CATÓLICA
INSTITUTO DE CIÊNCIAS DA SAÚDE
LISBOA·PORTO·VISEU

MIRNAS SALIVARES COMO BIOMARCADORES DA DIABETES MELLITUS TIPO II (DMTII)

Dissertação apresentada à Universidade Católica Portuguesa para obtenção do grau de mestre em Medicina Dentária

Por:
Alexandra Martins

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Orientador: Professora Doutora Maria José Correia
Coorientador: Professor Doutor Luís Silva Santos

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Resumo

Objetivo: Estudar o potencial dos miRNAs salivares como biomarcadores da T2DM, pela revisão sistemática da evidência disponível sobre alterações de expressão de miRNAs associadas a T2DM e identificar, os miRNAs que, por terem expressão alterada em T2DM e terem já sido detetados na saliva são candidatos promissores à utilização como biomarcadores salivares da T2DM.

Materiais e Métodos: Pesquisa sistemática nas bases de dados PubMed e Web of Science para identificação de estudos em que os miRNAs tenham sido quantificados em pacientes com T2DM e construção de uma base de dados com a anotação dos resultados desses artigos. Análise estatística dos resultados globais para verificar quais os miRNAs que podem configurar biomarcadores T2DM. Verificar dos miRNAs identificados se alguns já foram identificados em fluidos orais e/ou quais os que podem ser encontrados nestes fluidos.

Resultados: Foram encontrados 776 artigos não duplicados que referem miRNAs como biomarcadores para T2DM, dos quais foram selecionados 115 sendo apenas 31 incluídos nos resultados, pois preenchem os critérios de inclusão definidos. Da análise desses artigos, verificou-se que 53 miRNAs foram encontrados como estando alterados em amostras de pacientes diabéticos *vs.* amostras de pacientes com tolerância normal à glicose, 17 estão alterados quando se comparam pacientes com tolerância alterada à glicose (pré-diabéticos) *vs*. pacientes com tolerância normal à glicose e 13 estão alterados quando se comparam pacientes diabéticos *vs*. pacientes com tolerância alterada à glicose (pré-diabéticos). Entre estes três grupos de comparações há 49 miRNAs que propomos como potenciais biomarcadores da DMT2. Destes encontram-se na saliva 41 miRNAs, dos quais os melhores candidatos a biomarcadores são: miRNA 125b; miRNA 519c-3p; miRNA 519d-3p; miRNA 802; miRNA 486-5p; miRNA 136-5p, miRNA 369-3p, miRNA 411-5p, miRNA 487a-3p, miRNA 487b-3p, miRNA 655-3p, miRNA 656-3p, miRNA 432-5p, miRNA 593-3p e miRNA 128, porque apresentam expressão consistentemente alterada em pacientes com DMT2.

Conclusão: De momento, não há consenso sobre miRNAs específicos para a Diabetes Mellitus Tipo II. Contudo, a análise bioinformática realizada permite propor um conjunto de miRNAs que devem ser averiguados como potenciais biomarcadores de DMT2.

Palavras-chaves: miRNAs, Diabetes Melitos Tipo II, Saliva

Abstract

Objectives: Study the potential of miRNAs salivary as biomarkers of T2DM, by the systematic review of the evidence available on changes in the expression of miRNA associated with T2DM and identify the miRNAs which have their expression altered in T2DM and have been detected in saliva, making them promising candidates to be used as salivary biomarkers in T2DM.

Methods: Systematic search in PubMed and Web of Science databases to identify studies where miRNAs have been quantified in patients with T2DM and to build a database with the annotation of the results of these articles. Statistical analysis of the global results to determine which miRNAs can become T2DM biomarkers. Check if some of the identified miRNAs have been identified in oral fluids and / or which can be found in these fluids.

Results: Were found 776 unduplicated articles mentioning miRNAs as biomarkers for T2DM, of which 115 were selected but only 31 included in the results because they fulfilled the inclusion criteria.

From the analysis of these articles, 53 miRNAs were found to be altered in samples from diabetics *vs.* samples from patients with normal glucose tolerance, 17 are changed when comparing patients with altered glucose tolerance (prediabetes) *vs.* patients with normal glucose tolerance and 13 are altered when comparing diabetics *vs.* patients with altered glucose tolerance (prediabetes).

Among these three group comparisons, 49 miRNAs that we propose as potential biomarkers of T2DM exist. These 41 are in saliva, of which the best's potentials biomarkers are miRNA 125b; miRNA 519c-3p; miRNA 519d-3p; miRNA 802; miRNA 486-5p; miRNA 136-5p, miRNA 369-3p, miRNA 411-5p, miRNA 487a-3p, miRNA 487b-3p, miRNA 655-3p, miRNA 656-3p, miRNA 432-5p, miRNA 593-3p and miRNA 128, because they are involved in several pathophysiological processes of T2DM.

Conclusion: At the moment, there is no consensus about having exclusive miRNAs for Diabetes Mellitus Type II. However, the bioinformatics analysis done in this work allows the proposal of a group of miRNAs that should be investigated as potential biomarkers of T2DM.

Key Words: miRNAs, Diabetes Mellitus Type II, Saliva

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Index of abbreviators

NGT – Normal Glucose Tolerance

IFG - Impaired fasting glucose

T2DM - Type 2 diabetes mellitus

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Introduction

Diabetes Mellitus Type II

Diabetes Mellitus is the most frequent metabolic disease in the actual society (1). In 2014, according to the World Health Organization (WHO), 9% of adults over the age of 18 had diabetes. Diabetes Mellitus is expected to be the 7th leading cause of death worldwide in 2030 (2). In Portugal in 2014, according to the annual report of the National Observatory of Diabetes 2015, 13, 1% of the population had Diabetes Mellitus (where only 7, 4% is diagnosed), prevalence being higher in males (3).

Type 2 Diabetes Mellitus (T2DM) is clearly the most common form of diabetes mellitus (90-95%) (4). It is characterized by hyperglycemia, that it, fasting plasma glucose levels > 7.0 mmol/L (126 mg/dL), resulting from an inadequate response by insulin-sensitive tissues (liver, skeletal muscle and adipose tissue) to normal circulating levels of insulin – a phenomena usually referred to as insulin resistance (5).

T2DM etiology is multifactorial, resulting from both genetic and environmental/lifestyle-related factors: several genes (e.g. IRS1, TCF7L2, HNF4A, HNF1B, KCNJ11, PAX4, IGF2BP2, CAPN10, AKT2 and IL6) have been demonstrated to interfere with the management of glycemic control and other factors – such as over nutrition, hypertension, sedentary lifestyle, age over 40 years, previous gestational diabetes, medications and family history – also play a very important role. Interactions between several of these factors may occur (5–7).

Under normal circumstances, increased glucose availability in the pancreas – e.g. after a meal – stimulates pancreatic β-cells to secrete insulin, a hormone critical for the regulation of glucose transport and metabolism in target tissues such as liver, skeletal muscle and adipose tissue. In the liver, insulin will inhibit gluconeogenesis and glycogenolysis and increase hepatic glycogen synthesis, thus suppressing hepatic glucose output to the bloodstream and contributing to the return to a normoglycemic state. In skeletal muscle, insulin increases glucose uptake and utilization. Finally, in adipose tissue, insulin inhibits lipolysis and upregulates lipogenesis (5) (Figure 1).

