabetic animals. Results are conflicting with some studies reporting an increase [27–29] and other a reduction in miR-146a expression [22, 30–33]. This likely reflects variability in the compensatory anti-inflammatory miR-146a response in various stages and models of DMC. However, the observation that both NF-κB activity and inflammatory cytokine levels were elevated even when miR-146a was overexpressed suggests a state of relative miR-146a deficiency with insufficient activation of the NF-κB-miR-146a negative feedback loop [27]. Furthermore, a recent study in podocytes has described a feed-forward loop resulting in a progressive reduction in miR-146 expression. Specifically, MCP-1 upregulates the ribonuclease MCPIP1 that antagonizes miR-146a. This reduces miR-146 inhibition on its target gene ErbB4 and thus enhances signaling through the TGF-β1-ErbB4 pathway, which increases autocrine synthesis of MCP-1, further reducing miR-146a levels [33] (Figure 1).

In keeping with these data, intervention studies have consistently demonstrated an anti-inflammatory and protective effect of miR-146a in DMC. In experimental DSPN, systemic administration of a miR-146a mimic improved both functional and structural alterations of DSPN and induced a shift from M1 to M2 macrophages [31, 32]. In experimental DR, intravitreal injection of miR-146a ameliorated both microvascular leakage and retinal functional defects and reduced expression of intercellular adhesion molecule 1 (ICAM-1), a NF-κB downstream gene [34]. In experimental DN, deletion of miR-146a exacerbated proteinuria, fibrosis, and macrophage infiltration and induced an M2 to M1 macrophage shift with inflammasome activation [29], while treatment with erlotinib, a pan-ErbB kinase inhibitor, ameliorates podocyte injury and albuminuria by preventing ErbB4 induction secondary to miR-146 downregulation [33].

There is relatively little information on miR-146a in human DMC; however, a miR-146a polymorphism was associated with both DN and DSPN [35, 36]. Moreover, in a recent study comparing miRNA expression in human glomeruli from patients with various kidney diseases, the expression levels of miR-146a and miR-30a, used in combination, could effectively distinguish DN from all other renal conditions except IgA nephropathy [37], suggesting a potential relevance of miR-146a in the differential diagnosis of renal diseases.

MicroRNA-29b is of relevance in the context of inflammation because it can reduce NF-κB activity by targeting Sp-1, a transcriptional factor playing a key role in the activation of the NF-κB pathway. The expression of miR-29b was reduced in experimental DN, and treatments increasing miR-29b levels reduced microalbuminuria, renal fibrosis, and Sp-1/NF-κB-driven inflammation in diabetic animals [38]. However, this benefit cannot be entirely ascribed to miR-29b anti-inflammatory properties because Sp-1 can also modulate TGF-β1 signaling/apoptosis. miR-29 is also downregulated in both DR and DSPN [39, 40], but there are no data on a potential anti-inflammatory role of miR-29b in these complications.

miR-21 is overexpressed in both DR and DN [22, 41–45] and contributes to the pathogenesis of DR by enhancing inflammation. Indeed, intervention studies have shown that both miR-21 deletion and intravitreal injection of a miR-21 inhibitor ameliorated retinal leakage and inflammation at least in part via upregulation of peroxisome proliferator-activated receptor-α (PPAR-α) [22, 41], which is a miR-21 target and inhibits NF-κB by upregulating IκB-α. However, PPAR-α has also other beneficial effects particularly on metabolism that may explain its protective effect. There are

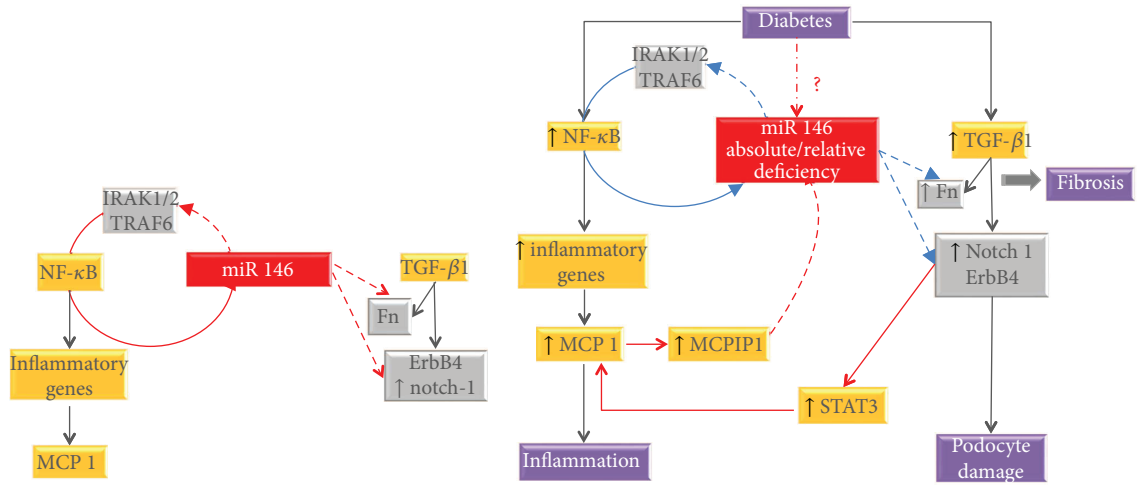

Figure 1: Role of miR-146a in diabetic microvascular complications. (a) In normoglycemic conditions, miR-146 is induced by NF-κB, and it inhibits NF-κB by suppressing its target genes interleukin-1 receptor-associated kinase 1/2 (IRAK1/2) and TNF receptor-associated factor 6 (TRAF6). Moreover, miR-146 represses expression of fibronectin (Fn) in retinal cells and ErbB4/Notch1 in podocytes. (b) In the presence of diabetes, there is an absolute/relative miR-146 deficiency leading to insufficient inhibition (light blue lines) of IRAK1/2/TRAF6 (enhancing inflammation), Fn expression (favoring fibrosis), and TGF-β1/ErbB4/Notch1 signaling (leading to podocyte damage). Enhanced signaling through the TGF-β1-ErbB4 pathway increases autocrine synthesis of MCP-1, further reducing miR-146a levels via MCPIP1 in a feed-forward loop. Grey boxes: miR-146 target genes; dotted lines: inhibition; continuous line: activation/induction.

no data on miR-21 expression in DSPN; however, miR-21 is upregulated in models of peripheral neuronal injury [46], and a recent study has described an unforeseen link between miR-21 and inflammation. Injured dorsal root ganglia (DRG) neurons release exosomes enriched in miR-21 that are phagocytized by macrophages in which miR-21 promotes a proinflammatory phenotype. Therefore, both upregulation and release of miR-21 appears to play a key role in sensory neuron-macrophage communication after damage to the peripheral nerve [47]. Likely future studies will explore if a similar mechanism is at play in DMC.

