microRNAs with different functions and roles in disease development and as potential biomarkers of diabetes: progress and challenges

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microRNAs with different functions and roles in disease development and as potential biomarkers of diabetes: progress and challenges

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ABSTRACT

Biomarkers inform us on early detection of diseases, in determining individuals at risk of developing complications or subtyping individuals for disease phenotypes. In addition, biomarkers may lead to better treatment strategies, personalize therapy, and improve outcome. A major gap in the field of biomarker development is that we have not identified appropriate (minimally invasive, life-style independent and informative) biomarkers for the underlying disease process(es) that can be measured in readily accessible samples (*e.g.* serum, plasma, blood, urine). miRNAs function as regulators in wide ranging cellular and physiological functions and also participate in many physiopathological processes and thus have been linked to many diseases including diabetes, metabolic and cardiovascular diseases, cancer, neurodegenerative diseases, and autoimmunity. Many miRNAs have been shown to have predictive value as potential biomarkers in a variety of diseases including diabetes, which are detectable in some instances many years before the manifestation of disease. Although some technical challenges still remain, due to their availability in the circulation, relative stability, and ease of detection; miRNAs have emerged as a promising new class of biomarkers to inform us on early detection of disease, monitoring disease progression, in determining individual's risk of developing complications or subtyping individuals for disease phenotypes, and to monitor response to therapeutic interventions. As a final note, most of the miRNAs reported in the literature have not yet been validated in sufficiently powered and longitudinal studies for specificity for that particular disease.

INTRODUCTION

Monitoring molecular and physiological changes associated with disease initiation; progression and onset, before, during, and after the therapeutic intervention, is essential for the early diagnosis and prediction of complex diseases and could be used to improve drug development [1]. Biomarkers are useful because they inform us on early detection of diseases, in determining individuals at risk of developing complications or subtyping individuals for disease phenotypes. In addition, biomarkers may lead to better treatment strategies, personalize therapy, and improve outcome. Biomarkers which can be objectively measured, experimentally validated, and clinically relevant to disease, can be used to predict molecular and phenotypical changes before they occur, to assess the efficacy and safety of drugs, and to stratify patients.

Biomarkers are useful molecular markers for disease detection and monitoring and may be treated as intermediate phenotypes as disease. They indicate the physiological status of a cell at a given time and vary during the disease development. Genetic mutations, epigenetic modifications, alterations in gene transcription and translation, and their protein products or metabolites, can all serve as molecular biomarkers for disease. A major gap in this field is that we have not identified appropriate biomarkers (minimally invasive, life-style independent and informative biomarkers) for the underlying disease process(es) that can be measured in readily accessible samples (*e.g.* blood).

Despite the advances, out of 150,000 biomarkers that has appeared in literature thus far, approximately only 100 have made it to the clinic [2]. This is because they either could not be validated or cannot be linked to a disease. One of the main challenges in biomarker development is the identification of robust molecular markers that can be measured in

readily accessible samples (*e.g.* blood) that are specific to diseased organ or tissue. Biomarkers should correlate with disease onset or pathogenic process, differentiate pathologies, be proportional to degree of severity of pathology, and readily released upon development of pathology. Additionally, biomarkers should be sensitive, specific, rapidly and readily detected, be predictive, should have long half-life, be robust and reproducible, with a high degree of cross-species selectivity, in a non-invasive fashion, in various biologic specimens, and should have clinical utility.

The development of protein-based biomarkers is challenging because of the complexity of protein composition in blood, the diversity of post-translational modifications and low abundance of many proteins, and the difficulties in developing highly sensitive and selective assays due to cross-reactivity of antibodies used in assays. Additionally, the scarcity of high-quality monoclonal antibodies as well as difficulty to identify low-abundance proteins afflicts the field. There are an estimated 10,000 unique proteins found in serum with concentrations spanning a dynamic range of greater than 10 orders of magnitude. Similar challenges afflict the development of other molecular biomarkers. Therefore, there is a need to develop alternative strategies to develop robust circulating biomarkers that can be validated and correlated with disease.

High-resolution fluorescence imaging techniques have been used to gain insights into dynamic changes of beta cell mass and function noninvasively *in vivo* by imaging techniques such as: magnetic resonance imaging (MRI) of magnetic nanoparticles, positron emission tomography (PET), and single-photon emission computed tomography. However, due to the small size of islets (~50–400 μm in diameter, current noninvasive *in vivo* imaging methods including PET, does not have the spatial power to resolve anomalies

in islets. Both the low spatial resolution of PET and islet/pancreas atrophy in diabetes are current challenges in this field. In addition, imaging methods, although proven valuable in numerous diseases, and all of these methods may help in diagnosis and evaluation of the protective effects of therapeutic drugs on functional beta cell mass. However, most of these methods can only detect changes after the onset of disease manifestation and cannot be used in early prediction of disease such as diabetes. Therefore, there is an unmet medical need to develop non-invasive life-style independent predictive biomarkers to predict diabetes before the onset of disease to enable physicians to stratify individuals at risk of developing diabetes.

MicroRNAs (miRNAs) as a new class of biomarkers

miRNAs are evolutionarily conserved across different species, endogenous, noncoding RNAs representing approximately 1–2% of the known genes in eukaryotes [3] which constitute a layer of epigenetic regulation of a broad spectrum of developmental, physiological, and pathological processes; sometimes entire pathways [4], and are abundant in many cell types [5]. Over 1000 human miRNAs have been identified [6,7] which target about 60% of mammalian genes [8,9] and the number of targets is likely to increase when considering unannotated transcripts [10]. Because of this, these molecules may play a powerful regulatory role with the potential to intercept a wide network of fundamental cellular processes.

Altered expression levels of miRNAs have been shown to be indicative of underlying pathophysiologic alterations, and often associates with a variety of conditions ranging from malignancies including cancer, cardiovascular, infectious, metabolic, neurodegenerative, fibrosis, and autoimmunity—to pregnancy and aging. Therefore,

profiling miRNA content in the circulation may reflect the activation state of circulating cells or tissue injury in response to disease states.

miRNAs have properties that are ideal for biomarkers, including miRNA expression levels closely correlated with the physiological or pathological state of an organism. miRNAs are relatively stable *in vivo* and *in vitro* and less susceptible to nuclease degradation compared to mRNAs. This is in part due to both their small size and the miRNA–protein complexes they form or they are protected because they are in lipid vesicles (*e.g.* exosomes, microvesicles (MVs), or apoptotic bodies). miRNAs can be detected in various specimen fluids including urine, saliva, and tissue samples and other specimens like formalin-fixed, paraffin-embedded (FFPE). Due to their smaller size, miRNAs cross link with formalin to a lesser extent than mRNAs where formalin chemically modifies nucleic acids rendering their detection difficult. Similarly, miRNAs have less sensitivity to differences in sample handling and processing, and they are less complex and less numerous than mRNAs or proteins. Contrary to challenges in the detection and monitoring of rare protein biomarkers in blood which are often modified post-translationally affecting the accuracy of measurement, miRNAs are relatively easy to detect, more sensitive, and specific to certain tissue types (*e.g.* liver specific miR-122 and heart specific miR-499). Thus, profiling miRNAs in circulation enables monitoring the health status of specific organs and can uncover the underlying physiological state of organism leading to early diagnosis of disease. Furthermore, recent evidence suggest that circulating miRNAs may also contribute to inter-cellular signaling and offer new insights into pathological mechanisms [11].

Role of miRNAs in human diseases

Several recent reports discuss circulating miRNAs and their association with various human diseases including cancer, and autoimmune diseases. A database (www.miR2Disease.org) providing a comprehensive resource of a panel of experimentally validated miRNAs (349) involved in 163 human diseases is available [12]. Among those, the circulating miRNA are particularly informative since they are highly stable and detectable in the serum. With an estimated 100 different miRNAs present in plasma, profiling these circulating miRNAs may provide a comprehensive analysis of pathologies in multiple organs [11].

Because close correlation of expression levels of miRNAs in serum and diseased tissues have already been shown for various cancers [13-15], the levels of miRNAs measured in formalin and frozen tissue samples are comparable; existing formalin samples with their associated clinical information can be a *bona fide* resource for rapid discovery of disease associated biomarkers. Likewise, increased levels of miR-122, miR-133a and miR-124 correlate to tissue injuries in the liver, muscle and brain, respectively suggesting their utility as diagnostic biomarkers [16].

