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Circulating microRNAs as novel biomarkers for diabetes mellitus

Running title: microRNAs as biomarkers in diabetes

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Competing interests

The authors declare no competing interests.

Abstract

Diabetes mellitus is a metabolic disorder characterized by insufficient insulin secretion from pancreatic β-cells to maintain blood glucose homeostasis. The disease is triggered by autoimmune destruction of β-cells (Type 1) or by conditions reducing insulin sensitivity and negatively impacting on β-cell activities (Type 2). Without proper management, diabetic patients develop serious complications that reduce life quality and expectancy. Although a definitive cure is not on the horizon, biomarkers permitting early detection of the disease and identification of individuals at risk of developing complications would greatly improve diabetes care. MicroRNAs are small non-coding RNAs controlling gene expression and participating in many physiopathological processes. Hundreds of microRNAs are actively or passively released in the circulation and can be used to evaluate health status and disease progression. Both Type 1 and Type 2 diabetes are associated with distinct modifications in blood microRNA profile, sometimes detectable years before the manifestation of the disease. Moreover, circulating levels of certain microRNAs appear to be predictive of long-term complications. As discussed in this review, there are still technical and scientific obstacles to be overcome, but circulating microRNAs may soon become part of the diagnostic arsenal to identify individuals at risk for developing diabetes and its devastating complications.

Introduction

Diabetes mellitus affects more than 350 million peoples worldwide and significantly contributes to morbidity and mortality in modern societies[1]. In industrialized countries, it is the leading cause of blindness, renal failure and nontraumatic lower limb amputations. Patients suffering from diabetes have also a higher risk of developing cardiovascular disorders and stroke rendering this disease a heavy socioeconomic burden[2]. Unfortunately, the prevalence of diabetes is increasing at a dramatic pace both in children and adults, as a result of modern lifestyle associated with reduced physical activity, overnutrition and obesity, but also as a consequence of population ageing. Indeed, according to estimates of the International Diabetes Federation, 552 million people are expected to be diabetic in 2030[1].

Although the origin and etiologies of diabetes mellitus can be diverse, the disease is invariably characterized by deregulation of blood glucose homeostasis resulting from the incapacity of pancreatic β-cells to secrete appropriate amounts of insulin to sustain the demands of the organism. In this review, we will focus on Type 1 and Type 2 diabetes mellitus (T1DM and T2DM, respectively), the two principal forms of the disease. T1DM is an auto-immune disorder in which pancreatic β-cells are attacked and eliminated by the immune system. During the immune response, leucocytes infiltrating the pancreatic islets secrete pro-inflammatory cytokines that recruits cytotoxic T lymphocytes and contribute to β-cell dysfunction and death[3]. The immune reaction leads to progressive destruction of β-cells resulting in severe or complete insulin deficiency. T1DM generally develops during childhood or in young adults and accounts for 5-8% of all diabetes cases. T2DM includes the vast majority of the other cases. The pathogenesis of this disease is closely linked to genetic and environmental/life style factors, such as hypercaloric nutrition, lack of exercise and obesity. T2DM is initiated by loss of insulin sensitivity of target tissues, including liver, skeletal muscles and adipose tissues. This insulin resistant state can normally be compensated by expansion of the functional β-cell mass and by an increase in their insulin secretory activity. However, in genetically predisposed individuals, the β-cells are unable to sustain the increased insulin demand, leading to chronic hyperglycemia and to the onset of T2DM[4].

Despite intensive research, the causes of diabetes mellitus remain incompletely understood and a definitive cure is still not available. The therapeutic arsenal available today permits, if not to cure, at least to delay the progression of the disease. The efficacy of these treatments would be drastically improved by implementing them during the initial phases of the disease and by

identifying individuals with the highest probability of benefitting from the therapeutic intervention. This goal can only be achieved by identifying new biomarkers predicting and/or monitoring the progression of T1DM and T2DM and their long-term complications. The aim of this reviewis to summarize the weaknesses of available blood parameters and biomarkers currently used to detect T1DM and T2DM and to discuss the potential use of circulating miRNAs as a novel class of biomarkers.

Classical biomarkers in diabetes

According to the World Health Organization, the diagnosis of diabetes mellitus is based on measurements of blood glucose levels in the fasted state and following an oral glucose tolerance test[5]. A diabetic state is defined by glucose levels above 7.0 mmol/L (126 mg/dl) in the fastedstate and higher than 11.1 mmol/L (200 mg/dl) after an oral glucose tolerance test[5]. Other serum parameters such as glycated hemoglobin (HbA1c) or residual C-peptide can also be helpful to diagnose diabetes mellitus.