4.3. MicroRNA and Cell Injury/Apoptosis in DMC. Cell damage eventually leading to apoptosis is a characteristic feature of DMC. Moreover, both podocytes and neurons are terminally differentiated cells; therefore, their damage has irreversible effects. Abnormally expressed miRNAs have been involved in diabetes-induced cell injury and herein we will focus in particular on miRNAs affecting neurons, podocytes, mesangial cells (MCs), and proximal tubular epithelial cells (TECs), as the role of miRNAs in microvascular endothelial cells has been recently reviewed elsewhere [48–50].

Several abnormally expressed miRNAs have been implicated in the pathogenesis of DSPN because they can enhance apoptosis and/or interfere with neuronal regenerative processes. *let-7i* was the most downregulated miRNA in a profiling study on diabetic DRG. Moreover, intranasal injection with a let-7i mimic improved experimental DSPN, and let-7i enhanced both growth and branching of cultured neurons [51], indicating a protective neurotrophic activity. Similarly, *miR-29b* expression was reduced in diabetic DRG and interventions normalizing its levels diminished neuron apoptosis and increased regenerative processes [40]. miR-29b also affects the neuronal component of the diabetic retina. Specifically, miR-29b prevents apoptosis of cultured retinal Müller cells by inhibiting its target gene Sp-1, and thus the downregulation of miR-29b observed in the diabetic retina may favor apoptosis [52]. On the contrary, another member of the miR-29 family *miR-29c* is overexpressed in both DRG neurons and sciatic nerve of diabetic mice and negatively regulates axonal growth by suppressing protein kinase C-iota [53].

Recent studies have explored the role of miRNAs in podocyte damage in diabetes. Nephrin, a major component of the junction connecting foot processes of adjacent podocytes, is crucial to prevent protein leaking, and miR-29, miR-155, and miR-93 have been causally linked to nephrin loss in diabetes. Lin et al. have shown that *miR-29a* was downregulated in the glomeruli from diabetic mice and that miR-29a overexpression attenuated nephrin downregulation, podocyte apoptosis, and proteinuria. The beneficial effect of miR-29a was due to inhibition of histone deacetylase (HDAC4) that causes nephrin deacetylation, ubiquitination, and loss. Not only was HDAC4 a miR-29a target, but also lowered miR-29a expression via an epigenetic mechanism [54], fueling a deleterious vicious cycle. Similarly, a reduction in *miR-93* expression was found in both diabetic glomeruli and podocytes exposed to high glucose [55]. Moreover, diabetic mice with inducible overexpression of miR-93 exclusively in podocytes exhibited significant improvements of albuminuria, nephrin downregulation, foot process effacement, and podocyte loss. miR-93 has an important role in chromatin reorganization by modulating its target MSK2, a histone kinase, and its substrate H3S10, and miR-93 constitutive expression is required to maintain podocyte health. Because miR-93 expression is reduced by hyperglycemia, miR-93 is a critical link between altered metabolism and

podocyte epigenetic alterations [56]. Finally, *miR-155* was overexpressed in both human and experimental DN [28, 57], and its deletion enhanced expression of nephrin, acetylated nephrin, and Wilms tumor 1 (WT-1), a marker of podocyte differentiation, through upregulation of suppressor of cytokine signaling 1 (SOCS1) that inhibits the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 1 (STAT1) pathway [57], indicating a deleterious effect of diabetes-induced glomerular miR-155 overexpression.

Besides nephrin downregulation, alterations of the podocyte cytoskeleton have also been implicated in proteinuria, foot process effacement, and podocyte loss. miRNAs abnormally expressed in response to hyperglycemia/TGF-β1 appear to contribute. In podocytes, high glucose induces *miR-27a* that activates β-catenin signaling by negatively targeting PPAR-γ. This leads to increased podocyte mesenchymal transition, disrupted podocyte architectural integrity, downregulated nephrin, and increased podocyte apoptosis. The *in vivo* relevance was proven in experimental DN and miR-27a upregulation confirmed in human diabetic kidney biopsies [58]. In cultured podocytes exposed to high glucose, *miR-29c* suppresses its target gene Spry1, resulting in both abnormal activation of Rho kinase, a key regulator of the podocyte cytoskeleton, and apoptosis. This is in agreement with *in vivo* studies showing miR-29c upregulation in the glomeruli from db/db mice and amelioration of DN in miR-29c knockdown mice [59]. Of interest, linagliptin inhibits the enzyme dipeptidyl peptidase-4 (DPP-4) that degrades miR-29, and the beneficial effect of linagliptin treatment in experimental DN may be partially ascribed to a reduction in miR-29 levels [60]. Finally, *miR-135a* is induced by TGF-β1 in cultured podocytes and causes severe podocyte injury and disarray of the podocyte cytoskeleton by downregulating transient receptor potential channel 1 (TRPC1) [61].