Several miRNAs have already been identified as potential biomarkers for cancers acting as oncogenes, tumor suppressors or modulators of metastasis [17,18], or as early-stage breast cancer diagnostics [19]. miRNA profiling of small-cell lung cancer tumors has shown miR-92a-2*, miR-147, and miR-574-5p are significantly associated with chemoresistance. Higher expression of miR-92a-2* in tumors is associated with decreased survival suggesting that these miRNAs can be used as biomarkers in screening patients at risk for chemoresistance [20]. Similarly, a recent study has shown the utility of circulating tumor

specific miRNAs (*e.g.* miR-10b, miR-34a, miR-141, and miR-155) in sera of breast cancer patients as diagnostic biomarkers [21]. Additionally, Liu and colleagues [22] have identified four miRNAs (-486, -30d, -1, -499) in sera of patients with non-small cell lung cancer (NSCLC). Furthermore, circulating miR-21, miR-155, and miR-210 have been shown to be elevated in diffuse large B-cell lymphoma [23].

Several other circulating miRNAs have also been identified as promising biomarkers in other diseases [24,25]. Additionally, miRNAs as circulating biomarkers in cardiac disease has been discussed by Gerald Dorn [26]. A recent study has shown a striking increase in miRNAs-208b and -499 after myocardial infarction (MI) and nearly undetectable in healthy controls [27,28]. Likewise, increased expression of miR-1 in serum has been shown to be associated with acute myocardial infarction (AMI) patients and positively correlated to serum creatine kinase-MB (CK-MB); and the levels decreased significantly after treatment and positively correlated to serum CK-MB [17,29]. Similarly, heart specific miR-208 levels increased significantly after isoproterenol-induced myocardial injury, suggesting its utility as biomarkers [30].

A variety of physiological and pathological conditions including obesity, stress, and pregnancy can affect insulin sensitivity of peripheral tissues such as liver, adipose tissue, and skeletal muscle; causing insulin resistance. Although exact molecular mechanisms leading to insulin resistance still are not known, many molecular factors including a potential involvement of miRNAs in metabolically involved tissues (**Table 1, 2**) in the context of diabetes (**Table 3**) has been proposed.

An emerging role of serum miRNAs in the pathogenesis of diabetes and the possible use of miRNAs as blood biomarkers to monitor diabetes development and as tools

for gene-based therapy to treat both type 1 and type 2 diabetes mellitus has been reported [31]. For example, plasma miRNA profiling has revealed loss of endothelial miR-126, miR-15a, miR-29b, and miR-223; elevated levels of miR-28-3p, and a reduction of miR-126, miR-15a, and miR-223 was already detectable years before the manifestation of disease [11,32].

Insulin, a conventional treatment for type 2 diabetes (T2D) was found to down-regulate the expression of miRNAs, particularly miR-1 and miR-133a, indicating their implication in the transcriptional action of insulin in diabetes patients [33].

In another study, serum miR-146a and miR-223 have been linked to sepsis [34], whereas, the expression of miR-150 correlated with the aggressiveness of sepsis suggested that miR-150 may serve as a prognostic biomarker in patients with sepsis [35].

Similarly, the expression of ~10% of miRNAs has been shown to be altered in idiopathic pulmonary fibrosis (IPF) including downregulation of miR-17 plus the Let-7, miR-29, and miR-30 families; and upregulation of miR-155, miR-21 and many others [36,37]. Additionally, identification of a number of mutations in precursor or mature miRNAs and decreased miR-132 have been linked to schizophrenia [38,39], suggesting that these mutations may be contributing to disease and be used for diagnosis. Similarly, altered expression of several miRNAs including miR-342-3p has been linked to late-stage prion disease [40-42].

miRNAs and drug response

Recent data suggest that miRNAs can play a critical role in regulating drug responses [24] through regulating key drug-metabolizing genes (*e.g.* the cytochrome P450 family) [43]. For example, the loss of miR-519c function caused by 3'UTR truncation resulted in drug-resistant cells which maintains high expression of ABCG2 (ATP-binding cassette, sub-

family G, member 2) [44], a transporter involved in the absorption, distribution, and excretion of drugs and cytotoxins [45]. Similarly, miR-1 has been shown to be suppressed by the beta-blocker propranolol in a rat model of MI, suggesting a new mechanism for ischaemic cardioprotection [46]. Likewise, miR-221 and miR-34a have been shown to be involved in the response to lithium treatment used for bipolar disorder patients [47].

miRNAs of the Let-7 family have been shown to induce radio-sensitivity [48] and inhibition of miR-21, and miR-200b increased sensitivity to gemcitabine in cholangiocarcinoma [48] or restoration of miR-34 activity in p53-deficient human gastric cancer which led to chemosensitization. Likewise, miRNA-210 expression is stimulated by hypoxic conditions and vascular endothelial growth factor (VEGF) induces expression of miR-296 implying that these miRNAs can be used as biomarkers for photodynamic therapy [49].

miRNAs as diagnostics

Because miRNAs play complex roles between genes and proteins, and contribute to the pathologic and physiologic processes in disease, some of the miRNAs are associated with distinct clinical *characteristics*, (*e.g.* diagnosis or disease activity) [13,50]. As mentioned earlier, a comprehensive list of 349 experimentally validated miRNAs in 163 diseases is available in the miR2Disease database (http://www.mir2disease.org/). For example, as diagnostics, miR-132 has been identified as a possible biomarker of joint inflammation, being expressed at considerably higher levels in the plasma of patients with rheumatoid arthritis (RA) and osteoarthritis (OA) than in healthy controls; and levels of miR-16, miR-146a, miR-155 and miR-223 were higher in synovial fluid of patients with RA than those with OA, suggesting their utility as disease-specific markers.

Recently, the first commercial miRNA diagnostic assays in oncology have become available [13,51]. Due to the selectivity of miRNAs, these assays can differentiate between malignant pleural mesotheliomas, peripheral adenocarcinoma of the lung, and metastatic carcinomas involving the lung and pleura, or to sub-classify non-small-cell lung carcinomas into squamous and nonsquamous histology subtypes.

For example, Prometheus Laboratories Inc. (Los Angeles, CA, USA) launched three new cancer diagnostic tests (ProOnc TumorSource Dx, ProOnc Squamous Dx, and ProOnc Mesothelioma Dx). ProOnc TumorSource Dx identifies the tissue-of-origin of a metastatic tumor (25 different tumor types, including colon, liver, brain, breast, kidney, lung, ovary, pancreas, prostate, and testis), and measures the expression level of 48 miRNA biomarkers. ProOnc TumorSource Dx uses a classifier to assign a primary site to the cancer sample based on the miRNA expression in the tumor and may assist in the detection of cancer of unknown primary (CUP). ProOnc Squamous Dx classifies non-small cell lung carcinoma tumors into cancers of squamous histology and non-squamous cancers by measuring the differential expression level of a squamous miRNA biomarker to differentiate patients that have squamous cell carcinoma of the lung from patients that have non-squamous non-small cell lung cancer. ProOnc Mesothelioma Dx uses miRNA to differentiate malignant pleural mesothelioma from peripheral adenocarcinoma of the lung and metastatic carcinomas involving the lung and pleura.

Rosetta Genomics (Rehovot, Israel) has three different tests to identify specific miRNA signatures that can identify the primary tumor site in metastatic cancer and cancer of unknown primary.

Asuragen (Austin, TX, USA) has a diagnostic miRNA test (miRInform^{R}) for pancreatic ductal adenocarcinoma (PDAC). The test measures expression levels of seven miRNAs comprised of miR-130b, -135b, -148a, -196a, -375, -96 and -24, in Fine Needle Aspirate (FNA) specimens of pancreatic masses and calculates a score between 0 and 1 using an algorithm resulting in a differential diagnosis between PDAC and benign pancreatic diseases (www.asuragen.com/Corporate/scientific literature.aspx#S7) [52].

Are circulating miRNAs cause or effect?

As discussed by Farr *et al.* [53], there is an ongoing debate whether circulating miRNAs is (1) an indicator of disease status, (2) are simply the products of an ongoing pathology due to leakage from damaged cells during tissue injury or tumorigenesis [54,55], or (3) whether the miRNAs play a role in disease pathogenesis as effectors through horizontal transfer [56].