Biomarkers for Type 1 diabetes mellitus

T1DM is generally diagnosed when more than 80 to 90% of the pancreatic β-cells have been destroyed by the immune system[6]. The progression of the disease is slow (months to years) providing a potentially long period to identify and treat individuals at risk. Recent progress has been made to preserve the functions of residual β-cells at the onset of T1DM using immunosuppressive medications[7, 8]. At present, the efficacy of these treatments is limited, but significant improvements could probably be obtained if therapies were initiated at earlier stages of the disease when a larger number of β-cells is still present. Autoantibodies against islet antigens are often used as biomarkers for T1DM, since their presence in the blood is a hallmark of the disease. Several autoantibodies have been described, but those directed against islet cells (ICA), insulin (IAA), the tyrosine phosphatase IA-2 and IA-2β, glutamate decarboxylase (GADA) and the zinc transporter 8 (ZnT8) are the most reliable for recognizing individuals at risk for developing T1DM (see Table 1)[6, 9]. However, the use of islet autoantibodies as biomarkers faces important limitations: 1) autoantibodies appear relatively late in the course of T1DM, 2) although most individuals at the onset of T1DM are positive for at least some autoantibodies, many autoantibody-positive individuals will never develop the disease, and 3) autoantibodies are not suitable for monitoring therapeutic outcomes[9]. There is therefore a

need for additional biomarkers for T1DM to complement the information obtained from the presence of autoantibodies and other risk factors (age, family history, susceptibility genes, and environmental triggers).

Biomarkers for Type 2 diabetes mellitus

Individuals at risk to develop T2DM are currently identified by a combination of easily accessible serum parameters (including glucose, triacylglycerol, cholesterol, lipoproteins and HbA1c), physical characteristics (body mass index, waist-to-hip ratio, blood pressure and sex) and lifestyle habits (food consumption, physical inactivity and smoking). By combining all these traditional/classical biomarkers and risk factors, the probability of predicting the development of the disease ranges from 0.85 to 0.90 in a period of 5 to 10 years before the onset of T2DM[10]. Other molecules have emerged as potentially useful biomarkers (see Table 1), including hormones, cytokines, adipokines, ferritin and C-reactive protein[11]. None of these so-called novel biomarkers can individually predict T2DM manifestation efficiently but in combination they can achieve predictive values similar to those with classical biomarkers[12].

All these serum parameters efficiently predict the development of T2DM few years in advance in individuals already displaying metabolic alterations, but they are not specific for diabetes and are unable to assess disease susceptibility in the general population. Genotypic analysis could potentially complement the biomarkers for the identification of individuals susceptible to develop T2DM later inlife. However, so far the predictive values of genotypic traits has not exceeded 0.60[11]. Thus, there is currently a need for early and life-style-independent predictive factors enabling physicians to recognize persons at risk of developing T2DM.

microRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules of 21 to 23 nucleotidesthat function as translational repressors by partially pairing to the 3'untranslated (UTR) region of target messenger RNAs (see Box 1). These regulators of gene expression were first discovered in *Caenorhabditis elegans*[13, 14] and later on in vertebrates and plants. According to recent estimates, the human genome encodes more than 1600 miRNA precursors, generating up to 2237 mature miRNAs (www.miRbase.org) each of whichhas the potential of controlling hundreds of targets. MiRNAs are now universally recognized as major regulators of gene expression and as key controllers of several

biological and pathological processes[15]. MiRNAs are produced from stem-loop precursor RNAs generated from independent transcriptional units or from introns of protein-coding genes (Box 1). These primary transcripts (pri-miRNAs) are initially processed to produce shorter RNA molecules (pre-miRNAs) and then exported to the cytosol where they are further cleaved to generate the mature forms of the miRNAs (Fig.1). The mature miRNAs can either be included in the RNA-induced silencing complex (RISC) to guide translational repression of target mRNAs or be released by the cells. In the latter case, the miRNAs associate to proteins, to lipoproteins or are loaded inside vesicles that are released in the extracellular space upon plasma membrane blebbing or after fusion of multivesicular bodies with the plasma membrane (Fig.1).

Role of miRNAs in diabetes pathogenesisPancreatic β-cells and insulin target tissues express a well-defined set of miRNAs. Most of them are not cell specific, but are widely distributed throughout the human tissues. A notable exception is represented by miR-375, a miRNA highly enriched in pancreatic islets that regulates the expression of genes involved in hormone secretion and in β-cell mass expansion in response to insulin resistance[16, 17]. The miRNA expression profile of β-cells and insulin target tissues is altered both in T1DM and T2DM most likely contributing to impaired function of these tissues under disease states [18-20]. Indeed, the islets of pre-diabetic NOD mice, a model of T1DM, contain increased levels of several miRNAs including miR-21, -34a, -29 and -146a which have deleterious impacts on β-cell functions [21, 22]. Most of these miRNAs and many others are altered also in the islets *ob/ob* and *db/db* mice, two models of obesity and T2DM[23, 24]. Interestingly, in these animals the expression of miR-29 and -34a is also increased in insulin target tissues, possibly contributing to insulin resistance[25, 26]. Other miRNAs dysregulated in insulin target tissues of *ob/ob* mice, dietary mouse models of obesity and diabetic GK rats include miR-143, miR-802 and two closely related miRNAs, miR-103 and -107 [25-28]. There is strong experimental evidence indicating a contribution of these miRNAs to the development of insulin resistance in these obesity models[26-28]. MiRNA changes related to diabetes have also been reported in human tissues. More than sixty differentially expressed miRNAs were detected in human skeletal muscle biopsies from T2D patients, including an up-regulation of miR-143 and down-regulation of two muscle-specific miRNAs, miR-206 and miR-133a[29]. Interestingly, the level of about 15% of these miRNAs was already modified in individuals with impaired glucose tolerance suggesting an involvement in the

early phases of the disease process. The expression of part of these miRNAs is controlled by insulin but this regulatory mechanism appears to be impaired in diabetic patients [30].