Several miRNAs modulate apoptosis in renal and/or retinal cells predominantly by affecting TGF-β1 signaling. The expression of *miR-21* is enhanced in both human and experimental DN [42–45], and recent work by Lai JY et al. suggests that both hyperglycemia and TGF-β1 induce miR-21 that in turn functions as a feedback inhibitor of TGF-β-induced podocyte apoptosis. Consistent with this notion, miR-21 deletion worsened albuminuria, podocyte loss, and renal injury in both diabetic and TGF-β1 transgenic mice. *In vitro* studies in podocytes have clarified that the beneficial effect of miR-21 is due to downregulation of proapoptotic target genes (Smad7, TGFR2, and Pdcd4) [42]. Sirtuins (SIRTs) are potent inhibitors of apoptosis and both *miR-195* and *miR-20b* have been shown to affect apoptosis in DMC by suppressing members of the SIRT family. Specifically, miR-195 was overexpressed in both the retina and the kidney of diabetic animals, and both miR-195 and miR-20 mediated high-glucose-induced apoptosis of renal cells by suppressing SIRT1 and SIRT7, respectively [62–65]. *miR-25* is downregulated in both human and experimental DN, and this deficiency has detrimental effects on both MCs and TECs as miR-25 prevents TEC apoptosis by modulating the phosphatase and tensin homolog (PTEN)/Akt pathway and reduces oxidative stress in MCs by targeting NADPH oxidase 4 (Nox4). Consistent with a protective role of miR-25, systemic administration of a miR-25 mimic ameliorated DN [66–68]. Several other miRNAs affect apoptosis; however, given their predominant effect on fibrotic processes, they will be described in the next session.

In the diabetic kidney, MCs undergo proliferation and then hypertrophy, a change in phenotype that preludes to enhanced expression of ECM components. Expression of *miR-34* is enhanced in DN and miR-34 induces MC proliferation by targeting GAS1 [69]. This is in contrast with the effect of miR-34 in retinal cells, where this miRNA reduces cell proliferation by inhibiting LGR4 [70]. MC hypertrophy is induced by *miR-21* through inhibition of its target gene PTEN and activation of the Akt/target of rapamycin complex 1 (TORC1) pathway. This together with the profibrotic effects of miR-21 in MCs may explain reports of amelioration of DN in diabetic miR-21 knockout mice despite the protective antiapoptotic effect of miR-21 on podocytes [43, 71, 72]. Another miRNA implicated in MC hypertrophy is *miR-200b/c*, which is overexpressed in experimental DN. Specifically, TGF-β1 induces expression of miR-200b/c that causes downregulation of the PI3K inhibitor FOG2, leading to MC hypertrophy through the PI3K/Akt pathway [73].

Collectively, these data underscore the relevance of miRNA in cell health and the contribution of miRNAs in the pathogenesis of the cellular injury induced by diabetes.

4.4. MicroRNAs and Fibrosis in DMC. Excessive deposition of ECM components, mainly collagen and fibronectin, leading to sclerosis, occurs in all DMC, but it is a predominant feature of DN, and thus most of the studies assessing the role of miRNAs on fibrosis were performed in this complication.

The prosclerotic cytokine TGF-β1, which is released by resident cells in response to diabetes-related insults, acts locally via autocrine/paracrine mechanisms and is a key mediator of fibrotic processes in both the glomeruli and the tubule interstitium. Several studies have thus investigated the complex interplay between miRNAs and TGF-β1 on renal fibrosis (Figure 2). TGF-β1 induces the expression of profibrotic miR-216 and miR-377, while it represses antifibrotic miR-29 and let-7. Data on the effect of TGF-β1 on miR-192 are more conflicting with contrasting results *in vitro* in MCs and TECs and also *in vivo* where miR-192 was found both upregulated and downregulated in experimental DN. This may be due to differences in animal models, disease stages, and/or *in vitro* experimental conditions. In MCs, miR-192 is induced initially through Smad3 signaling and then through a long-lasting epigenetic mechanism involving Ets1 and histone H3 acetylation by Akt-activated p300, and this is consistent with findings in the glomeruli from db/db mice [74]. On the contrary, in TECs, TGF-β1 represses miR-192 transcription by decreasing the binding of HNF to the miR-192 gene [75]. HNF expression is restricted to the tubules and this may partially explain the cell specificity of miR-192 expression [76].

The collagen gene has E-box regulatory elements placed in its far upstream region. In MCs exposed to TGF-β1 and in glomeruli from diabetic mice, upregulation of *miR-192* and *miR-200b/c* increases Col1a2 and Col4a1 expression by inhibiting the E-box repressors Zeb1 and Zeb2 [77–79].