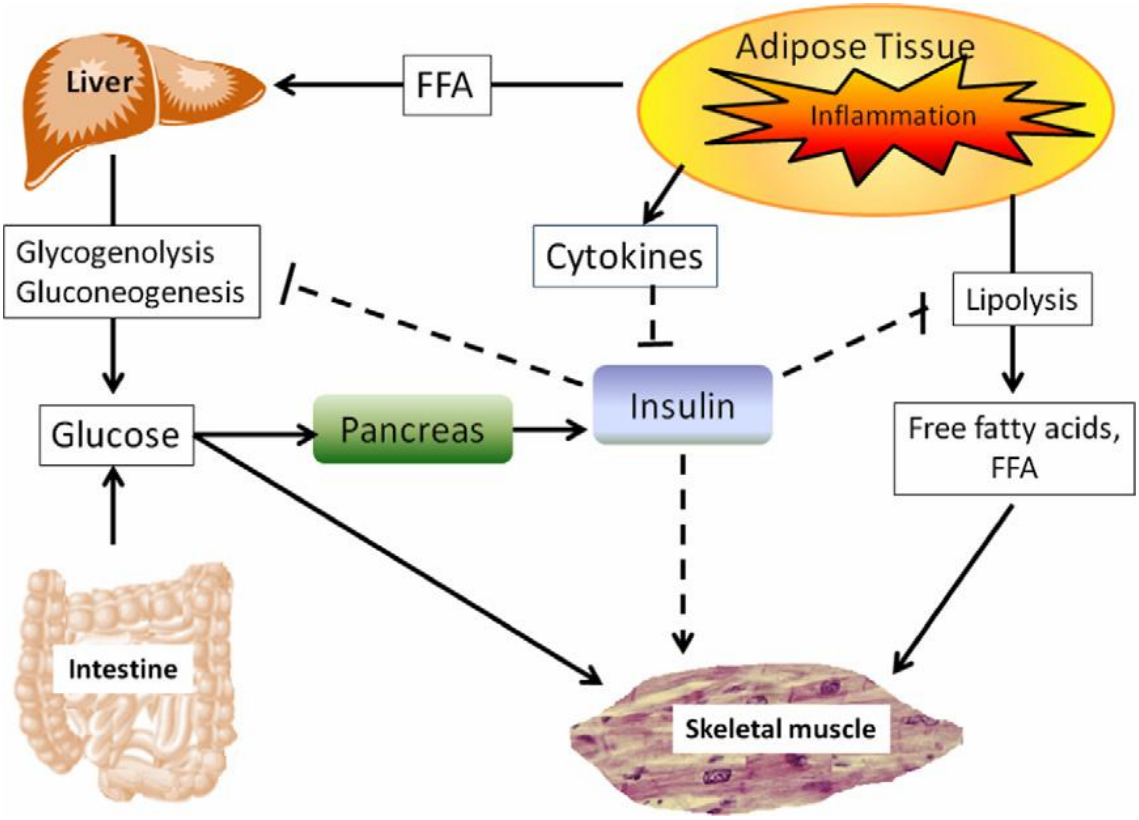

Figure 1 Mechanism of insulin and insulin resistance. Adapted from Marc Y. Donath (2011).

Chronically elevated plasma levels of glucose and free fatty acids – a consequence of over nutrition – will stress pancreatic islets and insulin-sensitive tissues, promoting insulin resistance in such tissues, mainly through downregulation of insulin receptor protein level (5). The pancreatic islets respond by enhancing their cell mass and insulin secretory activity (functional expansion of islet β-cells). As the disease progresses, β-cells gradually fail to produce sufficient levels of insulin and therefore to fully compensate for the degree of insulin resistance (8). A state of islet β-cell dysfunction develops where deficient insulin action results from inadequate insulin secretion and/or diminished tissue responses to insulin (2,3). Insulin resistance is therefore characterized by decreased glucose uptake in skeletal muscle, increased glucose production in the liver and increased lipolysis in adipose tissue, resulting in increased plasma levels of glucose and free fatty acids (FFA) (Figure 1). T2DM has an insidious progression (4), insulin resistance typically being present since early prediabetes stages (6). However, insulin resistance does not necessarily evolve to T2DM, as β-cells are seldom able to compensate for the increased request for insulin. The causes for this heterogeneity are incompletely understood, although genetics and epigenetics may play a role (8).

Inflammation is intimately associated with the development of insulin resistance. In fact T2DM pathogenesis: increased plasma levels of glucose and FFA – as a result of over nutrition or already established insulin resistance – induce a pro-inflammatory response through stimulating the production of cytokines and chemokines (e.g. IL-1β, IL-6, TNF-α) (5, 9) (Figure 2) in insulin-sensitive tissues (mainly adipose tissue) and their release into the circulation. These mediators impair insulin signaling in insulin-sensitive tissues and drive inflammation in other tissues such as pancreatic islets (8) (Figure 3). Among these cytokines, interleukin IL-1β plays a critical role, through stimulation of β cells apoptosis, inhibition of cell proliferation and stimulation of the production of other chemokines such as CC-chemokine ligand 2 (CCL2), CCL3 and CXC-chemokine ligand 8 (CXCL8). Also, IL-6 induces C-reactive protein, a well-known marker of systemic inflammation. On the contrary, the production of anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1RA) by β cells (and, as a consequence, of IL-10) is decreased. In result, immune cells will be recruited and contribute to tissue inflammation. Interestingly, a common pro-inflammatory metabolic profile is also observed in individuals with metabolic syndrome, a non-consensual concept characterized by hyperglycemia, abdominal obesity, hypertension, elevated plasma triglycerides and reduced plasma HDL cholesterol levels. Metabolic syndrome patients frequently present with insulin resistance and exhibit an increased risk of developing T2DM and cardiovascular disease, suggesting a strong interconnection between these diseases (5).

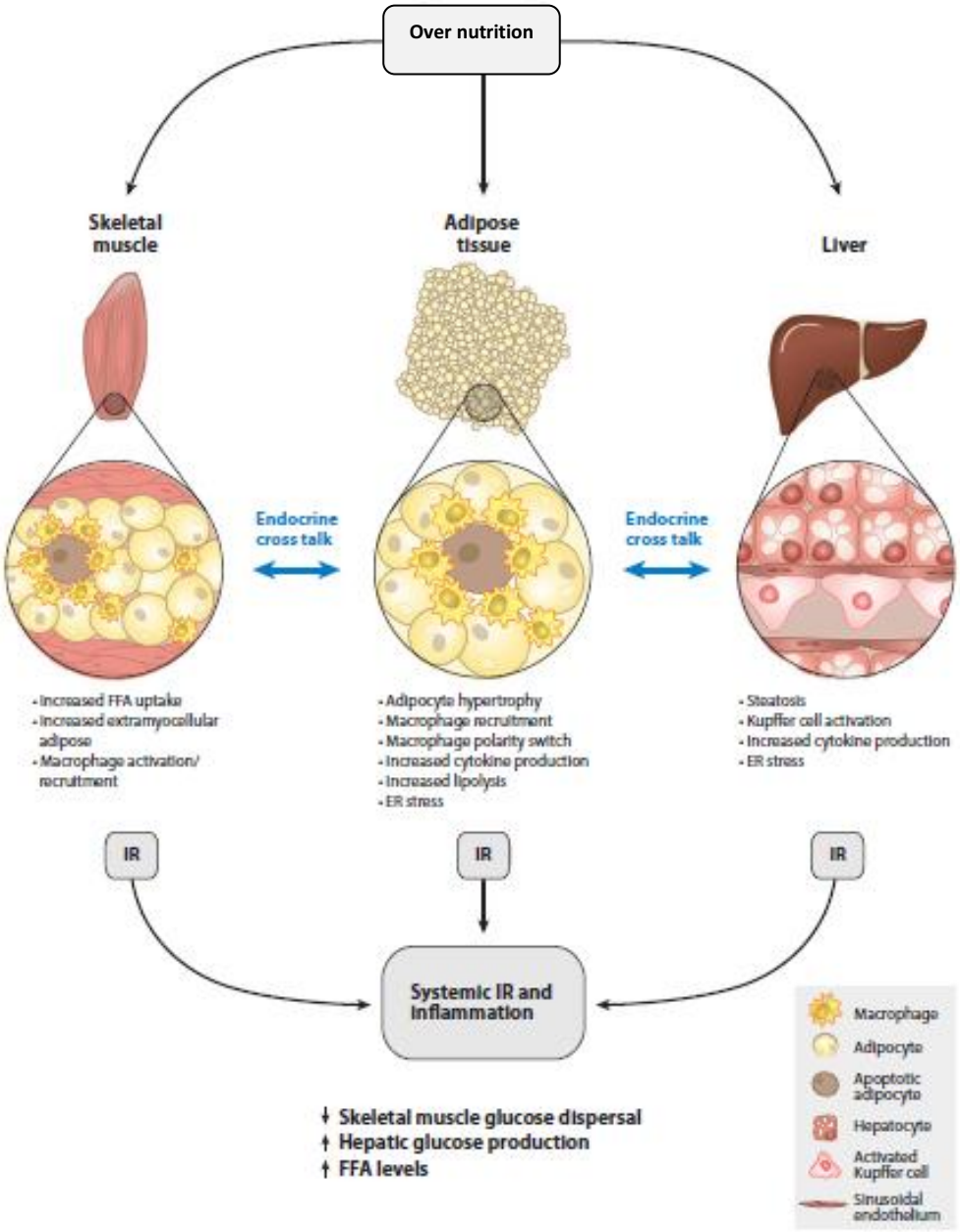

Figure 2 Mechanism of insulin resistance. Adapted from Olefsky (2010).

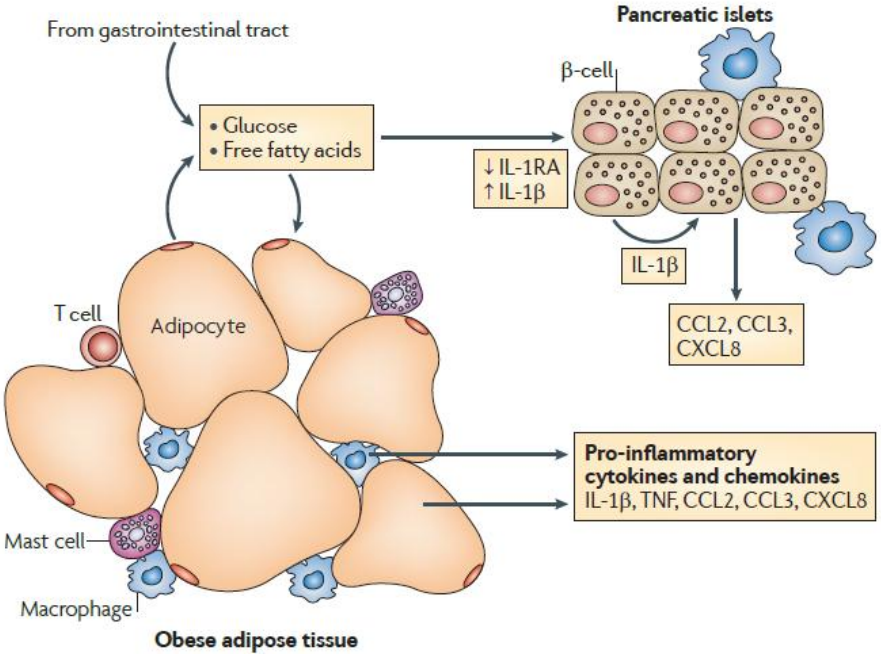

Figure 3 Activation in adipose tissue. Adapted from Marc Y. Donath (2011).