A number of miRNAs have been shown to be taken up by other cell types *in vitro* and have shown to play functional roles in target cells [53,57-59]. Exosomes loaded with membrane components and cytoplasmic contents are secreted from a variety of cell types into circulation and play an important role in intercellular communication, often inducing physiological changes in recipient cells by transferring macromolecules including nucleic acids (*e.g.* miRNAs and mRNAs), bioactive lipids, proteins [60,61] and regulate gene expression of recipient cells [58,62]. Data also demonstrated that exosomes are released through a ceramide-dependent secretory machinery [58] and furthermore, exosomes can function as intercellular containers to convey their macromolecular cargo, in particular, miRNAs [62-64]. Recent data reported that (1) exosomal miRNAs were taken up by other cells and that apoptotic bodies delivered miR-126 into endothelial cells [65] and (2) exosomes from breast cancer cells transfer miRNAs to normal cells and stimulate them to become

cancerous [66]. Another study identified nine miRNAs, including the oncogenic miRNAs (*e.g.* miR-21, miR-27b, and miR-29a) in nanovesicles originated from media of lung cancer cell lines, but not from normal cells [67].

Tumor-derived miR-21 and miR-29a was shown to bind to toll-like receptors (TLRs), murine TLR7 and human TLR8, to stimulate protumoral inflammation that results in tumor growth and metastasis [67].

Melo *et al.* demonstrated that exosomes play a critical role in cell-independent miRNA biogenesis that affects cancer progression [66]. The authors demonstrated that exosomes originating from cancer cells, but not those originating from normal cells, harbor proteins involved in miRNA biogenesis (*e.g.* Dicer, TRBP, and AGO2 as well as the membrane protein CD43 [66]. CD43 membrane protein plays a role in accumulating Dicer in cancer exosomes. The authors also demonstrated that Dicer-containing cancer exosomes process precursor miRNAs into mature miRNAs over time. Furthermore, exosome-miRNAs suppressed the expression of their respective mRNA targets (*i.e.* PTEN, a tumor suppressor protein and the transcription factor HOXD10 in the recipient epithelial cells and induced target cells to become cancerous [66]. These cells formed tumors when they were injected into nude mice.

These findings suggest a possible temporal oncogenic field effect induced by cancer exosomes harboring a variety of macromolecules that transforms surrounding normal cells to tumorigenic cells. These findings could be harnessed to design exosome-based diagnostics and therapeutics.

However, it is yet to be determined how this horizontal miRNA transfer occurs and whether specific cells are targeted. It has been proposed that miRNAs contained within

exosomes or some other forms of microvesicles are taken up by recipient cells by endocytosis, phagocytosis, or direct fusion with the cellular membrane; but there is no evidence showing that the horizontal miRNA transfer occurs through a specific receptor-mediated pathway mediated by protein-complexed miRNAs [68].

Recent data suggest that miRNAs both contained within exosomes [58,69,70] or complexed with HDL [71] are taken up by target cells *in vitro* where conditions for horizontal gene (in this case miRNA) transfer is much simpler (*e.g.* one cell type) than the *in vivo* environment. A recent study by Iguchi *et al.* showed that miR-16 when injected directly into tumors have function [72]; however, the findings does not support the notion that circulating miRNAs can be taken up by recipient cells and show function in target cells.

Because levels of miRNAs have been shown to be altered before diabetes onset both in circulation [32] and in metabolically involved tissues (*i.e.* muscle, adipose tissue, liver, pancreas, kidney, heart and vascular tissue, **Table 1**) [73]; it is tempting to suggest that miRNAs may not be the cause of the disease (*e.g.* diabetes) but may correlate with disease state. While *in vitro* studies have suggested that there is an active and selective release of a particular miRNA from the cell [74], *in vivo* studies in mice and human suggest that miR-375 is specifically expressed in beta cells and increased plasma levels of miR-375 are associated with beta **cell** death [75-79]. Plasma levels of miR-375 predict hyperglycemia in mouse models of type 1 diabetes (T1D), and T2D humans [31,77,78,80].

miRNAs in obesity and the metabolic disease

It has been suggested that a number of physiological and pathological conditions including obesity, stress, and pregnancy can affect insulin sensitivity of peripheral tissues (*e.g.* liver, adipose tissue, and skeletal muscle); leading to insulin resistance. Many molecular factors including a potential involvement of miRNAs in metabolically involved tissues (**Table 1, 2**) in the context of diabetes (**Table 3**) has been proposed. Many miRNAs have been shown to have predictive value in diabetes (**Table 3**), which are detectable in some instances many years before the manifestation of disease [32].

A recent study has identified 373 miRNAs involved in fat cell development, enlarged fat depots, obesity in a mice model, and in 3T3-L1 cells [81]. Another study has profiled the expression of 155 miRNAs in both subcutaneous and visceral human fat depots of overweight and obese individuals [82]. Although 16 miRNAs showed fat depot specific expression pattern, no miRNA was found to be exclusively expressed in either fat depot, suggesting common developmental origin of both fat depot [82]. The study also showed significant correlations between the expression of miRNA-17-5p, -132, -99a, -134, 181a, -145, -197; and both adipose tissue morphology and key metabolic parameters, including visceral fat area, HbA(1c), fasting plasma glucose, and circulating leptin, adiponectin, and interleukin-6 [82].

Likewise, differentially expressed miRNAs were identified in visceral and subcutaneous fat and in the circulation of obese and non-obese individuals. Researchers showed that visceral and subcutaneous fat specific miRNAs (miR-17–5p and miR-132) differed significantly between obese and non-obese visceral fat, and their expression pattern was reflected in the circulation in blood from obese subjects [83]. In addition, miRNAs altered in adipose tissue and circulation of the obese correlated significantly with

body mass index (BMI), fasting blood glucose, and glycosylated hemoglobin, therefore highlighting their potential as novel biomarkers for this complex syndrome [83]. Ortega *et al.* [84] profiled the expression miRNAs during adipogenesis of human adipocytes and in subcutaneous adipose tissue samples from lean and obese, with and without T2D subjects, and identified 70 miRNAs which were modulated in fully differentiated human adipocytes. Fifty miRNAs were differentially expressed between either pre or mature adipocytes from lean and obese individuals and 11 miRNAs were deregulated in subcutaneous fat from obese subjects with and without T2D [84]. Additionally, 17 miRNAs were correlated with BMI and/or metabolic (fasting glucose and/or triglycerides) parameters [84]. However, only miR-34a was shown to be positively correlated with the rate of adipocyte differentiation and development of the BMI [84].

A variety of miRNAs have been implicated in adipocyte differentiation, proliferation or growth (**Fig. 3**). miR-143 has also been shown to regulate genes involved in adipocyte differentiation suggesting miRNAs' role in fat metabolism and endocrine function [85]. The adipose tissue specific miR-103; and pancreatic specific miR-103, miR-107, and let-7; have been shown to reduce insulin sensitivity, whereas adipose tissue specific miR-132, miR-17-5p, and miR-29a are involved in insulin resistance and glucose transport [2,82,83,86,87].

Likewise, miR-143 has been shown to regulate genes involved in adipocyte differentiation suggesting miRNAs role in fat metabolism and endocrine function [85].

The brain, central and peripheral nervous systems have been implicated as key regulators of appetite, body fat content, and glucose metabolism [88]; and brain specific miRNAs (miR-132, miR-195) have been involved in appetite regulation [86]. **Fig. 3** summarizes some of the

miRNAs involved in a variety of metabolic processes including adipocyte differentiation (miR-103, miR-143); smooth muscle cell proliferation (colon specific miR-145) and growth (miR-132, miR-17-5p, miR-29a, b), lipid metabolism, and cholesterol biosynthesis (liver specific miR-33, miR-370 and miR-122), glucose metabolism and insulin synthesis and secretion (pancreatic islet cell specific miR-34a that blocks beta cell apoptosis).

A subset of miRNAs (let-7b, miR-103, miR-143, miR-146b, miR-148, miR-155, and miR-221) found in exosomoes [89], let-7b, miR-143, miR-155, and miR-221 were found to play roles in cell proliferation, apoptosis, inflammation and angiogenesis in both adipose and vascular tissues [90].

It has been hypothesized that adipose and vascular tissues communicate via exosomal miRNAs; however, only a few exosomal miRNAs have been shown to be differentially expressed in and released from adipose tissue during the development of obesity [91].

miRNAs and lipid and cholesterol biosynthesis

A number of miRNAs with regulatory function in cholesterol metabolism, fatty acid and triglyceride biosynthesis, and homeostasis have been identified (**Table 1**) [92]. For example, liver specific miR-33 plays a role in cholesterol homeostasis and inhibits fatty acid oxidation, while miR-122 and miR-370 have been shown to reduce fatty acid oxidation while promoting fatty acid and cholesterol synthesis. Interestingly, while miR-122 plays a key role in cholesterol and lipid biosynthesis, it is also required for hepatitis C virus (HCV) replication [93]. It has been used as potential therapeutic target for the treatment of hypercholesterolemia and HCV using locked nucleic acid (LNA) in animal and human studies [93].