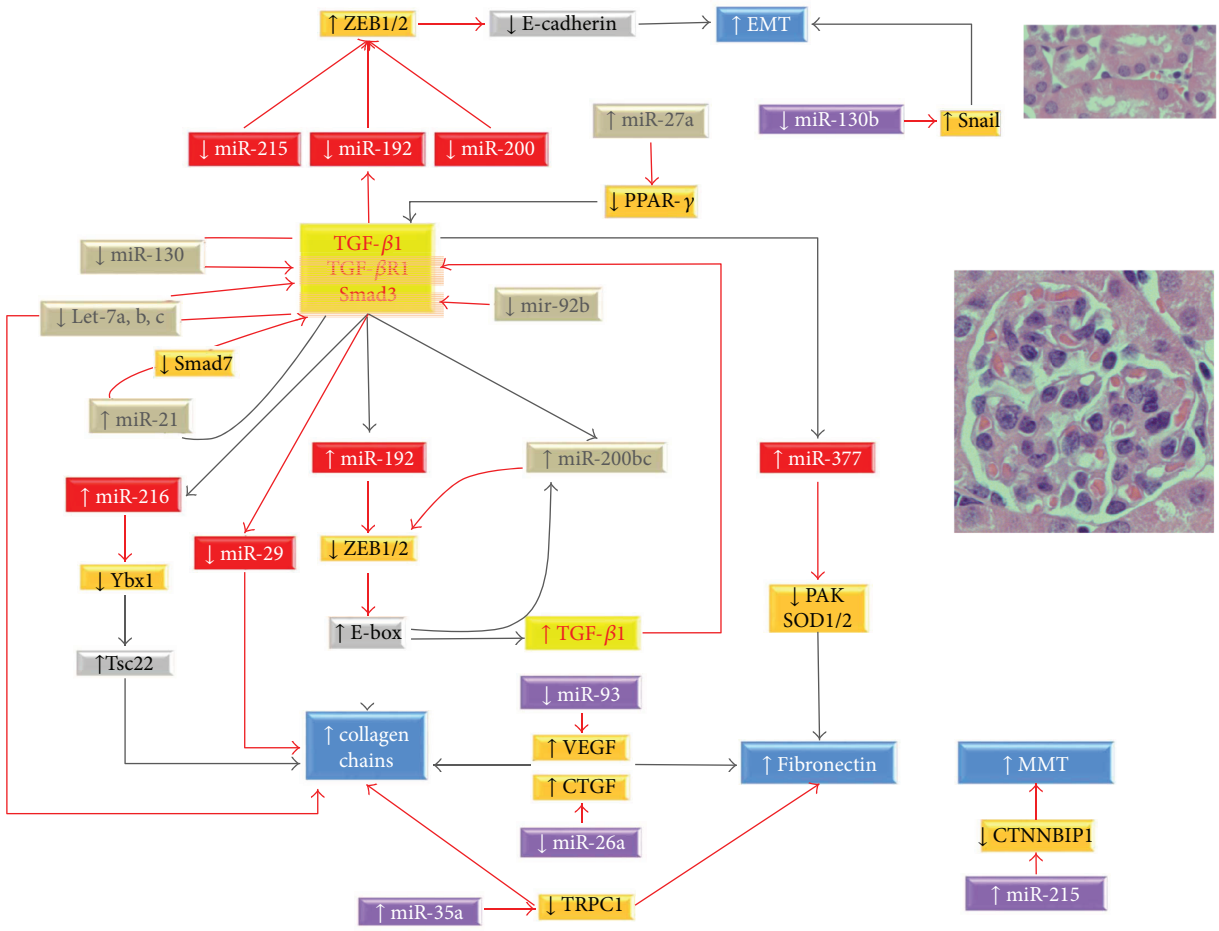

Figure 2: MicroRNAs involved in renal fibrosis in diabetes. MicroRNAs (miRNAs) implicated in glomerular (cream-coloured area) and tubule-interstitial (light purple-coloured area) fibrosis in diabetes. The image shows miRNAs in red boxes that are modulated by TGF-β1 and directly control collagen/fibronectin expression, miRNAs in bronze boxes that enhance TGF-β1 signaling, and miRNAs in purple boxes that affect fibrosis independently of TGF-β1. Target genes are shown in orange boxes. Grey lines indicate induction, while red lines indicate suppression of miRNA expression. EMT: epithelial mesenchymal transition; MMT: mesangial cell to myofibroblast transition; CTNNBIP1: catenin beta interacting protein 1; TGF-β1: transforming growth factor-β1; TGFB-R1: transforming growth factor type 1 receptor; VEGF: vascular endothelial growth factor; CTGF: connective tissue growth factor.

Consistent with this, a negative correlation between miR-192 and both Zeb1 and Zeb2 expression has been reported in patients with type 2 diabetes [80]. Moreover, diabetic mice either knockout for miR-192 or treated with miR-192 inhibitors had a less severe renal phenotype with amelioration of glomerular hypertrophy, fibrosis, and proteinuria [77, 78, 80]. In MCs, TGF-β1 also promotes expression of Col1a2 via upregulation of *miR-216a*. This effect is mediated by post-transcriptional upregulation of Tsc22 by Ybx1, which is a RNA-binding protein targeted by miR-216a. The interaction of Tsc22 with the transcription factor E3 increases Col1a2 expression and, in keeping with this, a significant increase of miR-216 and Tsc22 with parallel Ybx1 downregulation was observed in the glomeruli of diabetic mice [81]. Members of the *miR-29* and *let-7a*, *b*, *c* families suppress the transcription of collagen and have an antifibrotic effect. TGF-β1 counteracts this antifibrotic effect by downregulating both miR-29 and miR-7a, b, c [82–89]. Consistent with this, miR-29b overexpression ameliorates DN [38, 82]. Moreover, in a model of advanced DN, an angiotensin receptor blocker that enhanced miR-29 expression reduced both ECM deposition and renal fibrosis [84]. In MCs, TGF-β1 can also affect fibronectin expression through *miR-377*. Specifically, TGF-β1 enhances expression of miR-377 that increases fibronectin levels by both inhibiting PAK1 and increasing oxidative stress through suppression of superoxide dismutase (SOD)-1/2 genes [90].

As mentioned above, the interplay between miR-192 and TGF-β1 differs in TECs. In these cells, TGF-β1 induces a downregulation of *miR-200*, *miR-192*, and *miR-215*. As these miRNAs inhibit Zeb1 and Zeb2, this results in E-cadherin downregulation. E-cadherin is not only an epithelial marker, but is also involved in cell-to-cell adhesion, and E-cadherin dysregulation induces phenotypic changes contributing to epithelial mesenchymal transition (EMT) and thus to renal fibrosis [91–95].

A number of miRNAs affect fibrosis by enhancing TGF-β1 signaling. For instance, TGF-β1 downregulates *let-7a*, *b*, *c* and *miR-130* that are known to suppress expression of the TGF-β1 receptor of type 1 [85–89, 96]. The downregulation

of *miR-92b* enhances Smad3 expression, while TGF-β1-induced *miR-21* represses the expression of inhibitory Smad7 [43–45, 97]. Recently, Kato et al. have described a positive circuit whereby *miR-200b*/*c* enhances TGF-β1 expression in MCs. TGF-β1-induced miR-192 represses Zeb1/2, leading to TGF-β1 and miR-200b/c expression. In turn, miR-200b/c by inhibiting Zeb1/2 further enhances the effect of miR-192 on TGF-β1 [79]. Of interest, miR-200b/c has also been implicated in the fibrotic processes occurring in the diabetic retina. Specifically, miR-200b is increased in human PDR [98] and experimental DR [99] and promotes EMT both *in vitro* and *in vivo* [100]. Finally, diabetes-induced *miR-27a* overexpression promoted fibrosis in both TECs and diabetic rats by targeting PPAR-γ and indirectly enhancing TGF-β/Smad3 signaling [101].