Dietary measures and physical exercise comprise the first line options for the prevention and treatment of T2DM. Oral hypoglycaemic agents may also be used to control serum glucose levels, opposing insulin resistance in diabetic patient's cells. However, none of the pharmacological agents currently in use takes into account the molecular interaction networks leading to the long-term metabolic complications of the disease, which may arise as a consequence of glucose underutilization in tissues (12,3). Such complications – which include atherosclerosis, heart disease, stroke, neuropathy, kidney disease, vision disorder, peripheral vascular disease, ulcerations and amputations, infection, digestive diseases, oral complications and depression – are responsible for the increased morbidity of this disease. Considering the high prevalence of T2DM – especially in developed countries – its association with cardiovascular and inflammatory diseases and its overall burden to public health, it is important to clarify the molecular mechanisms that are involved in the development, progression and prognosis of this disease, so that better diagnostic and rational therapeutic tools may be developed. MiRNAs which have been demonstrated to influence insulin secretion (through the control epigenetic factors like DNA methylation) (8,9) and to contribute to pancreatic β-cell development, adiposity differentiation, energy homeostasis, and sugar and lipid metabolism (13), may be of great usefulness in this respect.

MiRNAs

Currently it is known that a large number of genes don't encode proteins but miRNAs. MicroRNAs (miRNA) are small molecules of non-coding RNAs (not translated into proteins) that function as gene regulators at the post transcriptional level (13, 14).

In the beginning, inside the nucleus, the nascent miRNA is transcribed (forming the pri-miRNA) by the enzyme RNA polymerase II. Afterwards Drosha (enzyme ribonuclease III) cleaves the stem-loop precursor from flanking pri-miRNA transcript sequences to form the pre-miRNA (14,15) (Figure 4 and 5).

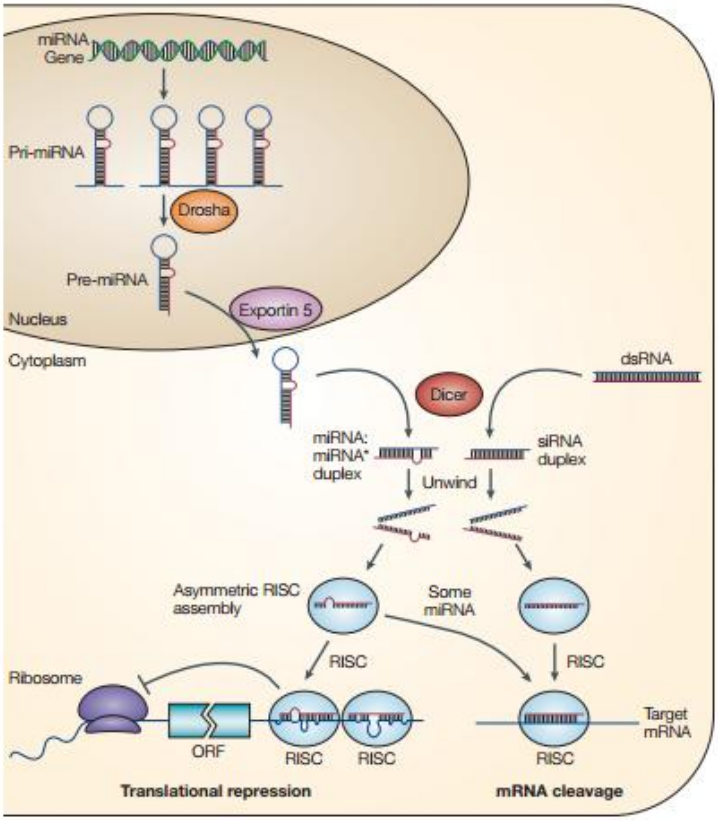

Figure 4 Biogenesis of miRNAs. Adapted from Lin He (2004).

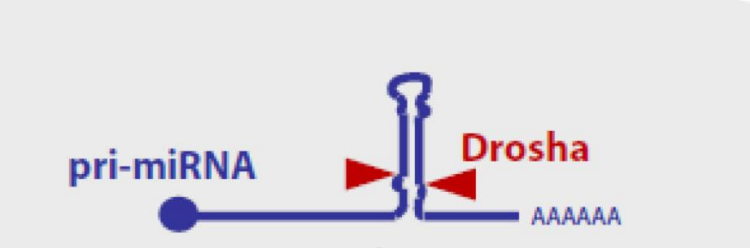

Figure 5 The pri-miRNA transcript is cut by the enzyme Drosha yielding the precursor pre-miRNA. Adapted from Hammond SM (2015).

Subsequently, the pre-miRNA is transported to the cytoplasm by the Exportin 5 protein Ran-GTP dependent nucleo/cytoplasmic cargo transporter (14,15) (Figure 4).

In the cytoplasm, the Dicer enzyme made up of the helicase domain, PAZ domain, RNase III domain, dsRNA binding, unknown domain, DUF 283 domain and Platform domain (Figure 6), cleaves the pre-miRNA. A miRNA duplex (imperfect stem-loop structures) is formed giving the miRNA duplex the capacity to unwind (14,16) (Figure 7).

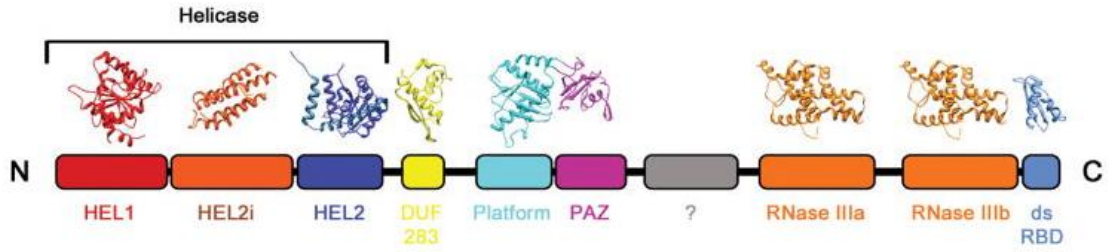

Figure 6 Domain structure for the Dicer protein. Adapted from Lau PW (2012).

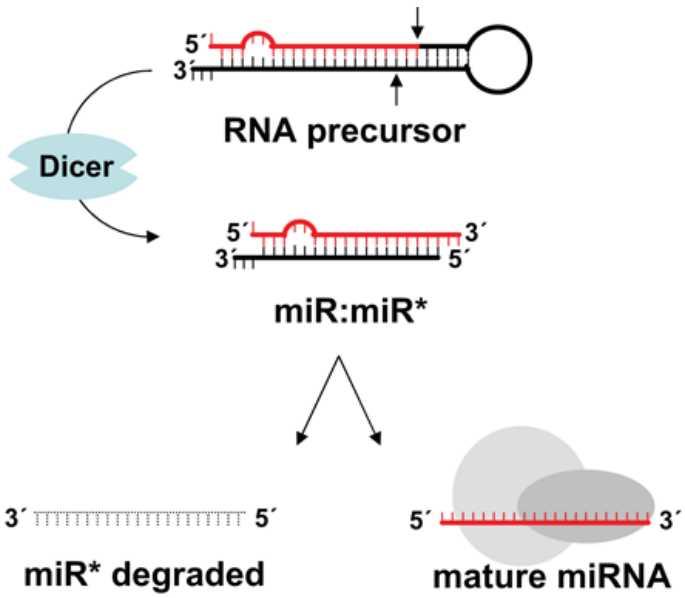

Figure 7 miRNA duplex. Adapted from Schwarz (2006).

The miRNA duplex is incorporated into the RNA induced silencing complex (RISC). This complex is includes the Argonaut, Dicer and TRBP proteins. (14,15) (Figure 8).