In addition to the changes in insulin target tissues described above, diabetes results in significant modifications in miRNA expression in blood vessels, heart, retina and kidneys indicating an involvement of these non-coding RNAs also in long-term diabetes complications (for review see [19, 31, 32]).

A functional role for circulating miRNAs?

Beside the gene regulatory activities accomplished inside the cells producing them, several miRNAs are found in blood and other body fluids in association with proteins, microvesicles and/or lipoprotein complexes[33-35] (Fig.2). The function of circulating miRNAs remains to be established, but *in vitro* studies indicate that miRNAs transported by exosomes (see Box 2) or high-density lipoprotein (HDL) can be transferred in active form to recipient cells[35, 36]. This raises the intriguing possibility of an involvement of miRNAs in a novel cell-to-cell communication mode. Circulating miRNAs are very stable and resistant to RNAse treatment, freezing/thawing cycles and other drastic experimental conditions[37, 38]. Consequently, serum or plasma samples can be stored at -20 °C or -80 °C for up to several months without significant miRNA degradation[39], suggesting that these small RNA molecules are sufficiently robust to serve as biomarkers. Circulating miRNAs present several other advantages as potential biomarkers: they are found not only in blood but also in other easily accessible biological fluids (like urine, saliva, amniotic fluid and maternal milk)[40], they can be detected by highly sensitive and specific quantitative PCR methods, and most of them are evolutionary conserved, facilitating the translation of results obtained from *in vivo* animal studies to human healthcare. Moreover, miRNA serum profiles of healthy donors are relatively homogenous and constant during the day and miRNAs can be measured in both serum and plasma[38, 41, 42].

miRNAs as diabetes biomarkers

The idea of using blood miRNAs as biomarkers is relatively new and was first proposed for detecting different forms of cancer[38, 43, 44], autoimmune diseases[45] and sepsis[46]. Recent studies have also analyzed the miRNA profile in serum, plasma or blood cells in attempt to develop new approaches to predict diabetes development and progression (summarized in Table 2). Zampetaki and colleagues were the first to identify a characteristic blood miRNA expression profile related to

T2DM[47]. In their prospective study, they analyzed blood samples of more than 800 individuals from the Bruneck cohort and identified a subset of 5 miRNAs (miR-15a, -28-3p, -29b, -126 and -223, and miR-28-3p) that display a characteristic deregulation in 80 pre-diabetic and diabetic subjects. Importantly, the level of these miRNAs was already modified years before the onset of the disease, providing initial evidence for the usefulness of circulating miRNAs as early predictors of T2DM and its vascular complications. The miRNA serum content of pre-diabetic and/or newly diagnosed T2DM patients was analyzed also by other groups. Kong and co-workers detected an increase in the expression of seven diabetes-related miRNAs (miR-9, -29a, -30d, -34a, -124a, -146a and -375) in T2DM patients compared to pre-diabetic or T2DM susceptible subjects[48]. However, no differences were observed between normal glucose tolerant and pre-diabetic individuals indicating that the level of these miRNAs is not suitable to predict T2DM susceptibility. More recently, Karolina *et al*. measured the miRNAs present in the blood and in exosomes of 265 patients with different health conditions associated with metabolic syndrome[49]. They detected an up-regulation of miR-27a, -150, -192, -320a, and -375 in T2DM individuals and observed a strong correlation between elevated fasting glucose concentrations and the alteration of miR-27a and miR-320a levels. These pioneering studies demonstrate the potential of miRNAs as biomarkers for T2DM. However, the heterogeneity of the results obtained underscores the need for large prospective studies to identify reliable miRNA signatures for T2DM.

A similar approach was attempted to identify new biomarkers predicting destruction or regeneration of residual β-cells in T1DM. Nielsen and colleagues compared two cohorts (Danish and Hvidoere) of newly diagnosed for T1DM to an age-matched control group [50]. Global miRNA sequencing, followed by qPCR verification, and regression analysis to adjust for age, sex and multiple testing, highlighted a group of miRNAs (miR-24, -25, -26a, -27a, -27b, -29a, -30a-5p, -148a, -152, -181a, -200a and -210) that were differentially expressed in diabetes cohorts compared to control groups. Several of these miRNAs modulate the expression of genes involved in apoptosis and/or important β-cell regulatory networks[50]. Moreover, miR-25 levels were found to correlate with residual β-cell function (C-peptide measurement) and adequate glycaemic control (HbA1c levels) 3 months after disease onset (Danish cohort). This correlation was not observed in the Hvidoere cohort, in which glycaemic control was evaluated 12 months after the diagnosis of T1DM. In a study presented at the last meeting of the European Association for the Study of Diabetes, Sebastiani and co-workers compared the blood miRNA profile of 20 newly-diagnosed T1DM patients with that of