There are also miRNAs that can affect fibrosis in a TGF-β1-independent manner. In podocytes, downregulation of *miR-93* and *miR-26* enhances both fibronectin and collagen expression by reducing the inhibitory effects of miR-93 and miR-26 on VEGF and connective tissue growth factor (CTGF), respectively [102]. In MCs, *miR-135a* induces synthesis of ECM components by inhibiting its target gene TRPC1, and miR-135a deletion restores levels of TRPC1 and reduces production of both fibronectin and collagen type I in experimental DN [103]. *miR-215* contributes to renal fibrosis in DN by suppressing its target gene catenin-β interacting protein 1 and thus favoring the transition of MCs to myofibroblasts [104]. *miR-130b* is downregulated in DN and miR-130 overexpression improves tubulointerstitial fibrosis via repression of Snail-induced EMT [105]. Deletion of *miR-146* worsens kidney injury at least in part by targeting the ErbB4 and Notch-1 pathway [33]. In addition, miR-146 can directly control fibronectin expression in retinal cells and *in vivo* a miR-146 mimic normalized fibronectin levels in the retina of diabetic mice [30].

Taken together, these studies prove the crucial role of miRNAs in regulating ECM production and have given us a deeper understanding of the complex mechanisms involved in diabetes-induced fibrotic processes.

5. Circulating miRNAs

miRNAs are also present in biological fluids, such as serum, plasma, saliva, urine, milk, and humor vitreous. Although their role is not completely understood, they are likely involved in cell-to-cell communication. In fact, after release by parental cells, circulating miRNAs can translocate into recipient cells in which they regulate gene expression.

miRNAs are very stable in biological fluids as they are either enclosed in microparticles or assembled into complexes that protect them from endogenous RNases. Cells can actively secrete miRNAs packed into exosomes, which are small vesicles released by cells through a tightly regulated active process [106]. Alternatively, miRNAs can be passively released in either apoptotic bodies or microvesicles (MV) by cells exposed to insults [107, 108].

miRNAs are increasingly recognized as a promising biomarker, given the ease with which they can be isolated and their structural stability under different conditions of sample processing and isolation. Data showing that circulating miRNA profiling can be disease-specific representing a molecular signature of the disease support the hypothesis that miRNAs can be valuable clinical biomarkers [109].

Several studies have investigated whether changes of miRNA levels in body fluids are associated with DN and DR, while no study on circulating miRNAs in DSPN is yet available despite evidence of alterations of intracellular miRNAs in DSPN. Clinical miRNA studies differ substantially in study design, patient sample size, type of diabetes, and body fluid analyzed (Table 5). Most studies performed miRNA profiling and validated highly differentially expressed miRNAs, while studies on candidate miRNAs focused on miRNAs known to be altered in relevant tissues.

5.1. Circulating MicroRNA and DMC. Based upon profiling results, Wang et al. validated 13 miRNAs and confirmed enhanced serum levels of miR-661, miR-571, miR-770-5p, miR-892b, and miR-1303 in subjects with type 2 diabetes and DMC. Among them, miR-1303, a miRNA involved in autophagy, is of particular relevance as high miR-1303 levels conferred an over threefold increased risk of DMC independently of body mass index and blood pressure [110]. Sebastiani et al. identified miR-31 as an upregulated miRNA in patients with DMC, and this miRNA is likely involved in angiogenesis and vascular permeability based on its predicted target genes (E-selectin, integrin-α5, and nitric oxide synthase 1) [111].

5.2. Circulating MicroRNAs and Diabetic Retinopathy. Three miRNA profiling studies have been performed on serum samples from patients with and without DR, allowing the identification of potential biomarkers of DR.

In a nested case-control study on two prospective cohorts of patients with type 1 diabetes from the DIRECT-1 trial (PROTECT-1 and PREVENT-1), Zampetaki et al. profiled 29 miRNAs and validated relevant miRNAs. They found that two miRNAs, miR-27b and miR-320a, were associated with the incidence and the progression of DR. Furthermore, proteomic analysis performed in endothelial cells showed that the antiangiogenic protein thrombospondin-1 was a target of both [112].

We have recently performed a miRNA profiling in pooled serum samples from type 1 diabetic patients with and without diabetic complications from the nested case-control study of the EURODIAB PCS. Among the 25 differentially expressed miRNAs, miR-126 was validated. miR-126 levels were lower in cases than in controls and inversely associated with all complications as well as with each complication examined separately. After adjustment for age, sex, A1C, and diabetes duration, a 25% risk reduction was still observed for PDR [113]. A significant decrease in serum miR-126 levels was also reported by another study performed in patients with type 2 diabetes and PDR [114]. Therefore, several studies on both intra- and extracellular miR-126 indicate a key role of this miRNA in PDR.

Qing et al. performed a differential miRNA profiling of serum samples from subjects with DR and identified 3 miRNAs (miR-21, miR-181c, and miR-1179) significantly

Table 5: Circulating microRNAs in diabetic microvascular complications.