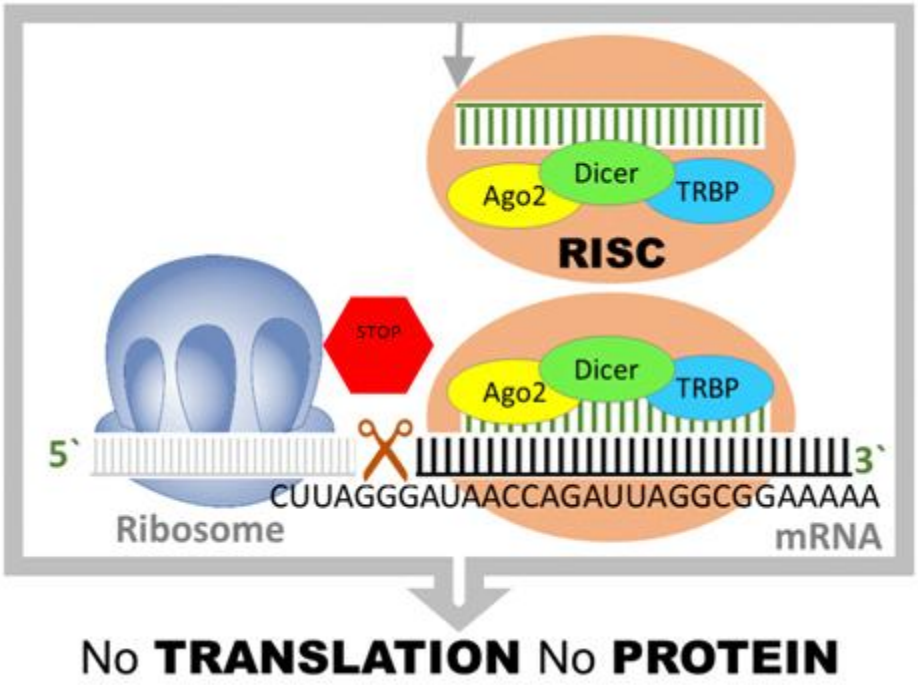

Figure 8 Complex RISC. Adapted from Mississippi State University (2016).

When the miRNA is incorporated in the RISC complex it interacts with the 3 UTRs of the target mRNA by imperfect base pairing. Besides that, the relative instability at the 5′ end of the mature miRNA might facilitate its preferential incorporation into the RISC (15).

However, the thermodynamic properties of the miRNA precursor determine the asymmetrical RISC assembly, and therefore, the target specificity for posttranscriptional inhibition (Figure 8). In rare cases in which miRNA and miRNA* have similar 5′-end stability, each arm of the miRNA precursor is predicted to be assembled into the RISC at similar frequencies. This prediction has been confirmed by similar recovery rates for such miRNAs and miRNA*s from endogenous tissues (14,16).

The molecular mechanism that explains the post transcriptional repression by miRNAs still unknown, but what happens is that the RISC complex targets the mRNAs (messenger RNA) which leads to translational repression (temporary) or destroys the mRNA by cleavage and degradation (15). On other hand, the RISC complex due to imperfect base pairing of its seed sequence with the target mRNAs, is capable of

regulating hundreds of mRNAs with a single miRNA. Therefore the miRNAs can inhibit the expression of several genes, preventing the translation of mRNAs and regulating various signaling pathways simultaneously (18,19,20).

On the other hand, when we have exogenous miRNAs such as dsRNA corresponding to virus or drugs (therapeutic), the process of protein translation inhibition is similar to endogenous miRNAs, albeit some differences. The structure of dsRNA is regular, without loops and is cleave by Dicer to form the precursor siRNA duplex. As the siRNA duplex has an uniform structure it is preferentially assembled into RISC, binding the mRNA more effectively, leading to post-transcriptional repression by miRNA cleavage and degradation (15) (Figure 4).

Functions

MiRNAs regulate gene transcription thus playing a key role in many biological processes. Each individual miRNA may be involved in the regulation of more than one mRNA and each mRNA in turn may be regulated by multiple miRNAs (21).

MiRNAs potentially regulate the expression of thousands of human genes, of which many are involved in major cell functions such as transcriptional regulation or other basic cellular functions such as proliferation, differentiation, apoptosis, immunity and stress response (22,23). The specific function of miRNAs may depend on the microenvironment of a particular cell type, which provides different repertoires of target genes (23). For example, miRNA let-7a is overexpressed in skeletal muscle reducing IL-13 secretion and consequently, glycogen synthesis is reduced in T2DM patients. In adipose tissue, overexpression of let-7a increases HMGA2 secretion, reducing adipogenesis in pre-adipocytes (24).

MiRNA application´s

Studies suggest that miRNAs control almost every biological process, and their aberrant expression leads to a disease state. Differential expression of miRNAs in a disease versus normal cells has generated enormous interest for the development of miRNA-based pathologic cell-targeted therapeutics (25).

MiRNAs have been found to exert a great influence on pathophysiological mechanisms of various diseases as cancer, viral infections, nervous system disorders, cardiovascular diseases muscular disorders, rheumatic diseases and kidney diseases (13,27).

Experimental studies propose the utility of nanoparticles as ideal carriers for miRNA molecule because of their ability to load miRNA molecules, protect miRNAs from serum environment and ultimately deliver them to target cells (25).

Additionally, since many of the proposed biomarkers for various diseases are proteins, it is important to understand how the expression of miRNAs can affect the levels of these biomarkers. Although, miRNAs have been found intracellular and their function has been described mainly within the cell, recently these molecules have been described in fluids such as blood, saliva, urine, breast milk, tears, cerebrospinal fluid, amniotic fluid and semen (20,25).

Salivary miRNAs as disease biomarkers

Extracellular and salivary miRNAs

Extracellular miRNAs have been found in all human body fluids (plasma, serum, saliva, urine, seminal plasma, breast milk, tears, amniotic fluid, and other examples). However, most RNAs in the extracellular environment are unstable because of the rich RNase activity. However, extracellular miRNA in the body can be encapsulated in small vesicles called exosomes, impermeable to RNases (28,29).

Many biological components in the blood, such as miRNAs, appear in the saliva by ultra-filtration through the salivary glands. Saliva is one of the body fluids with the highest number of detectable miRNAs. However, some miRNAs are specific to saliva, and not detectable in other body fluids (29).

Additionally, some studies referred the existence of exosome-associated miRNAs which are involved in intercellular communication to distant sites of the body. For example, some cells of the oral cavity may take up exosomal miRNAs from the saliva in order to regulate physiological conditions such as oral homeostasis. The exosomes are secreted from donor cells and taken up by recipient cells, resulting in the downregulation of target genes by function miRNAs in the exosomes (29).It is not clear if the best miRNAs biomarkers candidates are vesicle-free or in the exosomes (29).

Saliva as diagnostic fluid

The level of microRNAs in saliva and other body fluids (blood, urine, sweat) increases as a consequence of altered pathophysiological mechanisms and tissue insult (30).

Saliva has an advantage as a diagnostic fluid because it is simple to collect, convenient to store, non-invasive, it doesn´t need specialized individuals for collection, the equipment needed is simple and collection cost is low when compared to the blood, saliva doesn´t coagulate and contains high-quality DNA. However, it's important for future studies to understand the correlations between oral health and systemic health, because the environmental of oral cavity is distinct and immune biomarkers are influenced by processes of local immunity (34,34,35).

The study and quantification of these molecules exhibits some advantages when compared to protein markers that are related to the greater stability of the miRNA as

compared to the proteins, which makes easier to quantify them in the various types of sample. In pediatric patients or handicapped patients, saliva collection reduces anxiety and discomfort, something that would be difficult with blood collection (19,27).

For the health professional it is safer as there is a lower the risk of infection during collection (33). For the reasons mentioned it becomes important to check the potential of miRNAs present in saliva for comprehension and management of T2DM (19,27).

Salivary miRNAs data may eventually provide excellent and clinically relevant information that will add value to the processes of early detection of disease, treatment monitoring, recurrence prediction and other prognostic outcome assessments. Salivary biomarkers are well positioned for translational and clinical applications (development of omics-based tests and saliva-based point-of-care technologies) (35,36).

Rationale for assessing T2DM-associated miRNAs in saliva

Currently, there are some studies referring exclusive miRNAs as biomarkers in prediction, detection, monitoring of its complications and evaluation of treatment efficacy in T2DM (20).

Some authors suggest, that an abnormal activity of various miRNAs involved in pancreatic gene regulation, insulin secretion, beta-cell differentiation and regeneration, contribute to T2D pathophysiology. For example, the adipose tissue expression of some miRNAs associated with insulin sensitivity along with many other miRNAs and their targets – in both the pancreas and key insulin target tissues, likely play an important role in the pathogenesis of T2DM (37).

Furthermore, the serious impact of extracellular miRNAs on the development of diabetes needs also to be determined by studying their role in cell communication between insulin-sensitive tissues. During the beginning of the disease, secreted miRNAs derived from these tissues may be transferred to other cells for the induction of adiposity, insulin-resistance, alteration of pancreatic insulin release, inflammation, endothelium dysfunction or for the regulation of hepatic lipid homeostasis (38).

A study of Amanda Michael *et al*. (2010) showed that exosomes can be easily isolated from saliva, and that these exosomes contain microRNAs in quantities adequate for both qPCR and microarray. Additionally, exosomal microRNAs didn't suffer alteration by bacteria's, periodontal status and numbers nucleases in whole saliva (39).