Challenges with the classical biomarkers to predict diabetes

As illustrated in **Fig. 1**, classic T2D biomarkers [*e.g.* fasting blood glucose levels or levels of glucose following an oral glucose test (OGTT)] or serum parameters (*e.g.* HbA1c, C-peptide, triacylglycerol, cholesterol, lipoproteins), physical characteristics (BMI, waist-to-hip ratio, blood pressure and sex), and lifestyle factors (food intake, smoking, sedentary life style) can predict disease progression but cannot predict the disease before it develops. Even after combining all these risk factors and biomarkers, the probability of predicting the development of diabetes is 0.85-0.9 in a period of 5-10 years before the onset of diabetes [94]. Other molecular markers including incretins (*e.g.* glucagon-like peptide 1, cytokines, adipokines, ferritin, and c-reactive protein) individually cannot predict the development of diabetes; however, in combination they have similar predictive value as the classic biomarkers [95]. Despite their value to predict development of T2D a few years in advance of onset of T2D, many of these biomarkers are not specific for diabetes and cannot be used to assess the disease susceptibility in general population. Similarly, genotyping efforts have yet to produce genotypic traits that exceed the predictive value of 0.6 [94]. Therefore, there is a need to develop predictive biomarkers that are life-style independent and are in circulation to identify individuals at risk of developing T2D, monitor disease progress, and assess the therapeutic interventions.

miRNAs as novel biomarkers of diabetes

As illustrated in **Fig. 2**, a number of miRNAs have been identified that are involved in metabolically involved tissues in the context of diabetes [2,86]. Several miRNAs have been implicated in the regulation of key metabolic, inflammatory, and antiangiogenic pathways in T2D, insulin biosynthesis and secretion and action, and may contribute to common

disease complications. They are also associated with the development of diabetic complications such as nephropathy and cardiac hypertrophy [1]. For example, several miRNAs including miR-375, miR-9, miR-192, and miR-143 are involved in insulin secretion, glucose stimulated insulin release, and diabetic nephropathy [76]; whereas miR-375 is involved in beta cell growth and insulin synthesis; and miR-34a and miR-146 are associated with beta cell apoptosis [31]. The pancreatic islet-specific miR-375, miR-9, miR-96, and miR-124a were found to negatively regulate insulin secretion, whereas pancreatic specific miR-24, miR-26, miR-182, and miR-148 were found to suppress insulin synthesis [2,86]. Elevated expression of miR-375 was shown to reduce insulin secretion via reduction of beta cell number and viability whereas suppression of this miRNA expression reversed this effect. Likewise, miR-9 has been identified as the regulator of insulin release [96]. Similarly, pancreatic beta cell specific miR-124a has been implicated in mice where they play a role in pancreatic development and differentiation of pancreatic beta cells [97]. In a separate study, Stoffel *et al.* and his team showed that miR-375 is involved in the maintenance of pancreatic alpha and beta cell mass, and turnover is required for normal glucose homeostasis, and adaptive expansion of beta cell in response to increasing insulin demand in insulin resistance in mice [77]. miR-375 knockout mice are hyperglycemic, exhibit increased total pancreatic alpha cell numbers, fasting and postmeal glucose levels, and increased gluconeogenesis and hepatic glucose output and decreased beta cell mass as a result of impaired proliferation; whereas obese mice (ob/ob) increased expression of miR-375 as a consequence of increased beta cell mass [77].

A recent study showed that islet enriched miR-375 is a suitable blood marker to detect pancreatic beta cell death and predict type 1 diabetes mellitus (T1D) in sera of

streptozotocin (STZ) treated C57BL/6 mice and non-obese diabetic (NOD) mice. They showed that miR-375 levels were increased upon STZ treatment, prior to hyperglycemia and in the NOD mice 2 weeks before diabetes onset [75]. These data suggest that increased circulating miR-375 correlates with beta cell death and combined detection of miR-375 with other islet enriched miRNAs such as miR-7 [79] can be used as marker of beta cell destruction and potential predictor of T1D.

In another study, researchers examined correlations of the islet-specific miR-375 expression to islet amyloid formation and pancreatic islet damage in postmortem T2D patient samples [78]. There was a positive correlation between miRNA-375 level and the frequency and severity of islet amyloid formation, and negative correlation with proportions of islet beta cells and amylin-positive area, and islet mitochondria density [78].

More recently, Zampetaki *et al.* [32] profiled miRNAs in 80 T2D patients in the Bruneck study and compared these with 80 age-and sex-matched healthy control subjects (**Table 3**). Comprehensive network analysis showed a unique plasma miRNA signature for diabetes, which included reduced levels of miR-126, miR-15a, miR-29b, and miR-223; and elevated levels of miR-28-3p (**Table 2**). Among them, the expression of miR-126, miR-15a, miR-28-3p, miR-223, and miR-29b was already reduced 5-10 years before the manifestation of the disease. The determination of the level of this cluster of five miRNAs was sufficient to identify ~70% of the T2D patients. Among these miRNAs, the endothelial cell–derived miR-126 was most consistently associated with diabetes (**Table 3**). Notably, loss of miR-126 was observed before the onset of overt T2D and was associated with vascular complications. miR-126 was the only miRNA that was validated in a prospective study containing 822 individuals (Bruneck study) and in that study, the loss of miR-126

was observed before the onset of observable diabetes. miR-126 was also one of the identified downregulated miRNAs in atherosclerotic vascular complications (coronary artery disease (CAD). miR-126 is highly enriched in endothelial cells and facilitates VEGF signaling. miR-126 plays a critical role in maintaining endothelial cell homeostasis and vascular integrity by facilitating vascular endothelial growth factor signaling [98], suggesting that miR-126 can be used as a biomarker to predict microvascular and macrovascular complications of diabetes.

In another study, Kong *et al.*, [99] detected an increase in expression of miRNAs (miR-9, miR-29a, miR-30d, miR34a, mi124a, miR146a, and miR-375) in newly diagnosed T2D patients compared with prediabetics and/or susceptible to T2D (**Table 3**). However no difference was observed among individuals with normal glucose tolerance and those with prediabetes suggesting that the level of these particular miRNAs may not be suitable for predicting susceptibility to T2D.

Moreover, Karolina *et al.* [80] profiled miRNAs in the blood and exosomes of 265 patients with different health conditions (metabolic syndrome, T2D, hypercholesterolemia, hypertension) and detected an upregulation of miR-27a, miR-150, miR-192, miR-320a and miR-375 in patients with T2D. They also found a strong correlation between elevated fasting glucose and an increase in levels of miR-27a and miR-320a (**Table 3**) [80]. These studies show the potential of miRNAs as circulating molecular biomarkers for T2D. However, these findings must be validated in large prospective studies by different groups due to the heterogeneity of the results obtained, as there are differences in miRNAs as determined in these studies. These may, in part, be due to the different platforms or methods, demographics of the individuals, and data analysis in each study. Furthermore,

differences may have occurred due to the duration of disease, obesity status of individuals, gender or age.

A more recent study identified a panel of circulating miRNAs in T2D patients that change with insulin sensitization which were also validated in cross-sectional and longitudinal studies (with metformin and placebo control for three months) and were evaluated in healthy volunteers before and after a 6-h hyperinsulonemic euglycemic clamp and insulin plus intralipid/heparin infusion [100]. The study identified increased levels of circulating miR-140-5p, miR-142-3p, and miR-222 and decreased levels of miR-423-5p, miR-125b, miR-192, miR-195, miR-130b, miR-532-5p, and miR-126 in T2D patients.

A discriminant function of four miRNAs (miR-140-5p, miR-423-5p, miR-195, and miR-126) was specific for T2D with an accuracy of 89.2% ($P<0.0001$). Insulin infusion during the clamp decreased miR-222 whereas intralipid/heparin mixture increased circulating miR-222 and miR-140-5p. This study revealed a number of circulating miRNAs that associated with insulin action.