healthy individuals[51]. Out of 206 miRNAs detected in serum of both groups, 64 were found to be differently expressed in T1DM. Interestingly, some of these miRNAs regulate the functions of immune cells (miR-31, -146a -155, -181a, -199a) or of β-cells (miR-9, 34a). A miRNA abundantly expressed in the islets of Langerhans, miR-375, has been recently proposed as suitable biomarker to detect β-cell death and to predict the development of T1DM in animal models. Indeed, massive β-cell loss elicited by administration of streptozocin caused a dramatic rise in circulating levels of this miRNA[52]. Moreover, plasma levels of miR-375 were significantly increased in NOD mice two weeks before the onset of T1DM[52]. The changes in miR-375 levels consequent to β-cell death were short-lived. Thus, these promising findings obtained in mice will need to be verified in humans in which the decline of the β-cell mass spans much longer periods. Instead of analyzing plasma samples, other studies focused their attention on blood cells and measured the expression of specific miRNAs thought to play important roles in the immune reaction. Salas-Pérez et al. observed diminished expression of miR-21a and miR-93 in peripheral blood mononuclear cells (PBMC) of T1DM patients compared to healthy controls[53]. The reduction of miR-21a (but not of miR-93) could be reproduced by incubating PBMC from control individuals in the presence of 25 mM glucose, suggesting that it may be the consequence of chronic hyperglycemia. Finally, Sebastiani and colleagues analyzed miRNA expression in blood lymphocytes from T1DM individuals and observed a rise in miR-326 levels that correlated with the islet autoimmune attack[54]. The primary goal of the latter two studies was to identify miRNAs potentially involved in the development of the disease. However, since a large fraction of serum miRNAs are released by blood cells, it is possible that the miRNA changes in PBMC and/or lymphocytes observed in these two studies may yield detectable differences in plasma levels permitting to monitor the autoimmune reaction.

Prediction of diabetes complications

Both T1DM and T2DM are associated with long-term micro- and macrovascular complications with a devastating impact on life quality and expectancy. The discovery of biomarkers capable of identifying individuals at risk for experiencing serious complications such as retinopathy, nephropathy or cardiovascular disorders would permit to tailor the therapeutic approaches and minimize the expected impact of the disease. However, reliable biomarkers for these long-term complications are still missing.

Cardiovascular complications constitute a main concern since they account for up to 80% of premature mortality in diabetic patients[55]. Prevention, or even a delay, of these complications would represent a major advancement in the treatment of diabetes. As discussed above, Zampetaki and colleagues identified a unique plasma miRNA signature in T2DM patients[47]. Among the miRNAs displaying characteristic changes, the level of miR-126 showed the strongest association with T2DM, and correlated with the occurrence of subclinical and overt artery diseases. Interestingly, another study also reported down-regulation of miR-126 in blood samples obtained from patients suffering from coronary artery disease[56]. This miRNA is highly enriched in endothelial cells where it plays important roles in cell homeostasis and vascular integrity[57, 58]. Moreover, the level of miR-126 released in apoptotic bodies is reduced by chronic exposure of endothelial cells to elevated blood glucose levels[47] making this miRNA an ideal candidate biomarker for monitoring diabetic vascular complications. Another endothelial cell miRNA that deserves further attention is miR-503. The level of this small non-coding RNA is up-regulated in muscle biopsies and in peripheral blood-derived plasma of diabetic patients with limb ischemia[59]. Interestingly, local inhibition of miR-503 in a murine model of limb ischemia was able to improve vascular healing and blood flow recovery[59]. Other circulating miRNAs have also been suggested as diagnostic markers for various cardiovascular diseases (reviewed recently in[60, 61]), but their use in predicting or monitoring cardiovascular complications in diabetic patients remains to be investigated.

Kidney disease affects 20-30% of T1DM and T2DM patients. Microalbuminuria was previously proposed as a biomarker to predict the occurrence of this important complication, but recent studies revealed that a significant fraction of the diabetic patients undergo renal failure before, or even without, detectable microalbuminuria[62-64]. Circulating miRNAs represent a good alternative way of monitoring renal failure in diabetic patients. They are not eliminated by hemodialysis[65] and have already been tested in different renal diseases with promising results in both animal models and human patients[66-68]. Indeed, a correlation was observed between some specific circulating miRNAs and glomerular filtration rate, a well-known parameter of kidney disease progression[66]. Large scale prospective studies focusing on diabetic patients undergoing renal failure will be necessary to identify a specific miRNA profile in plasma or urine predicting the appearance of this complication. Urine represents an ideal source of miRNAs since it can be easily collected in a non-invasive manner and in large amounts. Moreover, urinary exosomes originate from various cell types spanning over the entire urinary track and would be ideally suited to monitor the progression of renal

diseases[69-72]. Indeed, urinary miRNA profiles were recently reported to differ across the stages of diabetic nephropathy[73], suggesting that they may be used as tools to follow the progressive alteration of the renal processes.

To our knowledge, there is at present no published report about the use of circulating miRNAs to predict diabetic retinopathy. However, recent studies have demonstrated an involvement of some specific miRNAs in the development of this complication (reviewed in[32]). These findings may open the door to future investigations aimed at assessing the potential use of miRNAs as predictors of this debilitating condition.