In spite of the fact that there are many studies focusing on miRNA expression in diabetes mellitus it is often difficult to compare them because of the heterogeneity of the methodology used (20).

Objectives

General objective:

- Identify a set of miRNAs to be evaluated as potential biomarkers for type II diabetes mellitus (T2DM).

Specific objectives:

- To study the potential of salivary miRNAs as biomarkers in T2DM.
- To revise the published results on miRNAs with altered expression in T2DM, building a database with the annotation not only with the measurements of the changes, but also of the types of samples and donors used.
- To execute a statistical analysis that allows, from the data gathered in this base, to propose which of the miRNAs are more promising as biomarkers for early diagnosis of T2DM.
- To use the miRNAs public databases to verify which proteins are regulated by miRNAs more differentially expressed in T2DM and which information can be inferred about the pathophysiology of the disease and / or the utility of the proposed protein biomarkers.

Relevance/potential utility:

- Translation of saliva-based biomarker assessment to clinical practice will allow the development of fast, noninvasive and inexpensive omics-based screening tests for early diagnosis and personalized T2DM management.

Material and Methods

Systematic review

Search strategy

PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) and Web of Science (WOS, https://login.webofknowledge.com) online databases were used to obtain studies identifying miRNAs differentially expressed between T2DM patients, prediabetes individuals and/or healthy controls.

The MeSH terms "Diabetes Mellitus, Type 2", "Prediabetic State" and "MicroRNAs", their MeSH synonyms, as well as the terms "Insulin resistance", "Hyperinsulinemia", "Glucose intolerance" and "Hyperglycemia" were used as keywords, combined in the following query: ((NIDDM) OR (Maturity-Onset Diabetes) OR (Diabetes Mellitus, Noninsulin-Dependent) OR (Diabetes Mellitus, Adult-Onset) OR (Adult-Onset Diabetes Mellitus) OR (Diabetes Mellitus, Adult Onset) OR (Diabetes Mellitus, Ketosis-Resistant) OR (Diabetes Mellitus, Ketosis Resistant) OR (Ketosis-Resistant Diabetes Mellitus) OR (Diabetes Mellitus, Maturity-Onset) OR (Diabetes Mellitus, Maturity Onset) OR (Diabetes Mellitus, Non Insulin Dependent) OR (Diabetes Mellitus, Non-Insulin-Dependent) OR (Non-Insulin-Dependent Diabetes Mellitus) OR (Diabetes Mellitus, Noninsulin Dependent) OR (Diabetes Mellitus, Slow-Onset) OR (Diabetes Mellitus, Slow Onset) OR (Slow-Onset Diabetes Mellitus) OR (Diabetes Mellitus, Stable) OR (Stable Diabetes Mellitus) OR (Diabetes Mellitus, Type II) OR (Maturity-Onset Diabetes Mellitus) OR (Maturity Onset Diabetes Mellitus) OR (MODY) OR (Type 2 Diabetes Mellitus) OR (Noninsulin-Dependent Diabetes Mellitus) OR (Insulin resistance) OR (Hyperinsulinemia) OR (Glucose intolerance) OR (Hyperglycemia) OR (Prediabetic States) OR (Prediabetic State) OR (State, Prediabetic) OR (States, Prediabetic) OR (Prediabetes)) AND ((MicroRNA) OR (miRNAs) OR (Micro RNA) OR (RNA, Micro) OR (miRNA) OR (Primary MicroRNA) OR (MicroRNA, Primary) OR (Primary miRNA) OR (miRNA, Primary) OR (pri-miRNA) OR (pri miRNA) OR (RNA, Small Temporal) OR (Temporal RNA, Small) OR (stRNA) OR (Small Temporal RNA) OR (pre-miRNA) OR (pre miRNA)).

Article selection

After removal of duplicated search results (retrieved from both the PubMed and the WOS databases), records were screened for compliance with predefined selection criteria (Table 1), in a 2-stage process: in a first step, record exclusion was based on title and abstract screening, followed by a second step where articles were excluded on full-text evaluation.

Table 1 Inclusion and exclusion criteria adopted for the selection of publications for data extraction.

Inclusion criteria	Exclusion criteria
Publications within the last 5 years	Other types of publications (Reviews, Comments, Editorials, Insights, Opinion articles)
Articles which the title, abstract and full text based in: Selection of original human studies reporting miRNA expression changes associated with diabetes mellitus type II	Articles in other languages (e.g. German, Chinese)
Published in English, French, Spanish, or Portuguese language	Studies using non-human models of disease (e.g. animal models, *ex vivo*, *in vitro*, *in silico* studies)
Studies in which the comparisons were established between the control groups: Normal Glucose Tolerance (NGT) or Impaired Fasting Glucose (IFG) and T2DM were included.	Studies without an appropriate control group (healthy subjects)
Studies with experimental results obtained / validated by qRT-PCR with statistical significance ($p<0.05$).	Studies focusing on complex metabolic and other multifactorial diseases (e.g. obesity, metabolic and wolfram syndromes, nonalcoholic fatty liver disease, cholesterol gallstone disease, heart and kidney diseases, cancer, aging-associated diseases)
	Other types of diabetes (e.g. T1D, MODY, gestational)
	Specific complications (e.g. diabetic-associated nephropathy, gastrocnemius skeletal muscle, retinopathy, cardiomyopathy, osteoarthritis)
	Studies evaluating the impact of drugs and other therapeutic interventions (e.g. lifestyle changes such as diet, food supplements, exercise, smoking cessation) on diabetes mellitus type II
	Studies focusing on the effect of miRNAs or miRNAs binding site polymorphisms

Data mining and database construction

All articles complying with predefined selection criteria were retrieved and manually curated for relevant information. The following data was extracted from each publication and incorporated in a comprehensive database: PubMed ID, publication title, sample type, sample size, country and ethnicity, study groups, clinical/pathological definitions (T2DM, IFG and NGT), miRNA quantification method, internal control (reference for normalization), miRNA ID, miRNA expression change (with statistical significance level and quantification through fold change, when available).

Biomarker evaluation (from literature data)

Identification of differentially expressed miRNAs and biomarker proposal

The database constructed from the literature review was used to build a final result table with 1) the list of miRNAs differentially expressed between any 2 of the 3 groups (T2DM *vs.* NGT, T2DM *vs.* IFG, IFG *vs.* NGT); 2) the annotation of the direction of the expression change for each miRNA (up or down regulated); 3) the annotation of their presence in saliva and 4) the final proposal of miRNAs as T2DM biomarkers. MiRNAs consistently reported (though different publications) to present concordant expression changes in any of the comparators were highlighted.

MiRNA nomenclature was standardized according to the identifiers used in the miRBase database (http://www.mirbase.org/) (40). Presence in saliva was evaluated though comparison with an external database – supplementary material of Bahn *et al.* 2014 (41). After excluding conflicting results (i.e. miRNAs with opposing expression changes reported by different articles for any of the comparisons), miRNAs differentially expressed between T2DM patients and NGT controls were proposed as T2DM biomarkers. Among these, those miRNAs that are also differentially expressed between T2DM patients and IFG individuals, as well as those that are counter regulated in T2DM and IFG individuals (differentially expressed between IFG and NGT controls in an opposing direction when compared to the expression change in T2DM patients *vs.* NGT controls), were proposed as differentiating T2DM biomarkers. Results were also graphically represented as Venn diagrams built with the Venny software (42).

Target validation

The previously obtained list of miRNAs proposed as T2DM biomarkers was introduced into the mir2disease.org (43) and HMDD v2.0 (44) databases, in order to verify which of these miRNAs are commonly recognized to be associated with T2DM. Mir2disease.org is a manually curated database of microRNAs that provides information on microRNA deregulation in various human diseases (43), while the HMDD v2.0 database is a collection of experimentally supported human miRNA-disease associations (45).

The list of miRNAs proposed as T2DM biomarkers (with the miRNAs not present in saliva) was also used to identify which genes were regulated by these miRNAs – their validated targets – a critical element for subsequent functional significance analysis. For this purpose, we used the mirWalk 2.0 database (46), a publicly available comprehensive resource hosting experimentally validated miRNA-target interaction pairs (as well as predicted ones).

Functional significance analysis

The Cytoscape v.3.4.0 bioinformatics software (47) – an open source tool for the visualization biological pathways and molecular interactions so that complex biological information can be bidirectionally integrated with other data (e.g. miRNA and gene expression) – was used to visualize and analyze the interacting network between T2DM-associated miRNAs and the corresponding genes (whose expression is, according to the miRWalk2.0 database, regulated by these miRNAs).

The list of interacting genes with degree 1 or 2 provided by the Cytoscape analysis was computed into the Disgenet database (7) to check for gene-T2DM associations. This database integrates human gene-disease associations (GDAs) from various expert curated databases and text-mining derived associations including Mendelian, complex and environmental diseases.