miRNA	Type of DM	Study design	Study population	Profiling	Source	Significant comparisons-details	Reference
Diabetes microvascular complications							
↑ 661 ↑ 571 ↑ 770-5p ↑ 892b ↑ 1303	DM2	Case Control	DMC+ ($n = 92$) DMC− ($n = 92$) Controls ($n = 92$)	Yes	Serum	DM versus controls DMC+ versus DMC−	[110]
↑ 31	DM2	Case Control	DMC ($n = 12$) DM-CVD ($n = 12$) DM ($n = 12$)	Yes	Serum	DMC versus others	[111]
Diabetic retinopathy							
↓ 27b ↑ 320a	DM1	Nested Case Control	Incidence of DR ($n = 62$) Progression of DR ($n = 93$) Controls ($n = 145$)	Yes	Serum	27b: OR: incidence of DR 0.57 (0.40, 0.82); 320: OR: incidence of DR 1.57 (1.07, 2.31), OR: progression of DR 1.43 (1.05, 1.94)	[112]
↓ 126	DM1	Nested Case Control	Complications+ ($n = 312$) Complications− ($n = 143$)	Yes	Serum	OR: PDR 0.75 (0.59–0.95)	[113]
↑ 21 ↑ 181c ↑ 1179	DM2	Case Control	PDR ($n = 90$) NPDR ($n = 90$) Controls ($n = 20$)	Yes	Serum	PDR versus NPDR	[115]
↓ 126	DM2	Case Control	PDR ($n = 39$) NPDR ($n = 42$) DR− ($n = 44$) Controls ($n = 59$)	No	Serum	PDR versus DR−	[114]
↑ 93	DM2	Case Control	DR+ ($n = 75$) DR− ($n = 65$) Controls ($n = 127$)	No	Plasma	DR+ versus DR−	[117]
↑ 21	DM2	Case Control	PDR ($n = 51$) NPDR ($n = 73$) DR− ($n = 65$) Controls ($n = 115$)	No	Plasma	PDR and NPDR versus DR−	[116]
↑ 23a ↑ 320a-b	DM2	Case Control	PDR ($n = 4$) ME ($n = 4$)	Yes	Vitreous/ serum	PDR versus ME	[138]
Diabetic nephropathy							
↓ let-7c ↓ 29a ↑ let-7b ↑ 21	DM1	Prospective: rapid progression to ESRF	Macro RP ($n = 38$) Macro NP ($n = 38$) Normo ($n = 40$)	No	Plasma	let-7c: OR: 0.23 (0.10, 0.53) 29a: OR: 0.39 (0.20, 0.76) let-7b: OR: 2.38 (1.31, 4.0) 21: OR: 5.87 (1.68, 20.46)	[118]

Table 5: Continued.

miRNA	Type of DM	Study design	Study population	Profiling	Source	Significant comparisons-details	Reference
↑ 217	DM2	Case Control	Normo ($n = 186$) Micro ($n = 169$) Macro ($n = 140$) Controls ($n = 195$)	No	Serum	DM2 versus controls Micro versus normo Macro versus micro	[121]
↑ 21 ↑ 29a ↑ 192	DM2	Case Control	Macro ($n = 21$) Micro ($n = 17$) Normo ($n = 12$)	No	Serum	Macro versus micro and normo CKD+ ($n = 18$) versus CKD− ($n = 32$)	[120]
↓ 130	DM2	Case Control	Normo ($n = 137$) Micro ($n = 122$) Macro ($n = 68$) Controls ($n = 131$)	No	Serum	Macro versus micro and normo Micro versus normo Controls versus others	[123]
↓ let-7a	DM2	Case Control	DN− ($n = 104$) DN+ ($n = 108$) Controls ($n = 62$)	Yes	Blood	DN+ versus DN−	[119]
↓ 126	DM2	Case Control	Normo ($n = 52$) DM2-micro ($n = 29$) DM2-macro ($n = 21$) Controls ($n = 50$)	No	Blood	Micro-macro versus controls and normo Macro versus micro	[122]
Urinary miRNA in diabetic nephropathy							
↓ 15	DM2	Case Control	CKD-IgAN ($n = 17$) CKD-DN ($n = 17$) CKD-HTN ($n = 22$)	Yes	Urinary sediment	DN versus others CKD	[124]
↓ 192	DM2	Case Control	DN ($n = 20$) MCN/FGS ($n = 21$) MGN ($n = 23$) Controls ($n = 10$)	No	Urinary sediment	DN versus others	[125]
↓ 2861 ↓ 1915-3p ↓ 4532	DM2	Case Control	DN+ ($n = 74$) DN− ($n = 71$)	Yes	Urine	DN+ versus DN−	[126]
↓ 323b-5p ↑ 122-5p ↑ 429	DM1	Case Control	Normo ($n = 10$) Intermittent micro ($n = 10$) Persistent micro ($n = 10$) Macro ($n = 10$)	Yes	Urine	Persistent versus intermittent micro	[127]
105, 1972, 28, 30b, 363, 424, 486, 495, 548o	DM1	Prospective: micro onset	Normo ($n = 27$)	Yes	Urine	9 miRNAs: molecular miRNA signature for microalbuminuria	[128]
29a	DM2	Case Control	Macro/micro ($n = 42$) Normo ($n = 41$)	No	Urine	Macro/micro versus normo	[139]
↑ 130a ↑ 145	DM1	Case Control	Micro ($n = 12$) Normo ($n = 12$)	Yes	Urinary exosomes	Micro versus normo	[129]

Table 5: Continued.

miRNA	Type of DM	Study design	Study population	Profiling	Source	Significant comparisons-details	Reference
↓ 155 ↓ 424 ↑ 320c ↑ 6068	DM2	Case Control	DN+ ($n = 8$) DN− ($n = 8$) Controls ($n = 8$)	Yes	Urinary exosomes	DN+ versus DN− and controls	[130]
↑ 133b ↑ 342 ↑ 30a	DM2	Case Control	Macro ($n = 44$) Micro ($n = 66$) Normo ($n = 56$) Controls ($n = 54$)	No	Urinary exosomes	Macro and micro versus normo (133b only macro versus micro)	[131]
↑ 192 ↑ 194 ↑ 215	DM2	Case Control	Normo ($n = 30$) Micro ($n = 30$) Macro ($n = 20$) Controls ($n = 10$)	No	Urinary MV	Micro versus normo, controls, and macro	[132]

DM1: type 1 diabetes; DM2: type 2 diabetes; ESRF: end-stage renal failure; OR: odds ratio; DMC: diabetic microvascular complications; CVD: cardiovascular disease; DR: diabetic retinopathy; PDR: proliferative diabetic retinopathy; NPDR: nonproliferative diabetic retinopathy; Micro: microalbuminuria; Normo: normoalbuminuria; Macro: macroalbuminuria; DN: diabetic nephropathy; CKD: chronic kidney disease; RP: rapid progressors; NP: nonprogressors; IgAN: IgA nephropathy; HTN: hypertensive nephrosclerosis; MCN: minimal change nephropathy; FGS: focal glomerulosclerosis; MGN: membranous glomerulonephropathy; MV: microvesicles; ME: macular edema.

increased in patients with PDR [115]. A rise in plasma miR-21 levels in patients with type 2 diabetes and DR has also been confirmed by another study [116], and these data are in line with studies showing miR-21 upregulation in experimental DR. Similarly, plasma miR-93 levels were greater in patients with type 2 diabetes and DR compared to those in patients without DR [117].