Gestational diabetes

Circulating miRNAs could in principle also be used to identify women at higher risk of developing gestational diabetes. At present, most screening protocols are based on a glucose challenge test performed around the 24-28th gestational weeks. Therefore, interventions such as diet, exercise or medication, are sometimes started as late as the 32nd week of gestation. In a multistage retrospective study, Zhao and colleagues screened miRNAs in serum of women at 16-19 weeks of gestation and identified three miRNAs (miR-29a, -122 and -132) that were deregulated in women developing gestational diabetes prior to detectable changes in blood glucose levels[74]. Placental specific miRNAs can be detected in maternal serum[41, 75]. Thus, it is possible that gestational diabetes may lead to changes in blood miRNAs that differ from those of T1DM or T2DM.

Future directions

The findings described above confirm the attractiveness of miRNAs as novel biomarkers for diabetes. Indeed, changes in the levels of a subset of these small RNA molecules in body fluids promises to provide new clues for early identification of individuals at risk to develop diabetes and their associated complications, for following disease progression or for assessing the efficacy of therapeutic interventions. However, as discussed below, before these goals can be achieved major scientific and technical advances need to be made.

Circulating miRNAs should substitute or complement other routine measurements. Thus, their efficacy in predicting the appearance of diabetes or its complications needs to be systematically compared to already available biomarkers. In particular, it will be essential to scrutinize whether miRNAs are indeed able to provide earlier and/or more precise detection of individuals at risk for

developing the disease or its long-term complications. The situation will hopefully evolve in the future, but for most published studies there is for the moment no obvious advantage of replacing other traditional biomarkers with measurements of circulating miRNAs levels.

Different papers have reported miRNA changes associated with diabetes or its complications but, for various reasons, we are still very far from a consensus about the most relevant miRNAs to be measured. This can in part be attributed to the heterogeneity of the approaches selected by the investigators. Some of the published studies carried out systematic profiling of a large number of miRNAs[47, 49, 50] while others focused on a small group of selected miRNAs that were supposedly more likely to be affected in diabetes[48, 52-54]. This yielded a large number of potentially interesting candidates but most of them still await confirmation by independent researchers and/or in larger cohorts. Standardized protocols for sample preparation, RNA extraction and miRNA analysis are urgently needed to facilitate comparison between different studies and to reach a consensus about the miRNAs most suitable to function as early diabetes biomarkers. In fact, the level of certain plasma miRNAs is greatly influenced by the degree of hemolysis and by cellular contamination, rendering them less suitable as clinical biomarkers[76].

Diabetes mellitus is a complex disorder involving major metabolic alterations and important adaptations in the activity of several organs. MiRNAs are proposed as potential biomarkers for an increasing number of disease states. Therefore, it will be essential to determine whether the detected miRNA changes are exclusively indicative of a prediabetic or diabetic condition or if they are observed also in other physiological or pathological situations[42] such as in response to modifications in the nutritional state, inflammation, autoimmunity, cancer, etc.

At present, the origin of blood miRNAs is largely unknown and their levels do not directly mirror changes in pancreatic β-cells or insulin target tissues occurring in diabetes. MiRNAs can be actively or passively released by a variety of cells and can be carried by membrane-bound vesicles, protein complexes or lipoprotein particles (Fig.1 and 2). So far, the vast majority of studies measured the level of the miRNAs directly in the plasma (or serum). This is obviously a convenient and straight forward approach. However, modifications in the level of miRNAs originating from specific groups of cells that are not in direct contact with the blood or are not very abundant are unlikely to have a significant impact on the pool of plasmatic miRNAs and will probably go undetected. Although technically demanding, protocols allowing a specific assessment of the miRNAs carried by exosomes, protein complexes or lipoproteins will probably furnish more detailed information about

the physiological or pathological status of the organs of interest. Furthermore, it may be possible to affinity-purify membrane-bound vesicles originating from a specific group of cells taking advantage of the presence of characteristic proteins on the vesicle surface[11]. This approach will be particularly appropriate to estimate the residual β-cell mass in newly diagnosed T1DM patients. Indeed, except in the case of a sudden loss of a significant number of β-cells, the miRNAs released by insulin-secreting cells represent only a negligible fraction of all non-coding RNAs circulating in the blood. This problem may be partially overcome by searching for miRNAs highly enriched in β-cells such as miR-375[16, 52]. However, the expression of this particular miRNA is modified in a variety of tumoral cells and several authors have suggested using circulating levels of this non-coding RNA for the diagnosis of different types of cancer[77-80]. Several insulin-secreting cell lines release significant amounts of exosomes[81-83] and we have initial evidence indicating that this is the case also for rodent and human islet cells[84]. Protocols permitting an enrichment of exosomes derived from islet cells would probably improve the detection of specific changes in insulin-secreting cells and provide a better evaluation of the functional β-cell mass.