Technical aspects of advances and challenges in miRNA detection and analysis

Advances in detection technologies have enabled the profiling of miRNAs in various biologic samples [101] using various methods [15,102] including microarrays [103], quantitative real-time PCR (qRT-PCR) [104,105], and RNA sequencing [106] with high specificity and sensitivity required for clinical applications [107]. Several recent articles describes methods for profiling and validating miRNAs as biomarkers [11,19,105,108]. A variety of issues such as sample types, miRNA extraction methods, quality and quantity assesments and miRNA

detection and profiling technologies, and data analysis and interpretation have also been extensively discussed in a recent review [102].

qRT-PCR enables measurements of small quantities of miRNAs, however, the primer design can affect the results, whereas, microarray-based methods require more material and it can be challenging to develop probes and assay conditions that work well to detect many different miRNAs simultaneously. Enzymatic miRNA-labelling approaches can be prone to substrate sequence bias [109]. Alternative chemical approaches to miRNA labelling, including chemical alkylation-based labelling along the miRNA (*e.g.* Mirus Bio Label IT) and approaches based on platinum coordination chemistry with nucleic acids (*e.g.* Kreatech ULS) can be used to reduce sequence-specific labelling biases. Initial size fractionation of small RNA using column- or gel-purification-based methods should also be used to reduce background noise caused by other cellular RNAs that are labelled by both enzymatic and chemical labeling approaches.

Another challenge for profiling miRNAs in parallel is that, because of their short length and variance in their sequence content, these often result in a wide variance in melting temperatures (Tm) for annealing reactions, creating miRNA-specific biases. One effective strategy has been the incorporation of locked nucleic acids (LNAs) into primers. This enables the standardization of optimal miRNA primer hybridization conditions across hundreds of PCR assays that are to be run simultaneously.

Sequencing produces direct sequence information that can be used to compare single nucleotide variants, map terminal sequences of transcripts, detect novel splicing events, and copy number. Moreover the precision and sensitivity of sequencing allows the sequencing of low abundance RNA isolated from rare cells (*e.g.* circulating tumor and stem

cells) providing insights on underlying disease pathophysiology. Furthermore, RNA sequencing can detect all classes of RNA thus likely generate information on the entirety of the transcriptome. Contrary to others, RNA sequencing is not biased by thermodynamics, a drawback of quantitative PCR and microarray platforms [110] and do not require the design of primers or probes specific to each miRNA enabling more comprehensive and complete analysis of miRNA measurements [111]. However, RNA sequencing requires high quantity and quality of starting total RNA. (*e.g.* several hundred ng of total RNA/ >10,000 cell equivalents) and additional enrichment steps may be required to reduce the content of ribosomal RNA (rRNA) before library construction. If small RNA enrichment is required, small RNA species (*e.g.* in the 18–24 nt range to capture miRNAs) can be recovered by size fractionating and gel purifying the desired size range fraction on a denaturing polyacrylamide gel [112]. Several amplification tools (*e.g.* The Ovation® RNA-Seq System, NuGEN) can be used for the preparation of cDNA library from small amounts of RNA (*e.g.* ~500 pg of total RNA/~50−100 cell equivalents).

Other potential limitations of next-generation sequencing include the high cost, although this is dropping rapidly, and the use of DNA 'barcoding', which permits multiplexing of many samples in a single run. Additionally, limitations include the computational infrastructure required for data analysis and interpretation, and additional challenges include adapter attachment and sequence-specific biases related to enzymatic steps in small RNA cDNA library preparation methods that favor capture of some miRNAs over others [113-115].

Additionally, RNA sequencing shows high degree of sequence heterogeneity at the 3' and 5' ends (isomirs), probably as a result of imprecise processing of miRNAs and from

posttranscriptional additions of uridines and adenosines [116,117] further complicating the accurate profiling of miRNAs. Sequencing data has also revealed that the most abundant miRNAs do not match the sequence listed in public databases, which suggests that probe or primer design may not be optimal for all miRNAs [110]. Hence, there is a need for rigorous control of pre-analytic and analytic variables when considering potential circulating miRNAs as biomarkers [118].

Consequently, recent data suggest poor correlation and reproducibility between different miRNA detection platforms [16,119]. This is partly due to short length and high sequence homology among various miRNAs and different probe or primer designs and difficulty to distinguish mature miRNAs from precursor miRNAs.

Other challenges related to miRNA biomarker development include variations in sample collection, processing, quantification, detection, pre-analytical (*i.e.* hemolysis) and analytical methods, internal and external normalization controls and data analysis; poor experimental design; and lack of validation of candidate miRNAs in properly designed large human studies [120]. Contrary to mRNAs, where ribosomal RNAs are used to assess RNA integrity and as control, assessment of the integrity, quality and quantity of miRNAs and sample-to-sample and cross platform comparisons are challenging emphasizing the need for standardized methods for sample preparation, processing and analysis.

Currently, data normalization is done by using spiked in synthetic exogenous miRNAs derived from *Caenorhabditis elegans* which do not account for differences in extraction efficiency or endogenous miRNAs (miR017-5p, miR-1249, miR-454) or small non-coding RNAs (RNAU6b) [120]. Alternatively the average expression levels of all measured miRNAs or a panel of miRNAs that are detectable at similar levels in all study

subjects [120] or biomolecules such as creatinine levels in blood and urine have also been used as controls to normalize miRNAs [14].

Often, because the whole tissue with a mixture of various cell types is used instead of pure cell populations, this limits information derived from miRNA profiling studies and affects the accurate measurement of circulating miRNAs. Additionally, the circulating miRNAs are often confounded by varying levels of cellular miRNAs of different hematopoietic origins [121]; therefore, it is necessary to segregate the specificity of measured signals into cell-free and cellular miRNAs that can help distinguish circulating miRNAs from cellular counterparts. A recent study profiled *bona fide* circulating miRNA species in the context of cellular miRNAs in healthy individuals [121]. By segregating the specificity of measured signals into cell-free and cellular miRNAs, researchers generated different categories of fluid-derived miRNA signatures which can help distinguish circulating miRNAs from cellular counterparts and help mitigate the inherent ambiguity in the interpretation of these profiles [121]. miRNAs with the greatest and statistically most significant changes are then selected as candidate biomarkers that can be validated in subjects from different cohorts of studies[105].

Variations in sample collection, processing, quantification, detection, pre-analytical (i.e. hemolysis) and analytical methods, internal and external normalization controls and data analysis; poor experimental design; and lack of validation of candidate miRNAs in properly designed large human studies all contribute to data variability.

As discussed by Chau *et al.* [122] and elsewhere, the technical validation of miRNA biomarkers depends on all aspects of the analytic method including specificity, selectivity, precision, repeatability, reproducibility, assay sensitivity, specificity, reliability, and

reproducibility [123-125]. Specificity describes the ability of the assay to clearly distinguish the analyte of interest from structurally similar substances, whereas selectivity measures the degree to which unrelated matrix components cause analytic interference.

Precision is defined as the repeatability and reproducibility of the assay, which are factors used to quantitatively express the closeness of agreement among results of measurements done under specific conditions [126]. Repeatability refers to the measurements that are done under the same conditions. Reproducibility on the other hand addresses measurements done under different conditions. The reproducibility of the assay relies on assay reliability or variability, levels of technical, instrumental, and biological noise. The reproducibility lso relies on different validation phases of the method such as pre-study and in-study validation of the method.

Furthermore, performance characteristics of miRNA biomarkers in samples collected at different time points (time variation), effects of multiple freeze-thaw cycles of samples, and inter-sample variations must be established. Data suggest that miRNAs are highly stable in blood and other bodily fluids [54,127-129] for multiple freeze-thaw cycles [130]. Another factor that must be considered is the effect of metabolic stressors such as hyperglycemia on the levels of circulating miRNAs. A recent report showed that there was no effect of post-load glucose in circulating miR-375 [75].

Likewise, statistical analysis of the association between circulating miRNA levels and disease endpoints are challenging because miRNAs are highly correlated and traditional statistical methods show poor performance [120]. Consequently, large numbers of miRNAs tested in studies leads to an increase in the type I error and a high risk of false discovery [120]; therefore, better analytical standards are a prerequisite for miRNA research.

Other challenges in validating miRNAs as biomarkers

Although many miRNAs have been identified as potential biomarkers, only a few of these have been validated in well-designed cohorts in large longitudinal studies specifically designed to validate miRNAs as biomarkers.

Therefore, identification and validation of candidate miRNAs as clinically relevant biomarkers is the most challenging issue. Accordingly, a large number of clinical samples (50-200) in separate cohorts should be employed. miRNAs identified in pilot studies must be validated on a more extensive scale in separate, parallel design (placebo or comparator controlled), appropriately powered larger, longitudinal, and ideally interventional clinical studies, ideally in a predicted responder group of subjects; for the determination of its clinical applicability such as correlation between the expression level of miRNAs and factors including age, gender, or disease severity.