Other salivary miRNAs with biomarker potential in T2DM

Identification of miRNAs regulating proteins with altered salivary expression in T2DM

OralCard (48) – a manually annotated database that aggregates data from proteomic studies of the oral cavity and therefore provides essential information on the molecular basis of bio-pathological mechanisms of the oral cavity – was used to obtain a list of proteins whose salivary expression has been demonstrated to be altered in T2DM, the Oral Proteome in T2DM. This protein list was converted to the corresponding list of genes through the UniProt web-site (49) which, in turn, was introduced into the mirWalk 2.0 database (46) so that the list of miRNAs that target these genes could be obtained. By this way, we were able to obtain a new set of miRNAs that may also have their expression altered in saliva as a result of T2DM.

Target validation and functional significance analysis

The interacting network between the new set of T2DM-associated proteins and the corresponding miRNAs was analyzed in Cytoscape v.3.4.0. All miRNA-protein interactions with degree 1 were retrieved and computed into the mirWalk 2.0 database (46), so that univocal miRNA-protein interactions could be identified. The mir2disease.org (43), HMDD v2.0 (44) and Disgenet (7) databases were then used to check whether these miRNA-protein pairs have been previously associated with T2DM or not.

Results and Discussion

Systematic review

547 records were retrieved from our PubMed search while, in WOS, 673 records were identified. After removal of duplicated records (n=444), a total of 776 records were selected and screened for eligibility. Of these, 661 were rejected on title and/or abstract reading and 85 after full-text analysis. Overall, 31 articles fulfilled the inclusion/exclusion criteria and were therefore annotated (Figure 9).

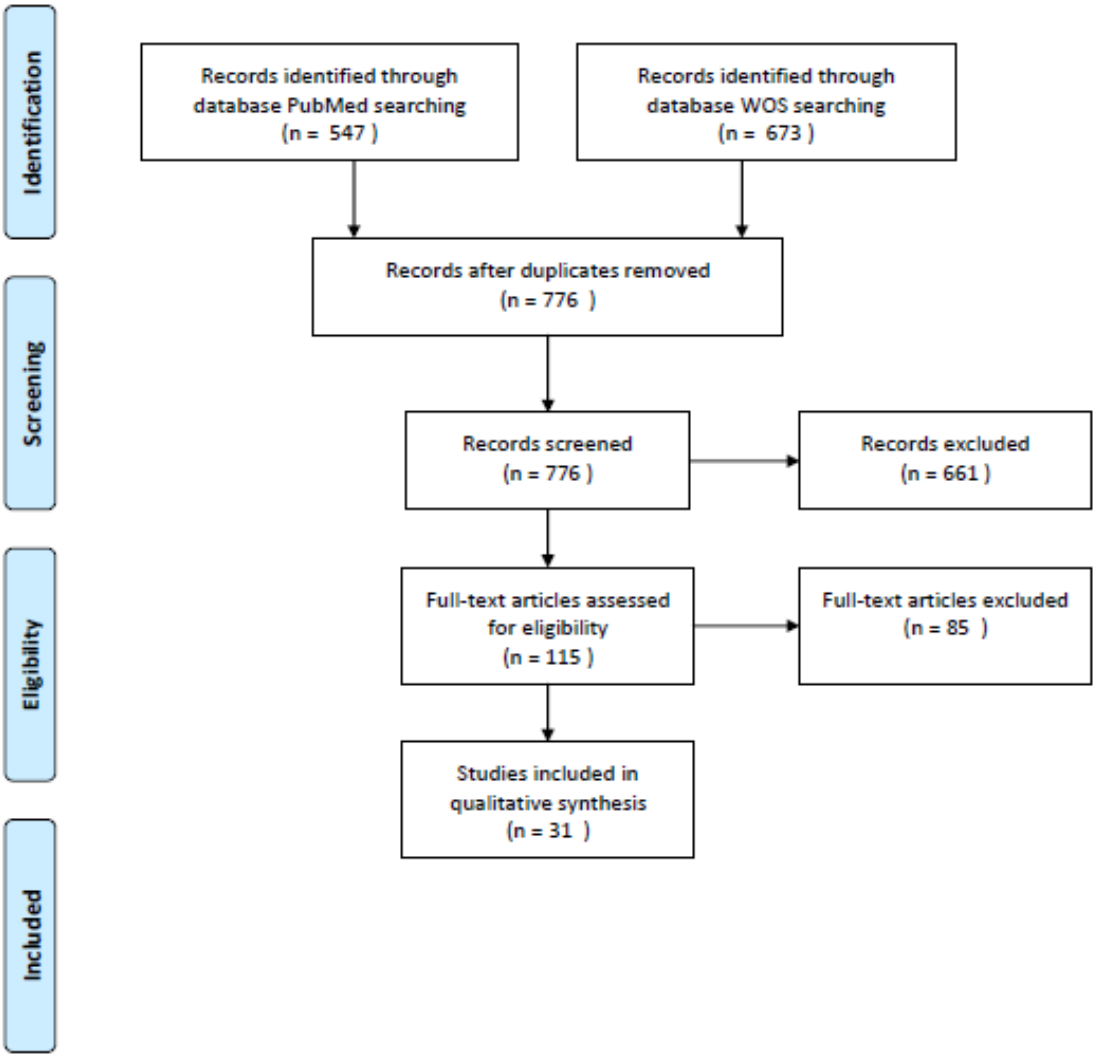

Figure 9 Prisma Flow Diagram. Records excluded after read title or/and abstract, Full-text articles excluded after read article.

From the analysis of these 31 articles, a database with the most relevant data was built. The general characteristics of the studies are presented in Annex 1. Blood samples were used for most studies (Figure 10) and none of the studies included was performed in saliva samples.

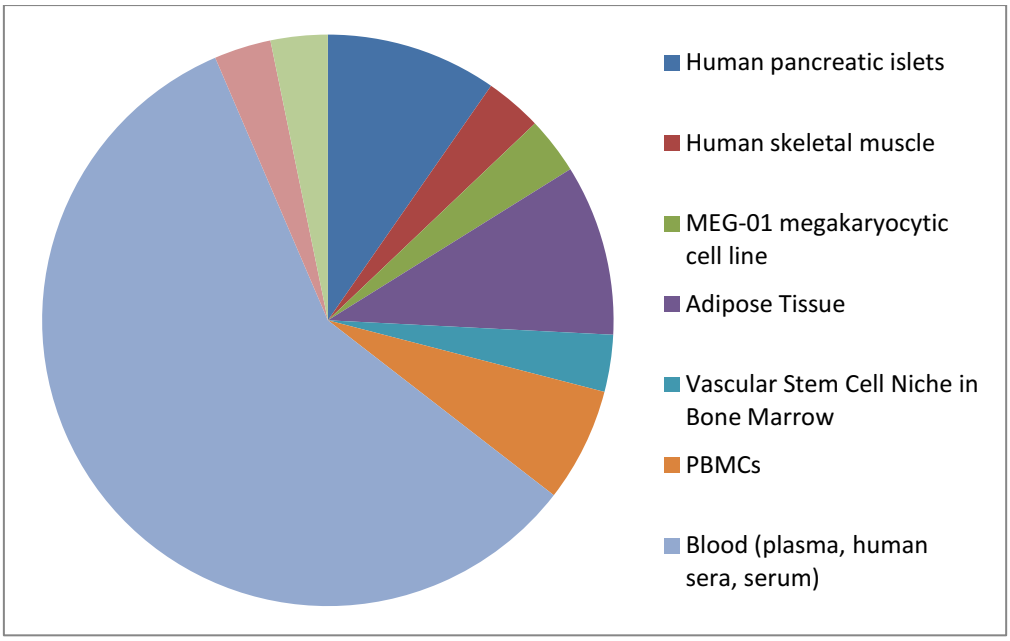

Figure 10 Number of samples in all articles (31articles).

Biomarker evaluation (from literature data)

Identification of differentially expressed miRNAs and biomarker proposal

Overall, from our systematic search, 59 miRNAs were found to be differentially expressed between any 2 of the 3 groups: 53 in T2DM *vs.* NGT, 13 in T2DM *vs.* IFG and 17 in IFG *vs.* NGT (Figure 11) (Annex 2).