Conclusions

MiRNAs are emerging as important regulators of gene expression and central players in many physiological and pathological processes. Their presence in extracellular fluids in astonishingly stable forms has led to the idea of using them as biomarkers for a variety of diseases. These properties have attracted also the attention of diabetologists who have initiated the search for miRNAs allowing early detection of T1DM and T2DM and their associated complications. There are certainly still a number of scientific and methodological issues to be addressed before circulating miRNAs can attain the status of diabetes biomarkers, but the available data suggest that they will soon serve as valuable new blood parameters that will help physicians to refine their therapeutic interventions.

Key points

- There is a need for new biomarkers to improve the identification of individuals at risk of developing diabetes and its associated complications, to follow disease progression and/or to assess the efficacy of therapeutic interventions.

- Circulating miRNAs are very attractive biomarker candidates since they can easily be collected, are stable under different storage conditions and can be measured using assays that are specific, sensitive and reproducible.

- Pioneer studies have identified characteristic changes in blood miRNA levels in samples of diabetic patients from different cohorts. However, definitive consensus signatures for T1DM, T2DM or their associated complications remain to be defined.

- Although measurements of circulating miRNAs promise to improve the identification of individuals prone to develop diabetes and its complications, a number of key issues including the determination of the most appropriate blood sampling protocols (serum, plasma, exosomes, etc.) remain to be addressed.

Boxes

Box 1 miRNA biogenesis

miRNAs are generated from intergenic genomic sequences or by intronic regions of protein-coding genes. miRNA precursors belonging to the latter category are therefore co-transcribed with their hosting gene[85]. Most mammalian miRNAs are transcribed by RNA polymerase II as long precursor molecules containing a characteristic stem-loop structure[86]. These primary transcripts are cleaved by the RNase III-type enzyme Drosha to produce hairpin-structured pre-miRNAs of about 70 nucleotides. These molecules are then transported via an Exportin-5-dependent process into the cytoplasm, where they are further cleaved by the endoribonuclease Dicer to generate imperfect miRNA/miRNA* duplexes of ~22 nucleotides. The guide miRNA strand of this duplex is incorporated together with Argonaute proteins into the RNA-induced silencing complex (RISC). The passenger miRNA* strand is usually degraded but can sometimes also be loaded into the RISC complex and be functional. The majority of the miRNAs are generated by this canonical pathway, but alternative pathways have recently been described[87, 88]. Mature miRNAs exert their action by guiding the RISC complex to complementary sequences within the 3'UTR of target mRNAs, leading to translational repression and/or transcript degradation[89]. Target recognition is mainly determined by miRNA complementarity with the so-called "seed sequences" (i.e. residues 2-8) of target mRNAs[89, 90]. Interestingly, a single miRNA can potentially bind and regulate the expression of hundreds of targets, whereas a single 3'UTR region can be targeted by numerous different miRNAs[90].

Box 2 Exosomes

Exosomes are microvesicles of 30 to 120 nm that were first observed to be released by reticulocytes in 1980s[91-93]. Since then, exosomes have been detected in the culture media of most cell types and were abundantly found in several body fluids like blood, urine, saliva and breast milk[40]. Vlassov et al. have estimated the concentration of exosomes in the blood to be around 3 million per microliter[94]. These vesicles display a characteristic size, form and lipid bilayer structure that can be visualized by electron microscopy. Exosome markers, such as the tetraspanins CD63 and CD81 and the Escort proteins Alix and Tsg101, are also routinely used to distinguish exosomes from other vesicles secreted by cells. Detailed analysis of the exosome content revealed that they can carry proteins and

nucleic acids (mRNAs, miRNAs and long non-coding RNAs)[36, 95]. The interest in exosomes increased dramatically in the past 5 years, after the discovery that their cargo can be transferred in active form to recipient cells[36, 96, 97], leading to the exciting concept of exosomes as messengers of a new cell-to-cell communication mode. However, the precise mechanisms leading to exosome release and/or uptake remain to be elucidated. Exosomes are produced inside cells via the multivesicular endosomal pathway[92, 93] but very little is known on the selective packaging of nucleic acids and proteins composing the exosome cargo. A better understanding of these mechanisms may reveal new biological roles for exosomes in healthy state, but also in pathophysiological conditions.

Figure legend

Fig.1 : Biogenesis and release of miRNAs

MiRNA precursors (pre-miRNAs) are generated in the nucleus by the RNase III enzyme Drosha after cleavage of primary RNA transcripts (pri-miRNAs) (1). The pre-miRNAs are then transported in the cytoplasm through a process involving Exportin-5 and the GTP-binding protein Ran (2) and further cleaved by Dicer to yield 21-23 nucleotide duplexes (3). One strand of the miRNA duplex can either associate to the RISC complex and guide translational repression of target mRNAs (4) or be released by the cells. In the latter case, the mature miRNA binds to RNA-binding proteins such as Argonaute 2 (5) or to lipoproteins (6). Alternatively, the miRNAs can be loaded in microvesicles formed by plasma membrane blebbing (7) or in exosomes that are released in the extracellular space upon exocytic fusion of multivesicular bodies with the plasma membrane (8).