Furthermore, there have to be convincing preliminary data demonstrating that the miRNA assays are truly specific and can discriminate patients from controls on an individual rather than group basis.

The controls must also include subjects from disease categories such that biomarkers that are being investigated are specific for that particular disease. For example, in studies that we have conducted with T1D subjects, we have included subjects from LADA, T2D, subjects with pre-T2D, and healthy matching controls. It would be highly valuable to include subjects from other disease groups such as autoimmune, inflammatory, and metabolic disease to further validate these candidate biomarkers for specificity for that particular disease.

miRNAs identified as potential biomarkers of a specific disease must be validated in a different and sufficiently powered study cohort with proper controls to demonstrate

specificity for that particular disease. Clinical validation of biomarkers is the evidentiary process of linking biomarkers with biological processes, and clinical end points for that particular disease [1,131,132].

For example, we conducted a cross-sectional assessment of circulating biomarkers of beta cell injury by measuring plasma levels of beta cell specific or enriched miRNAs among subjects with different types of diabetes including subjects with prediabetes, T2D, latent autoimmune diabetes of adults (LADA), and T1D as compared with healthy controls (*e.g.* age, gender, BMI) (**unpublished data)**. As a follow up validation of our findings from the cross-sectional study, we evaluated the levels of miRNA biomarkers of beta cell injury in a placebo controlled, parallel designed, longitudinal and interventional study (**unpublished data**).

In addition, if appropriate with the therapeutic intervention (*e.g.* if drug effect can washout quickly, duration of treatment period is relatively short, and ethical to do, and response to the treatment by miRNA biomarkers follow the changes with treatment and washout response), a randomized-cross-over design can be used for the assessment of these circulating miRNA biomarkers to demonstrate the specificity of these biomarkers for that particular disease. This will allow testing the reproducibility and durability of these candidate miRNAs, to be assessed at different stages of disease development and progression, and their responses to therapeutic interventions.

If appropriate, samples for measurement of miRNAs should be collected prior to disease manifestation (prospective design) since comparisons between patients and healthy controls are likely to be confounded by medication and common disease risk factors. If a constant expression level during different stages of disease is measured, the miRNA may

serve as a biomarker for the diagnosis of early-stage of a disease, whereas if the expression level varies during the development of the disease, the miRNA may serve as a biomarker for disease classification and prognosis.

Additionally, the analysis of such miRNAs must be assessed for the differences in level of miRNA abundance in normal (healthy) and diseased tissues using multiple *ex vivo* (*e.g.* primary islet culture), *in vivo* (*e.g.* non-obese diabetic (NOD) mice), and clinical testing (serum samples from T1D, T2D, predisposed individuals and healthy normal volunteers).

Because understanding the functional roles of miRNAs on the regulation of genes and pathways and their relevance to disease is critical for developing miRNAs as biomarkers, additional studies are recommended to determine miRNAs roles in patients as most of the current findings are indirect.

Likewise, miRNAs tend to be organized in a related cluster and appears to target multiple mRNA transcripts within common pathways (*e.g.* proliferation, apoptosis), suggesting that miRNA clusters have a capacity for coordinated regulation of multiple steps within a specific pathway [25]; therefore, integrating miRNA profiles with mRNA profiles and other large-scale genomic data sets in gene regulatory networks associated with disease pathogenesis, will help to develop better systems-level understanding of disease development. Accordingly, by combining several miRNAs into a panel and applying machine-learning techniques may provide greater accuracy to distinguish individuals with disease from controls or from individuals with other diseases, than can be expected from the assessment of a single miRNA. By evaluating changes of several miRNAs in the context of the overall miRNA network, disease specific signatures with

diagnostic and prognostic value can be identified consequently increasing the chances of successful treatment in a heterogeneous patient population. For example, miR-885-5p has been shown to be elevated in liver-associated diseases including hepatocellular carcinoma, liver cirrhosis, and chronic hepatitis B [133]; therefore, a panel of miRNAs may provide greater specificity for the diagnosis of each disease. This will improve sensitivity and specificity leading to more comprehensive and functional inference information to characterize both early diagnosis and response to treatment studies, and for patient stratification.

Furthermore, representation of biological data as networks facilitates data integration across multiple levels of biological complexity; and relationships between genes, RNAs, proteins, and diseases, may unravel contribution of specific biological components to systems-wide properties of disease. Therefore, network inference algorithms could be effective to characterize miRNA networks, to decipher rewiring of miRNA profiles under pathological conditions, and to test for potential associations of miRNA clusters with disease endpoints.

The mechanistic disconnect between preclinical and clinical research is one of the major issues that interfere with successful drug development. Identifying informative biomarkers is essential for evaluating clinical trial outcomes and for selecting appropriate treatment options. Thus, identifying the intricate relationships between disease and miRNA signatures could help not only to understand the mechanism behind the pattern of disease associations, but also provide information on the pathological processes because miRNAs in turn influence the expression of multitude of genes. Consequently, evaluation of miRNA

and disease signatures can provide guidance for clinical trials conducted in mechanistically defined patient groups.

Likewise, incorporating miRNAs and associated single nucleotide polymorphisms (SNPs) and copy number variations into pharmacogenomic studies may provide more insights into the mechanisms of drug response. Furthermore, combining miRNAs and associated epigenetic, genetic and genomic, transcriptomic, and proteomic variations and clinical data, may identify better predictor biomarkers for disease diagnosis and the treatment outcomes of individual patients as exemplified in a recent study [108] where significant correlations between the genomic location of disease-associated genetic variants and deregulated miRNAs have been observed.

Moreover, since miRNAs are highly correlated, traditional statistical approaches show poor performance when allowing for highly correlated variables. Consequently, current statistical methods should be supplemented by more sophisticated approaches suitable for high-dimensional and collinear data analysis (*e.g.* L1-penalized Cox regression analysis) and more statistical rigor must be applied for the successful development of miRNA biomarkers.

CONCLUSIONS

The development of minimally invasive and highly specific biomarkers that could inform disease progress prior to clinical manifestation would be advantageous in the management of disease, segmentation of patients populations, and monitoring of therapeutic interventions.

Due to their roles in gene regulation and their association with a variety of diseases and phenotypes such as individual response to therapeutic treatments, miRNAs provide an opportunity to personalize medicine.

In addition, their rich information content, their exclusivity to specific tissue of origin, their presence in specimen types in stable forms, and their potential for sensitive measurement make them ideal candidates for minimally invasive and cost-effective biomarkers for the early diagnosis, progress, and prognosis of diseases.

Successful assessment of the levels of multiple miRNAs within circulation will provide us with an exciting opportunity to closely monitor disease progress and therapeutic interventions with minimal invasive sampling.

miRNAs could, if validated appropriately, be used as a first line of test to stratify patients or to identify high risk populations in conjunction with more traditional tests. Furthermore, because of their contribution to disease development, miRNAs could be targets for early intervention.

However, a number of scientific and technical considerations must be addressed before circulating miRNAs become biomarkers of disease diagnosis and prognosis and be used to monitor therapeutic intervention.

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DISCLOSURE SUMMARY

AS has nothing to disclose.

SEARCH STRATEGY AND SELECTION CRITERIA

PubMed and internet were used to identify articles published on the subject discussed in this review. The following search terms were used to identify relevant articles: ‘‘biomarkers, RNA, miRNA, microRNA’’ AND ‘‘metabolic disease’’, ‘‘diabetes’’, “obesity”, and “lipid and cholesterol biosynthesis”. The search was restricted to the recent studies involving miRNA and their potential as biomarkers for metabolic disease with particular interest given to diabetes and all searches were limited to human and animal studies published in English. Articles resulting from these searches and relevant references cited in those articles were reviewed.

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TABLE/FIGURE LEGENDS

Graphical abstract. Composite biomarkers of beta cell injury (miRNAs, autoantibodies, or cytokines) may lead to better biomarkers of diabetes.

Table 1. miRNAs play role in glucose and cholesterol homeostasis and vascular integrity.

Table 2. miRNAs involved in pancreatic beta cell function.

Table 3. Circulating miRNAs as diagnostic biomarkers in diabetes.