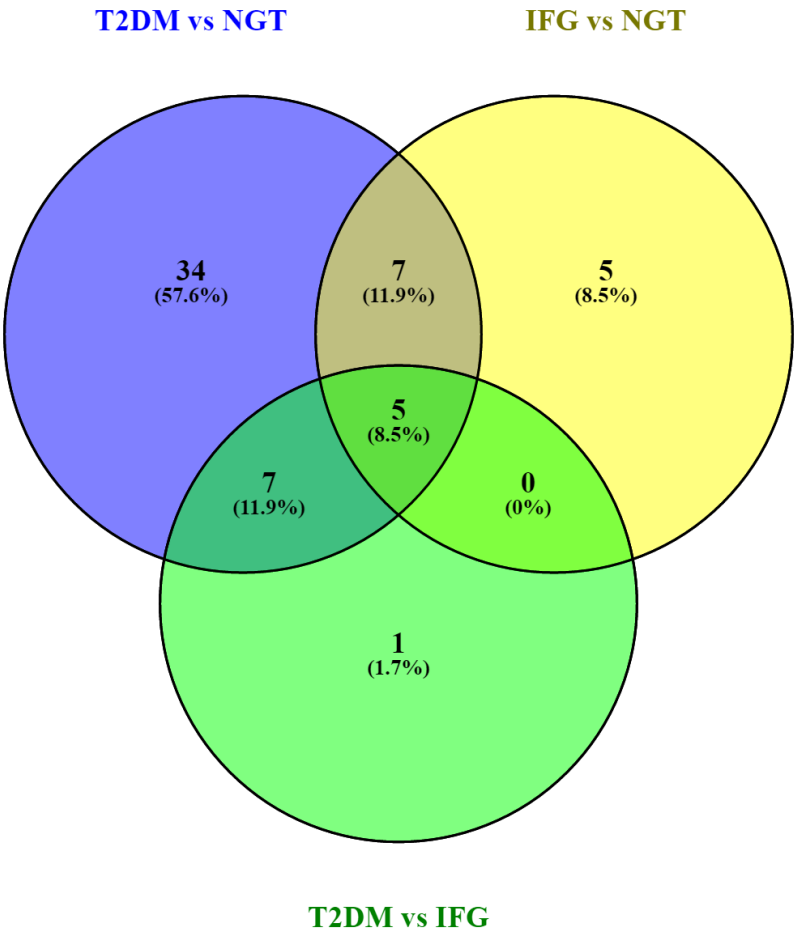

Figure 11 Venn diagram – Blue circle represents the number of individuals with Diabetes Mellitus Type II (T2DM) compared with healthy individuals (NGT), yellow circle is the number of individuals with Prediabetes (IFG) in relation with healthy individuals (NGT) and the green circle is the number of individuals with T2DM in relation with individuals with Prediabetes (IFG).

Among those that are differentially expressed between T2DM and NGT (n=53), 4 miRNAs (Table 2) present conflicting results among different sources. The origin of conflicting miRNAs may is because of different expression of miRNAs in different tissue samples.

Such conflicting miRNAs were excluded from further analysis since there is no consistency in their expression change.

Table 2 Conflicting miRNAs in T2DM.

miRNAs	Increase	Decrease
has-miR-130b-3p	Rome *et al.* (2015)	Ortega *et al.* (2014)
has-miR-146a-5p	Kong *et al.* (2011); Rong *et al.* (2013)	Yang *et al*. (2014); Karolina *et al*. (2011); Paramasivam *et al*. (2016); Baldeo´n *et al.* (2014)
has-miR-30d-5p	Kong *et al.* (2011)	Karolina *et al.* (2011)
has-miR-192-5p	Karolina *et al.* (2011)	Ortega *et al.* (2014); Yang *et al.* (2014)

Among the remaining 49 miRNAs, 4 (hsa-miR-124-3p, hsa-miR-487a-3p, hsa-miR-593-3p, hsa-miR-802) have not yet been detected in saliva samples, according to data from Bahn *et al.* (2014) (41), leaving 45 miRNAs as potential salivary biomarkers for T2DM (Table 3). These 4 miRNAs are not described as detectable in saliva, but futures investigations can detect them.

Among these potential salivary biomarkers, 9 miRNAs (hsa-miR-126-3p, hsa-miR-186-5p, hsa-miR-191-5p, hsa-miR-23a-3p, hsa-miR-29a-3p, hsa-miR-34a-5p, hsa-miR-375, hsa-miR-9-5p, hsa-miR-96-5p) are also differentially expressed between T2DM patients and IFG individuals and 5 miRNAs (hsa-miR-150-5p, hsa-miR-182-5p, hsa-miR-223-3p, hsa-miR-320a, hsa-miR-375) are counter regulated in T2DM and IFG individuals. Since hsa-miR-375 observes both criteria, 13 T2DM-associated salivary miRNAs may be regarded as differentiating T2DM biomarkers, being potentially useful for assessing disease progression from the prediabetic state to T2DM.

Of notice, also, 6 miRNAs (hsa-miR-124-3p – not present in saliva, hsa-miR-126-3p, hsa-miR-144-3p, hsa-miR-222-3p, hsa-miR-29a-3p, hsa-miR-375) among our defined set of potential salivary biomarkers for T2DM have been consistently reported (by 2 or more independent studies) to be altered (Annex-2).

Table 3 Proposed as potential salivary biomarkers T2DM. Bold: consistently reported.

	Expression change					
MiRNAs=45	IFG *vs.* NGT	T2DM *vs.* NGT	T2DM *vs.* IFG	PubMed ID	T2DM biomarker	Differentiating T2DM
hsa-let-7i-5p		decrease		(50)	YES	NO
hsa-miR-101-3p		increase		(51)	YES	NO
hsa-miR-125b-5p		decrease		(52)	YES	NO
hsa-miR-126-3p	**decrease**	**decrease**	decrease	(34,52–56)	YES	YES
hsa-miR-130a-3p		decrease		(57)	YES	NO
hsa-miR-133a-3p		decrease		(58)	YES	NO
hsa-miR-135a-5p		increase		(59)	YES	NO
hsa-miR-136-5p		decrease		(60)	YES	NO
hsa-miR-140-5p		increase		(52)	YES	NO
hsa-miR-142-3p		increase		(52)	YES	NO
hsa-miR-144-3p	increase	**increase**		(61,62)	YES	NO
hsa-miR-150-5p	decrease	increase		(61)	YES	YES
hsa-miR-155-5p		decrease		(63)	YES	NO
hsa-miR-181a-5p		increase		(64)	YES	NO
hsa-miR-182-5p	increase	decrease		(61)	YES	YES
hsa-miR-184		decrease		(65)	YES	NO
hsa-miR-186-5p		decrease	decrease	(50)	YES	YES
hsa-miR-187-3p		increase		(66)	YES	NO
hsa-miR-191-5p		decrease	decrease	(50)	YES	YES
hsa-miR-195-5p		decrease		(52)	YES	NO
hsa-miR-199a-5p		increase		(67)	YES	NO
hsa-miR-21-5p		decrease		(57)	YES	NO
hsa-miR-221-3p		increase		(68)	YES	NO
hsa-miR-222-3p		**increase**		(52,68)	YES	NO
hsa-miR-223-3p	increase	decrease		(62,69)	YES	YES
hsa-miR-23a-3p	decrease	decrease	decrease	(50)	YES	YES
hsa-miR-27a-3p		decrease		(57)	YES	NO
hsa-miR-27b-3p		decrease		(57)	YES	NO
hsa-miR-29a-3p	increase	**increase**	increase	(61,70)	YES	YES
hsa-miR-320a	decrease	increase		(61)	YES	YES
hsa-miR-34a-5p		increase	increase	(70)	YES	YES
hsa-miR-369-3p		decrease		(60)	YES	NO
hsa-miR-374a-5p		increase		(71)	YES	NO
hsa-miR-375	decrease	**increase**	increase	(51,70,72,73)	YES	YES
hsa-miR-411-5p		decrease		(60)	YES	NO
hsa-miR-423-5p	decrease	decrease		(52,71)	YES	NO
hsa-miR-432-5p		decrease		(60)	YES	NO
hsa-miR-486-5p		decrease		(50)	YES	NO
hsa-miR-487b-3p		decrease		(60)	YES	NO
hsa-miR-503-5p		decrease		(74)	YES	NO
hsa-miR-532-5p		decrease		(52)	YES	NO
hsa-miR-655-3p		decrease		60)	YES	NO
hsa-miR-656-3p		decrease		60)	YES	NO
hsa-miR-9-5p		increase	increase	(70)	YES	YES
hsa-miR-96-5p		decrease	decrease	(50)	YES	YES

Target validation

From the 45 miRNAs proposed as potential salivary biomarkers in T2DM some are referred in the HMDD v2.0 database (44) and/or in the mir2disease.org database (43) as being related to T2DM (Table 4).

Table 4 miRNAs with altered expression in T2DM by HMDD V.2.0 database and mi2disease.org.

miRNA	T2DM expression change	Detected in saliva	mir2disease.org	HMDDV.2.0
hsa-miR-21	Decrease	+		20651284
hsa-miR-29a	Increase	+	17652184	
hsa-miR-34a	Increase	+	19259271	
hsa-miR-126	Decrease	+	23144172	20651284
hsa-miR-144	Increase	+		21829658
hsa-miR-191	Decrease	+		20651284
hsa-miR-181a	Increase	+	19259271	
hsa-miR-223	Decrease	+		20651284
hsa-miR-486	Decrease	+		20651284

It is surprising that only one of the miRNAs (hsa-miRNA-126-3p) is found in both bases and, from our revision it is reported by two different studies. The miRNA-126-3p regulates 30 different genes (46) and many of these are related with angiogenesis and vascular inflammation (75). Its expression and secretion from circulating CD34+ and CD14+ PBMCs promotes proangiogenic effects and alterations in type 2 diabetics (44). Also, this miRNA has been implicated in coronary artery disease and myocardial infarction (52,53), and even proposed as a biomarker for endothelial dysfunction in type 2 diabetes (78). Considering that inflammation is an element common to both diabetes and cardiovascular dysfunction, the role of miRNA-126-3p in vascular inflammation and its involvement both in T2DM and cardiovascular disease is not surprising.