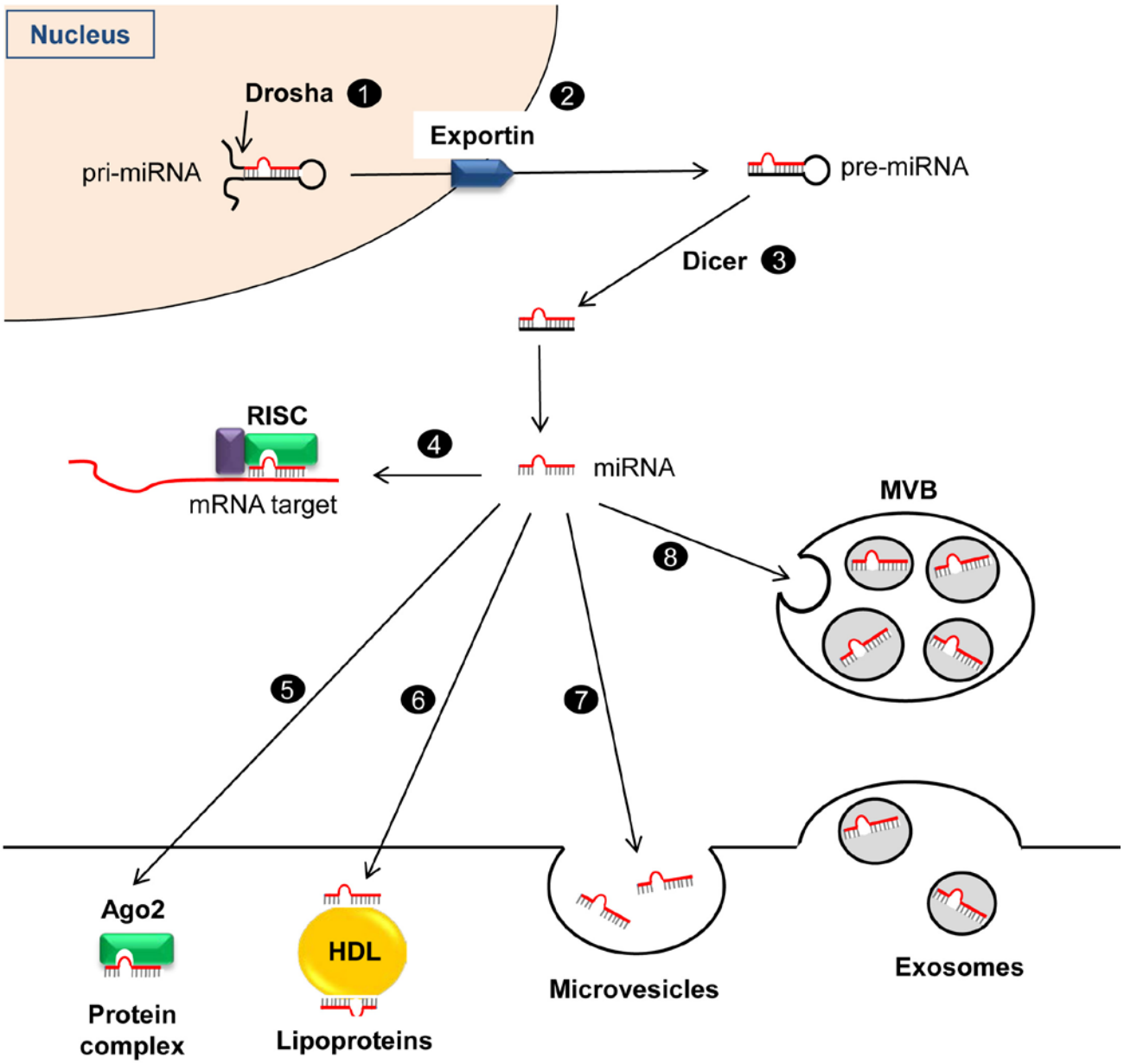
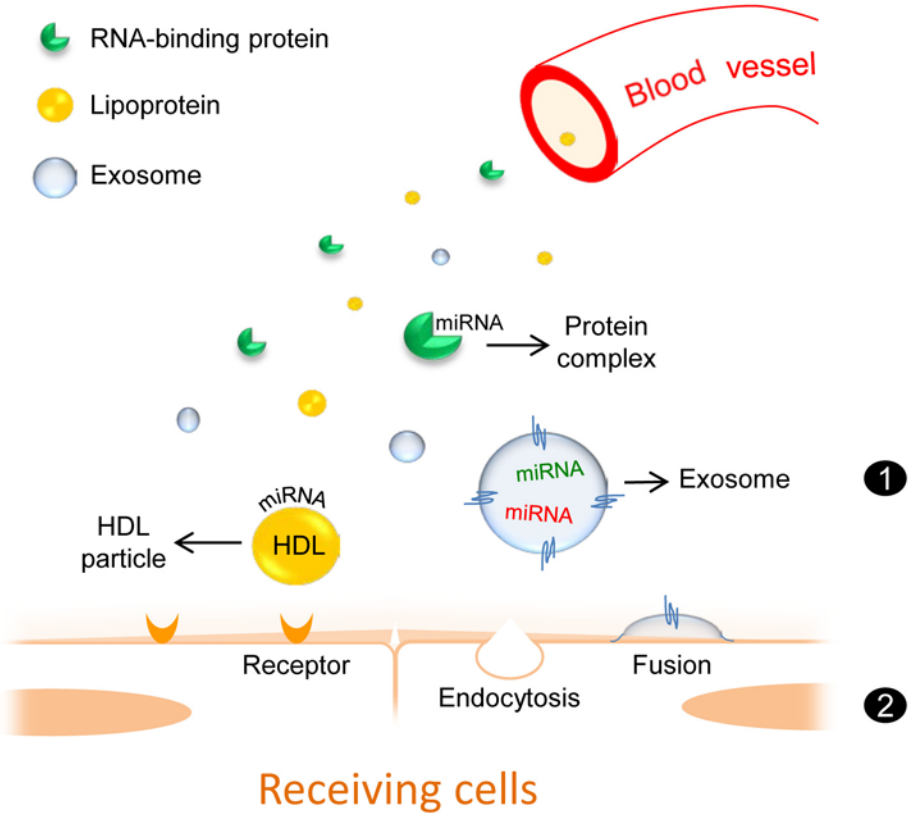