Figure 1. Illustration of tests for assessing the loss of beta cell mass and function and opportunity for predictive markers for assessing beta cell destruction. Deterioration of FPIR in the IVGTT and OGTT responses are currently the best indicators of impending diabetes. Imaging methods for monitoring beta cell mass are still in development. Little is known about the pattern of insulitis and decline in beta cell mass in humans during the prediabetic prodrome. Opportunity to develop beta cell-specific biomarkers is highlighted with a box. OGTT: Oral glucose tolerance test. IR: Insulin resistance. FPIR: first-phase insulin response. PI/C-peptide, proInsulin-to-C-peptide ratio; T_{eff}, T effector cells. Adapted from [134].

Figure 2. Known roles of miRNAs in metabolically involved tissues in the context of diabetes. Adapted from [2,86].

Table 1, Seyhan

MiRNAs	Target tissue / organ	Function	Confirmed Targets	References
miR-33	Liver	Cholesterol metabolism and homeostasis	ABCA1, ABCG1, CROT, CPT1A, HADHB, PRKAA1, SREBF1, ACLY, ACC1, FASN	135-137
miR-122	Liver	Cholesterol and fatty acid biosynthesis	ACC1, ACC2, SCD1, ACLY	93
miR-370	Liver	Cholesterol and fatty acid biosynthesis	CPT1A, SREBP1c, and DGAT2	138
miR-378/378*	Adipose	Adipocyte differentiation and metabolism	KLF5, FABP4, SCD1, Resistin	139
miR-17-5p	Adipose	Adipocyte clonal expansion, insulin resistance	RBL2	82,83,140
miR-132	Adipose	Adipocyte clonal expansion, insulin resistance	CREB	82,83
miR-29a	Adipose/skeletal muscle	Insulin resistance, glucose transport		141
miR-103	Adipose	Adipocyte differentiation	PANK1	81,142
miR-143	Adipose	Preadipocyte differentiation	MAPK7	143
miR-9	Pancreas	Impairs insulin secretion	OC2	96
miR-375	Pancreas	Insulin secretion and apoptosis by lipoapoptosis	PDK1, Mtpn, Vti1a MTPN, USP1, JAK2, ADIPOR2	75,76,144 76,86,145
miR-96	Pancreas	Insulin secretion	Noc2, Granuphilin,	146
miR-124a	Pancreas	Regulates insulin secretion	Rab3A, SNAP25, Synapsin 1A, Noc2	146
Let-7	Pancreas	Insulin sensitivity, adipogenesis, angiogenesis, inflammation	IGF1R, INSR, IRS2	147
miR-34a	Pancreas	Beta **cell** apoptosis	VAMP2, Bcl2	146
miR-146a	Pancreas	Beta **cell** apoptosis	BRCA1, BRCA2, CD40LG, CFH, FADD, FAS, IRAK1, IRAK2, MMP16, STAT1, TRAF6	146,148-151
miR-103 miR-107	Pancreas	Insulin sensitivity	Caveolin-1	87
miR-24 miR-26 miR-182 miR-148	Pancreas	Insulin synthesis	Sox6, Bhlhe22	152
miR-126	Pancreas, skeletal muscle	Pancreatic development	SPRED1, PI3KR2	92
miR-132 miR-195	Brain	Adipocyte proliferation and growth, insulin resistance	CREB	82
miR-192	Kidney	Kidney and diabetic nephropathy development	SIP1	1
miR-133	Heart	Smooth muscle survival and proliferation	Sp1	153
miR-21	Heart and vascular tissue	Smooth muscle survival and proliferation	ERK, PTEN, RhoB, PPAR, Bcl2, PDCD4	11,14,154,155
miR-221 miR-222	Heart and vascular tissue	Smooth muscle survival and proliferation	cKit, p27Kip1, p57Kip2, STAT5A	156-159
miR-143 miR145	Vascular tissue	Smooth muscle survival and proliferation	KLF4, KLF5, Myocardin, CaMKII, Elk1, PDGF-Ra, PKCe	160-163
miR-192 and miR-193b	Pancreas, liver, fat	miR-193b contributes to brown adipocyte differentiation, decreases inflammation	CCL2	164,165
miR-182	Pancreas	Insulin synthesis		152
miR-184	Pancreas	Beta cell expansion	Ago-2	166

Table 1. miRNAs play role in glucose and cholesterol homeostasis and vascular integrity.

Table 2, Seyhan

miRNA	Target tissue / organ	Function	Confirmed Targets	References
miR-9	Pancreas	Insulin secretion	OC2	96
miR-375	Pancreas	Insulin secretion, and apoptosis	PDK1, Mtpn, Vti1a	75-77,144,145
miR-96	Pancreas	Insulin secretion	Noc2, Granuphilin,	146
miR-124a	Pancreas	Insulin secretion	Rab3A, SNAP25, Synapsin 1A, Noc2	146
Let-7	Pancreas	Insulin sensitivity, adipogenesis, angiogenesis, inflammation	IGF1R, INSR, IRS2	31,147
miR-34a	Pancreas	Beta cell apoptosis	VAMP2, Bcl2	146 31
miR-146a	Pancreas	Beta cell apoptosis		146,148-151
miR-21	Pancreatic islet, Heart and vascular tissue	Smooth muscle survival and proliferation	ERK, PTEN, RhoB, PPAR, Bcl2, PDCD4	167
miR-30d	Pancreas, beta cell	Insulin biosynthesis		31
Mir-126	Endothelial cells	Vascular integrity	SPRED1, PI3KR2	92
miR-376a	Pancreas, beta cell			145,168
miR-103, miR-107	Pancreas	Insulin sensitivity	Caveolin-1	87
miR-24, miR-26, miR-182, miR-148	Pancreas	Insulin synthesis	Sox6, Bhlhe22	152

Table 2. miRNAs involved in pancreatic beta cell function.

Table 3, Seyhan

Disease	Study Design	miRNA Biomarkers	Source	Method of analysis	Multivariate analysis	Observations	References
T2D	800 individuals with normal glucose tolerance, impaired glucose tolerance, manifest T2D	miR-126↓ miR-15a↓ miR-29b↓ miR-223↓ miR-28-3p↑	Plasma	Microarray profiling (validated by qPCR)	Endpoints: normal (NGT fasting and 2-h glucose), prediabetes (IFG/IGT), onset T2D	Dysregulation of these miRNAs antedated the manifestation of T2D	[32]
T2D	56 individuals with prediabetes, T2D susceptible normal individuals and newly diagnosed T2D	miR-9↑ miR-29a↑ miR-30d↑ miR-34a↑ miR-124a↑ miR-146a↑ miR-375 ↑	Serum	qPCR on a panel of miRNAs	Endpoints: T2D susceptible with NGT, prediabetes (IGT), newly diagnosed T2D	Dysregulated in T2D	[99]
T2D	265 individuals with metabolic syndrome, T2D, hypertension, hypercholesterolemia , and healthy controls	miR-150↑ miR-192↑ miR-27a↑ miR-320a↑ miR-375↑	Blood and exosomes	Microarray profiling (followed by qPCR validation)	metabolic syndrome, T2D, hypertension, hypercholeterolemia, and healthy controls	Correlation between increased levels of fasting glucose and increase in miR-27and miR-320a	[80]
T2D	12 T2D patients without any chronic complications, 12 T2D patients with macrovascular complications and 12 with microvascular complications	miR-31↑	Serum	qPCR on a panel of miRNAs	Endpoints: T2D with macrovascular complications; T2D with microvascular complications; T2D without complications	Correlates with diabetic microvascular complications	[169]
T1D	Newly diagnosed children with T1D and normal controls	miR-24↑ miR-25↑ miR-26a↑ miR-27a↑ miR-27b↑ miR-29a↑ miR-30a-5p↑ miR-148a↑ miR-152↑ miR-181a↑ miR-200a↑ miR-210↑	Serum	RNA sequencing (validated by qPCR)	Endpoints: Beta cell function (stimulated C-peptide) and glycemic control (HbA1c)	miR-25 correlates negatively with residual beta cell function and positively associated with glycemic control (HbA1c) 3 months after onset of T1D	[170]
T1D	20 newly diagnosed patients with T1D and 20 control subjects	miR-9 miR-31 miR-34a miR-146a miR-155 miR-181a miR-199a	Serum	Taqman Low Density Array cards (validated by qPCR)	NA	Dysregulated in newly diagnosed T1D. Involved in immune processes (miR-31, miR-146a, miR-155, miR-181a, miR-199a) and in the regulation of the beta cell mass and function (miR-9, miR-34a)	[171]
T1D	20 patients with T1D	miR-21a↓ miR-93↓	PBMCs	qPCR on a panel of miRNAs		Decreased in T1D	[172]
T1D	19 patients with T1D	miR-326↑	Lymphocytes	qPCR on a panel of miRNAs		Positive correlation with islet immune attack	[173]

Multivariate adjustments for BMI, waist/hip ratio, smoking, social status, alcohol, physical activity, and C-reactive protein.

miR-126 is the only miRNA that was validated in a prospective population of 822 individuals (Bruneck study) [32]. In that study, loss of miR-126 was observed before the onset of diabetes and was also downregulated vascular complications (atherosclerotic coronary artery disease).