5.3. Circulating MicroRNAs and Diabetic Nephropathy. Pezzolesi et al. performed a prospective study on circulating miRNAs in DN. Specifically, they assessed if the circulating levels of 5 miRNAs, which are under the control of TGF-β, predicted the development of ESRD during follow-up in patients with type 1 diabetes and proteinuria but normal renal function at baseline. They found that let-7c-5p and miR-29a-3p were associated with an over 50% reduction of the risk of rapid progression to ESRD, while let-7b-5p and miR-21-5p were associated with a 2.5-fold increase in ESRD risk independently of HbA1c and other confounders [118]. This finding is of particular relevance given the lack of clinical biomarkers to predict ESRD and supports the hypothesis that measurement of miRNA circulating levels may be of relevance in clinical practice to identify subset of patients at high risk.

Recently, Zhou et al. performed a miRNA microarray assay in patients with type 2 diabetes and found that let-7a was differentially expressed in patients with DN as also confirmed by PCR. In addition, they discovered that the rs1143770 variant of the let-7a-2 gene was associated with an increased risk for DN [119].

Other studies focused on selected miRNAs and reported significant changes in the circulating levels of miR-217, 21, 29a, 192, 130, and 126 in type 2 diabetes patients with and without albuminuria [120–123]. However, given the cross-sectional design of these studies, it is unknown whether these miRNAs can help predicting either the development or the progression of DN. Moreover, levels of miRNAs were often correlated with HbA1c, and it is thus unclear whether these changes in miRNA levels were specific of DN or simply mirrored worse metabolic control in patients with albuminuria.

5.4. Urinary miRNAs in DN. Urine is another body fluid that has been used to identify miRNA biomarkers in DN. Two studies on the urinary sediment from patients with chronic kidney diseases showed that miR-15 and miR-192 levels were lower in patients with DN than those in subjects with other kidney diseases [124, 125], suggesting that circulating miRNA may be of practical value in the differential diagnosis of renal diseases.

Other groups have measured free miRNAs into urine. A recent study found that urinary miR-2861, miR-1915-3p, and miR-4532 levels were enhanced in patients with DN and correlated with both renal function and tubulointerstitial injury [126]. Argyropoulos et al. performed a miRNA profiling in patients with type 1 diabetes and worsening stages of albuminuria and found 27 differentially expressed urinary miRNAs [127]. Importantly, the same group also carried out a prospective study assessing the expression of 723 urinary miRNAs in patients with type 1 diabetes who were normoalbuminuric at baseline. Eighteen miRNAs were found associated with microalbuminuria development, and 9 of them were used to define a miRNA signature for microalbuminuria [128].

More recently, several studies have measured/profiled miRNAs enclosed in urinary exosomes/MVs. In type 1 diabetes, we reported that urinary exosomes from patients with microalbuminuria were enriched in miR-130a and miR-145, while their content in miR-155 and miR-424 was reduced. Of interest, exosomes either released by MCs exposed to high glucose or isolated from the urine of animals with DN were also enriched in miR-145 [129]. Other studies on urinary exosomes/MVs were performed in patients with type 2 diabetes. Delić et al. identified 16 differentially expressed miRNAs in urinary exosomes from patients with DN and confirmed that exosomal miR-320 and miR-6068 content was greater in patients with DN [130]. Another study showed increased levels of miR-133b, miR-342, and miR-30a in patients with DN [131]. Finally, a study performed on urinary MVs from patients with type 2 diabetes and different degrees of albuminuria revealed that miR-192, miR-194, and miR-215 levels were specifically increased in patients with microalbuminuria and that there was a correlation between miR-192 and TGF-β1 levels as expected based on their close relationship in renal tissue [132].

6. Future Perspective and Limits of This Line of Research

In the last decade, much progress has been made in our understanding of miRNA role in the pathogenesis of DMC. Today, we know that several miRNAs are deregulated and play a major pathogenic role in DMC. Moreover, vascular complications of diabetes share, at least in part, insults and underlying pathogenic mechanisms, and some miRNAs have been implicated in multiple diabetes complications (Figure 3). The best example is miR-146 that is involved in all DMC and also in the pathogenesis of atherosclerosis [133]. Another miRNA that has attracted much attention in multiple areas of diabetes research is miR-126. Besides playing a role as both mediator and marker of PDR, this miRNA is also a biomarker of the risk of developing type 2 diabetes [134, 135] and has been implicated in the pathogenesis of CVD [136]. These overlaps may be advantageous as therapies targeting single miRNAs may have beneficial effects on other vascular beds.

In other fields, such as oncology, research on miRNAs has already moved from gaining a better knowledge of miRNA involvement in pathogenic mechanisms to the use of miRNAs as targets for intervention. Gene therapy designed to modulate miRNA expression can be applied to either increase or decrease miRNA levels in order to obtain desirable clinical outcomes. However, delivery to relevant tissues/organs is still a major issue. Chemically modified oligonucleotides, sponges, MV, viruses, and gold nanoparticles have been tested for both effectiveness and specificity of delivery; however, many problems still need to be addressed. Besides delivery, treatments targeting single miRNAs can affect many genes, as a single miRNA controls the expression