Nucleus
Drosha
1
2
Exportin
pri-miRNA
pre-miRNA
Dicer
3
RISC
4
mRNA target
miRNA
MVB
8
5
6
7
Ago2
HDL
Microvesicles
Exosomes
Protein complex
Lipoproteins

Fig.2: Blood and other body fluids contain active miRNAs

MiRNAs can be released or shed by cells and are found in stable form in body fluids. The miRNAs present in the blood are associated with protein complexes including Argonaute 2, or with HDL particles, or are transported inside membrane-bound vesicles such as exosomes (1). There is evidence that circulating miRNAs can be taken up in active form through different mechanisms including receptor-mediated capture, endocytosis or fusion of exosomes with the plasma membrane of receiving cells (2). Transfer of miRNAs between distantly located cells constitutes a potentially new communication mode.



Tables

Table 1 : Blood parameters and biomarkers currently used to predict and diagnose T1DM and T2DM

Type of diabetes	Blood parameters/Biomarkers		
	Type	Name	Symbol
T1DM and T2DM	Blood parameters	Glycaemia	-
		Glycated hemoglobin	HbA1c
T1DM	Autoantigens	Glutamate decarboxylase	GADA
		Insulin	IAA
		Islet cells	ICA
		Tyrosine phosphatases	IA-2, IA-2β
		Zinc transporter 8	ZnT8
T2DM			
	Traditional biomarkers	Cholesterol	-
		Creatinine	-
		Free fatty acids	FFA
		α-hydroxybutyrate	α-HB
		Lipoproteins	HDL
		Triacylglycerol	Tg
	Novel biomarkers	Adipokines	-
		C-reactive protein	CRP
		Cytokines	-
		Ferritin	-
		Incretins	GLP-1
		Linoleoylglycero-phosphocholine	L-GPC

Table 2 : Blood miRNA changes associated with diabetes mellitus

Study design	Source	Method of analysis	miRNAs identified	Observations	Ref.
T2DM					
800 ind. from Bruneck cohort	Plasma	microarray profiling (confirmed by qPCR)	miR-15a, -28-3p, -29b, -126 and -223	First study to suggest a blood miRNA signature for T2DM	47
56 ind. with health conditions related to DM	Serum	qPCR on specific miRNAs	miR-9, -29a, -30d, -34a, -124a, -146a and -375	Deregulated in T2DM	48
265 ind. with health conditions related to metabolic syndrome	Blood	microarray profiling (confirmed by qPCR)	miR-150, -192, -27a, -320a and -375	Correlation between elevated FG and altered levels of miR-27a and -320a	49
T1DM					
Danish and Hvidoere cohorts of newly diagnosed children	Serum	microarray profiling (confirmed by qPCR)	miR-24, -25, -26a, -27a, -27b, -29a, -148a, -152, -181a, -200a and -210	miR-25 negatively correlated with residual β-cell function	50
20 newly diagnosed T1DM patients	Serum	microarray profiling (confirmed by qPCR)	miR-9, -31, -34a, -146a, -155, -181a and -199a	Deregulated in T1DM	51
20 patients with T1DM	PBMC	qPCR on specific miRNAs	miR-21a and miR-93	Underexpressed in T1DM	53
19 patients with T1DM	Lympho-cytes	qPCR on specific miRNAs	miR-326	Correlation with islet autoimmune attack	54

Abbreviations: Ref = references, ind.= individuals, DM = diabetes mellitus, T1DM = Type 1 diabetes mellitus, T2DM = Type 2 diabetes mellitus, FG = fasting glucose and PBMC = peripheral blood mononuclear cells.

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Review criteria

We systematically searched Pubmed using different combinations of the following words: diabetes, type 1 diabetes, type 2 diabetes, gestational diabetes, diabetes complications, diabetic retinopathy, diabetic nephropathy, cardiovascular complications, biomarkers, microRNAs, circulating miRNAs and exosomes. We also scrutinized the appropriate references cited in the selected papers. Two abstracts from the EASD meeting 2012 held in Berlin were also included. Because of space limitations, not all original articles could be listed in the present review. Whenever possible, the most recent and complete reviews on the topic were chosen. All selected articles were in English.

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