Table 3. Circulating miRNAs as diagnostic biomarkers in diabetes.

Graphical Abstract, Seyhan

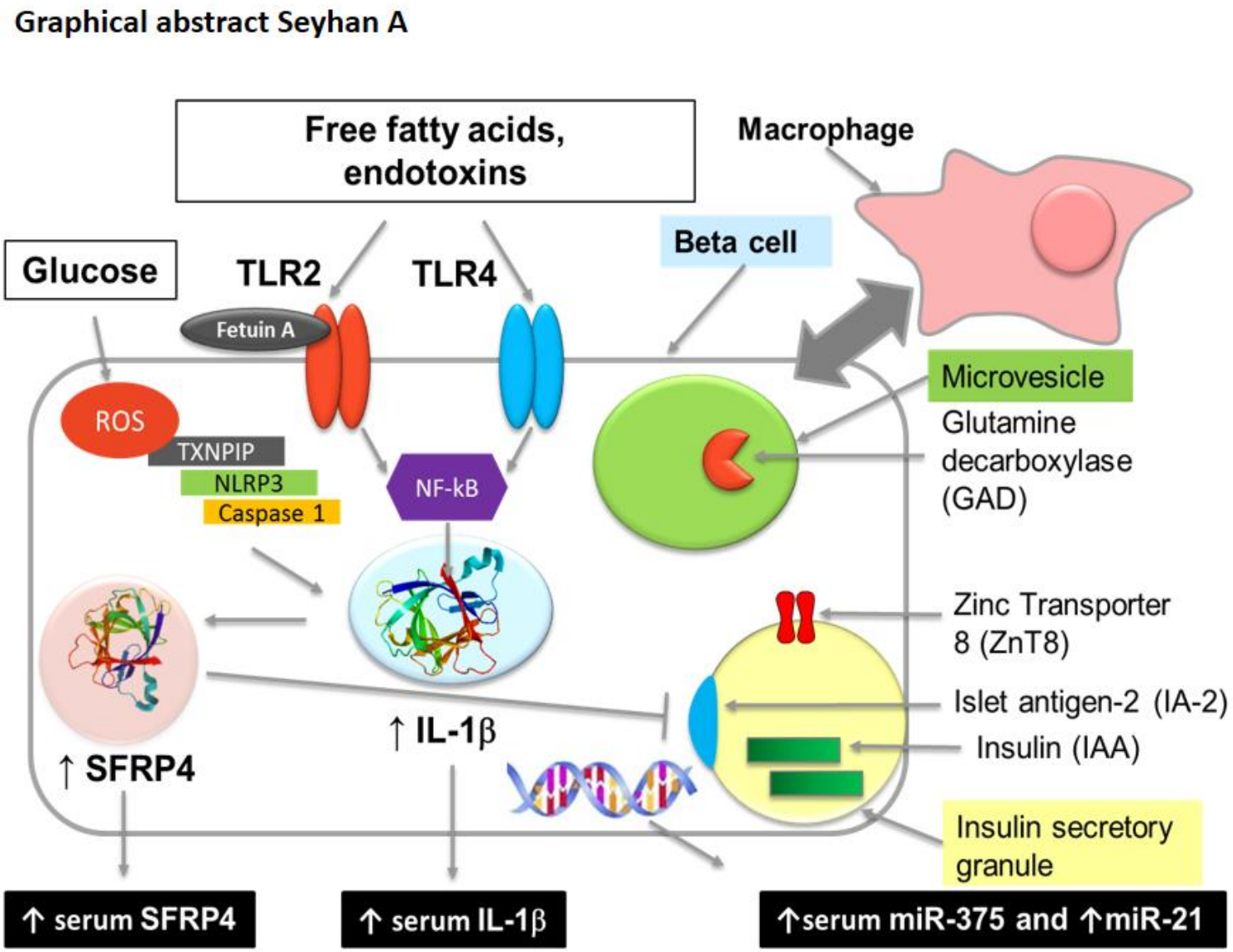

Figure 1, Seyhan

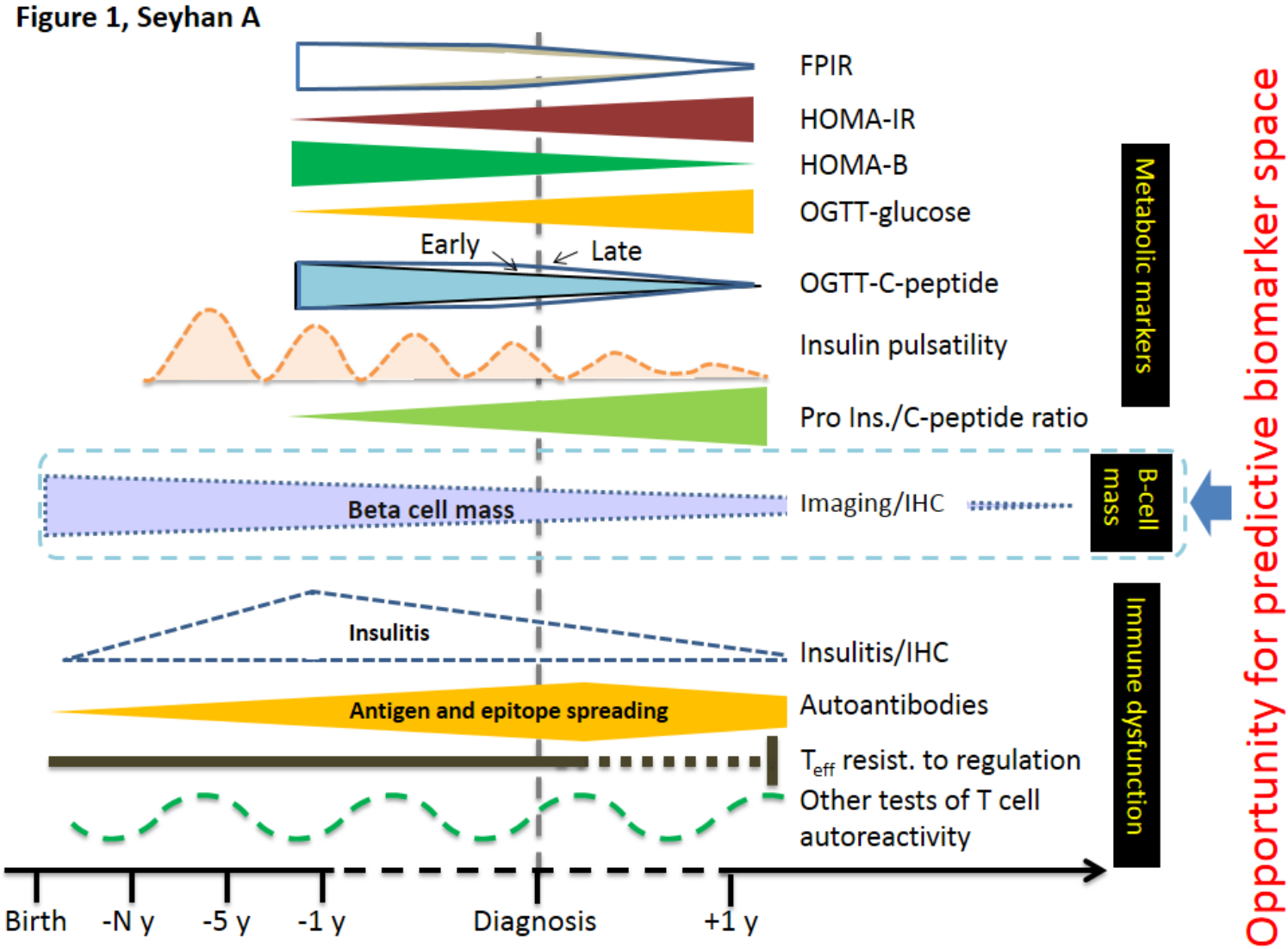

Figure 2, Seyhan