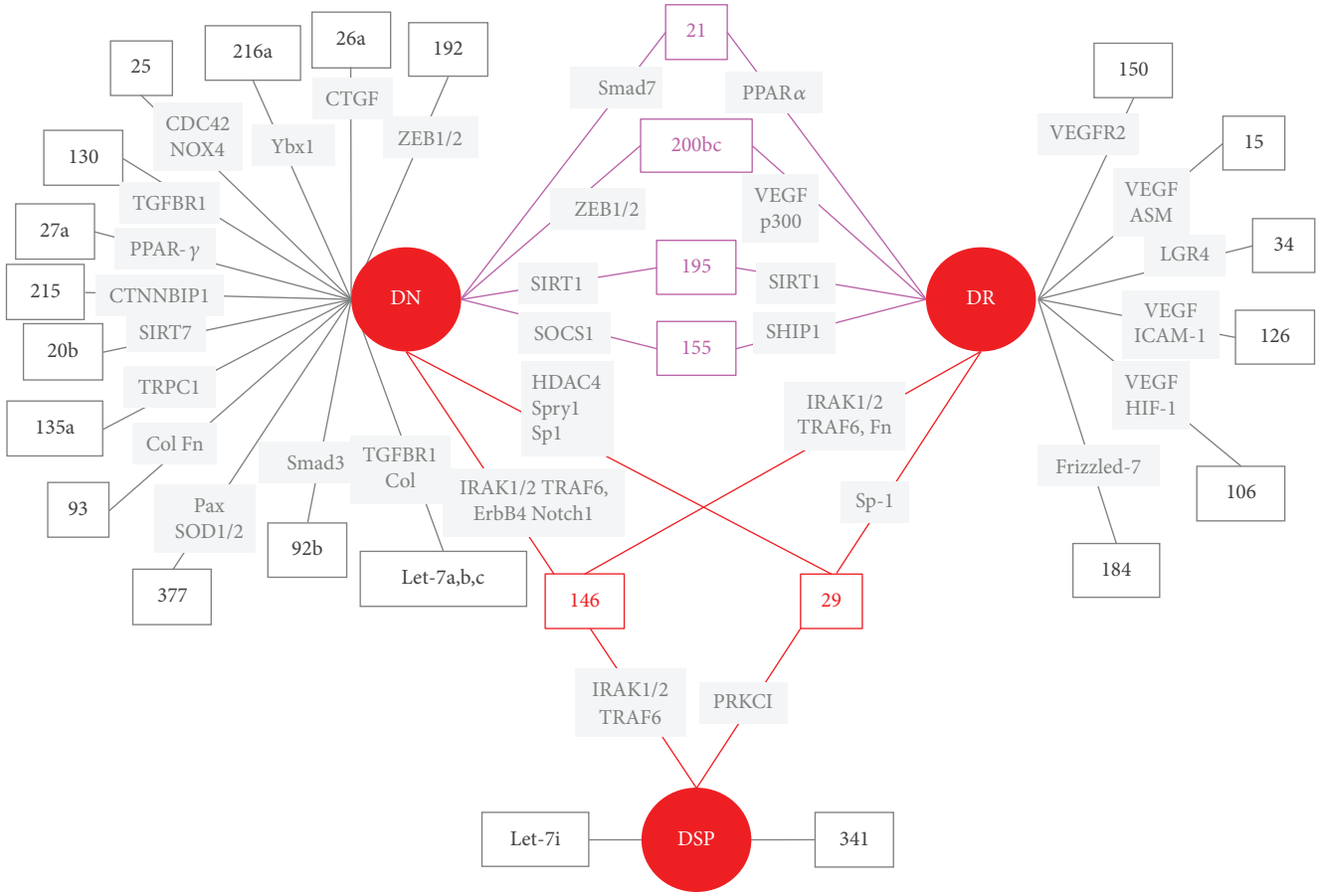

Figure 3: MicroRNA involved in diabetes microvascular complications. miRNAs and miRNA targets (in italics) abnormally expressed in diabetic nephropathy (DN), diabetic retinopathy (DR), and diabetic neuropathy (DSP) are shown. Pink lines connect miRNAs involved in both DN and DR. Red lines connect miRNAs involved in all diabetes microvascular complications.

of multiple mRNAs, possibly causing undesired off-target effects, and this is an important pitfall of potential new therapies manipulating miRNAs.

Available data on circulating miRNAs in DMC are still scarce, but they are likely to increase in the near future as the number of studies assessing miRNAs as biomarkers is growing exponentially. However, there are several limitations in this area of research. First, reproducibility of results in other series of patients and using different methodologies is very poor and this underscores the importance of a coordinate effort to standardize both collection and analyses of biological samples for miRNA biomarker discovery and to use appropriate and homogenous endogenous controls. Second, circulating miRNAs derived from damaged/necrotic cells are a possible confounder and measurement of miRNAs enclosed in exosomes/MV may be a preferable option. However, the procedure of exosome/vesicle isolation is both time-consuming and expensive, making miRNAs less attractive as biomarkers. Third, despite the growing number of studies measuring miRNAs in body fluids, there is relatively little knowledge on how environmental variables, including drug therapies, affect circulating miRNA levels in normal subjects, and this can significantly hamper our understanding of the significance of miRNA changes in pathological conditions. Therefore, studies covering this gap in knowledge are desperately needed. Fourth, prospective studies in longitudinal cohorts are required to evaluate whether potential novel biomarkers of DMC have a predictive value, and their addition to currently available clinical markers and scores improves identification of subgroup of patients at high risk of DMC development/progression.

Most of the research on miRNA biomarkers in DMC has been hypothesis driven and non-hypothesis-driven research is still in its infancy in the field of diabetes. However, both improved availability of biobanks that store samples from clinical cohorts, and increasing experience with the use of both omic technologies and biostatistics is likely to change this scenario in the next future. "Molecular signatures," obtained using an open omic approach, are likely to outperform research based on individual biomarkers as single biomarkers can hardly reflect the biological complexity of the underlying microvascular injury.

7. Conclusions

Lately, our understanding of the importance of miRNAs in the pathogenesis of DMC has grown substantially, and intervention studies in experimental animals indicate that treatments targeting miRNAs can be beneficial. Moreover, there is increasing evidence for a potential role of miRNAs as

clinical biomarkers of DMC. Discovery of new set of miRNA biomarkers might help to guide diagnostic and therapeutic decisions and to facilitate the implementation of personalized medicine into the clinical setting. However, novel miRNA biomarkers must be rigorously validated in adequately powered, prospective, independent clinical studies prior to implementation in clinical practice.

Conflicts of Interest

F. Barutta, S. Bellini, R. Mastrocola, G. Bruno, and G. Gruden declare that they have no conflict of interest.

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