MiRNA-144 also plays an important role in impairing insulin signaling, through inhibition of the expression of insulin receptor substrate 1 in T2DM patients (44).

Through introducing the 49 miRNAs candidates biomarkers in T2DM into the mirWalk 2.0 database (46), it was possible to identify 9692 genes targeted by 41 of these miRNAs. The remaining 8 miRNAs (without validated gene targets, according to miRWalk) were further investigated, though an additional PubMed search, for functions potentially related with T2DM.

All the 8 miRNAs are related to T2DM. The miRNAs 369-3p, 411-5p, 487a-3p, 487b-3p, 655-3p, 656-3p and 432-5p are expressed in T2DM human islets (60). The miRNA 593-3p is down-regulated in blood samples of patients diabetic (79).

Functional significance analysis

The Cytoscape v.3.4.0 was used to visualize and analyze the interacting network between T2DM-associated miRNAs and the corresponding genes (whose expression is, according to the miRWalk2.0 database, regulated by these miRNAs) (Figure 12).

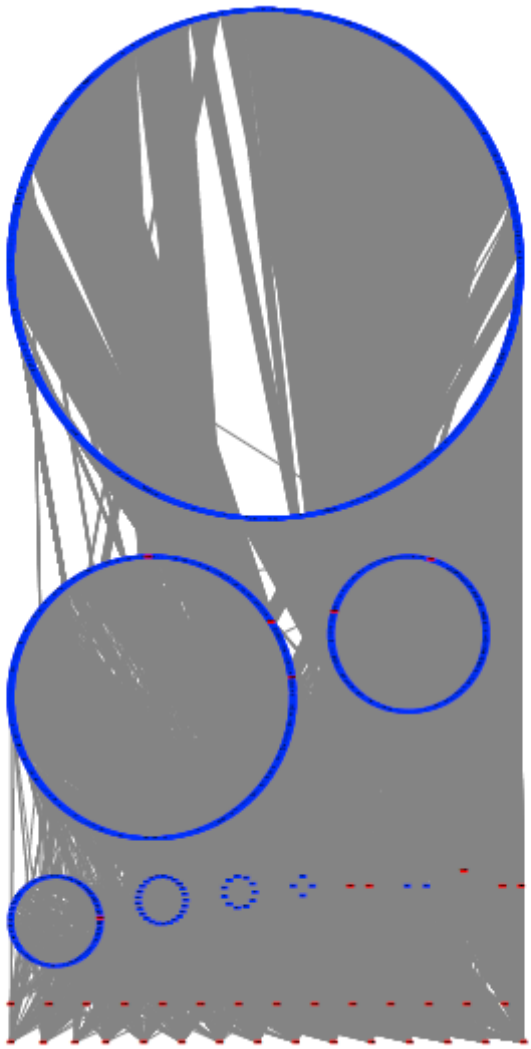

Figure 12 Cytoscape v.3.4.0 representation of the miRNA-gene interaction network. Network of interactions between the miRNAs proposed as biomarkers (red) and the genes which they interact with (blue). Circles are organized by increasing degree from top to bottom and left to right.

Figure 12, demonstrates that most proteins are regulated by only one miRNA of the 41 included in the study, indeed the hsa-miRNA-124-3p regulates over a thousand proteins in involved in many conditions. This miRNA is the most abundant miRNA in the nervous system, but has other function as; reducing the levels of pentose phosphate pathway enzymes in colorectal cancer cells (36); enhancing the expression of autophagy related protein in breast cancer cells (77); inhibiting the cell migration and invasion in bladder cancer cells by targeting ROCK1 (39).

In therapy it is used in Oncolytic Virotherapy, because the miRNA 124-3p targets within the 5' NCR suppressing virus replication in the central nervous system (40) and in Friedreich's ataxia targets at the level of FRDA-3′-UTR (35). As this miRNA is related to many proteins and different pathophysiological processes, is not a good candidate biomarker.

On a finer analysis we can observe that there are 4 miRNAs which have a degree equal to 2, meaning that there are 4 miRNAs from the 41 proposed which, although not exclusive, may be important markers. These miRNAs and the genes they regulate are presented in table 5.

Table 5 miRNAs and the genes/proteins they regulate by mirWalk database. In bold are the proteins with direct relation to the physio-pathology of T2DM.

MiRNAs	Gene	Protein
hsa-miR-802	**MECP2** AGTR1	**-Methyl-CpG-binding protein 2** -Type-1 angiotensin II receptor
hsa-miR-486-5p	**CD40** TMED1	**-Tumor necrosis factor receptor superfamily member 5** -Transmembrane emp24 domain-containing protein 1
hsa-miR-187-3p	TUBG1 MAD2L2	-Tubulin gamma-1 chain -Mitotic spindle assembly checkpoint protein MAD2B
hsa-miR-136-5p	**BCL2** MTDH	**-Apoptosis regulator Bcl-2** -Protein LYRIC

The genes MECP2, CD40 and BCL2 may be related to the physio-pathology of T2DM, these genes regulate processes related to T2DM (Table 5).

The MECP2 gene is involved in DNA methylation (49), some studies demonstrated differential DNA methylation of some candidates genes in T2DM patients in human pancreatic islets indicating a potential role of DNA methylation in the pathogenesis of this disease (12). The miRNA-802 is increased in the serum of T2DM patients *vs.* NGT controls and therefore it seems reasonable to assume that this increase is at least partially responsible for the hypermetilation of MECP2 gene (51). However, Elin Hall *et al*. concluded the protein MECP2 has the same expression in T2DM patients and non-diabetic patients which weakens the hypothesis of a methylation deregulation via MECP2 (72), but leaves open the possibility that miRNA-802 is also responsible for regulating other proteins responsible for methylation.

The CD40 gene is involved in the induction of immunoglobulin secretion (49) and therefore is related to the response to infection, a process often compromised in T2DM patients (80). Studies have found that miRNA-486-5p is down regulated in T2DM patients (50) and consequently it might be linked to a lack of response to infection.

The BCL2 gene suppresses apoptosis and may attenuate inflammation by impairing NLRP1-inflammasome activation, hence CASP1 activation and IL1β release (43). It is well known that IL1β is associated with the mechanism of insulin resistance in T2DM (8,10).

The miRNA 136-5p is down regulated in pancreatic islets donors of T2DM patients in relation with non-diabetic patients (44) and therefore it seems reasonable to assume that this decrease is responsible to promote apoptosis process in β cells.

The list of interacting genes with degree 2 provided by the Cytoscape analysis was computed into the Disgenet database (7) to check for gene-T2DM associations. The genes CD40 and BCL2 are related to T2DM and other diseases which further supports that the miRNAs identified as being related to them should be evaluated as important markers.

Other salivary miRNAs with biomarker potential in T2DM

Target validation and functional significance analysis

In the second analysis, 552 proteins identified as altered in T2DM patients were converted to the corresponding genes resulting 491 genes, which in turn, was introduced into the mirWalk 2.0 database (46). Of these 491 genes, we obtained 333 genes with correspondence with 722 miRNAs.

The interacting network between the new set of T2DM-associated proteins and the corresponding miRNAs was analyzed in Cytoscape v.3.4.0. All miRNA-protein interactions with degree 1 were retrieved and computed into the mirWalk 2.0 database (46), so that univocal miRNA-protein interactions could be identified. We found 108 miRNAs with degree 1 of which 5 have univocal miRNAs – proteins links (Table 6) (Figure 13).

Finally, the mir2disease.org (43), HMDD v2.0 (44) and Disgenet (7) databases were then used to check whether these miRNA-protein pairs have been previously associated with T2DM or not.

The miRNA 128 is increased in blood both in prediabetic subjects and T2DM patients compared to control subjects (71). This miRNA is also related with neuropathology diseases. The corresponding gene-DBI is related to T2DM and other diseases (e.g. neoplasm diseases and mental diseases) (7,43).

The miRNA 125b has been identified in pancreatic β-cells (81). The corresponding gene - IL1RN inhibits the activity of interleukin-1 (7) and the miRNA 125b in T2DM patients is down regulated, preventing the inhibition of IL1RN, promoting the physio-pathology of T2DM (52). This gene is also related to other inflammatory diseases (7,43).

The miRNA 519c-3p and the miRNA 519d-3p are not related to T2DM, but with gout and neoplasm diseases. T2DM and gout disease share some common pathogenic factors, mainly associated with the metabolic syndrome (82). The corresponding gene – TIMP2 inactivates the MMP´s involved in degradation of extracellular matrix (65,7). High blood glucose levels in diabetic's individuals causes deregulation of the MMPs/TIMPs (TIMP´s inactive the MMP´s) system, which considerably upsets the equilibrium between synthesis and degradation of vascular extracellular matrix (66,65).

The miRNA 616-3p is not related to T2DM, but with prostatic neoplasm. The corresponding gene-PON1 is related to vascular complications in T2DM (7,44).

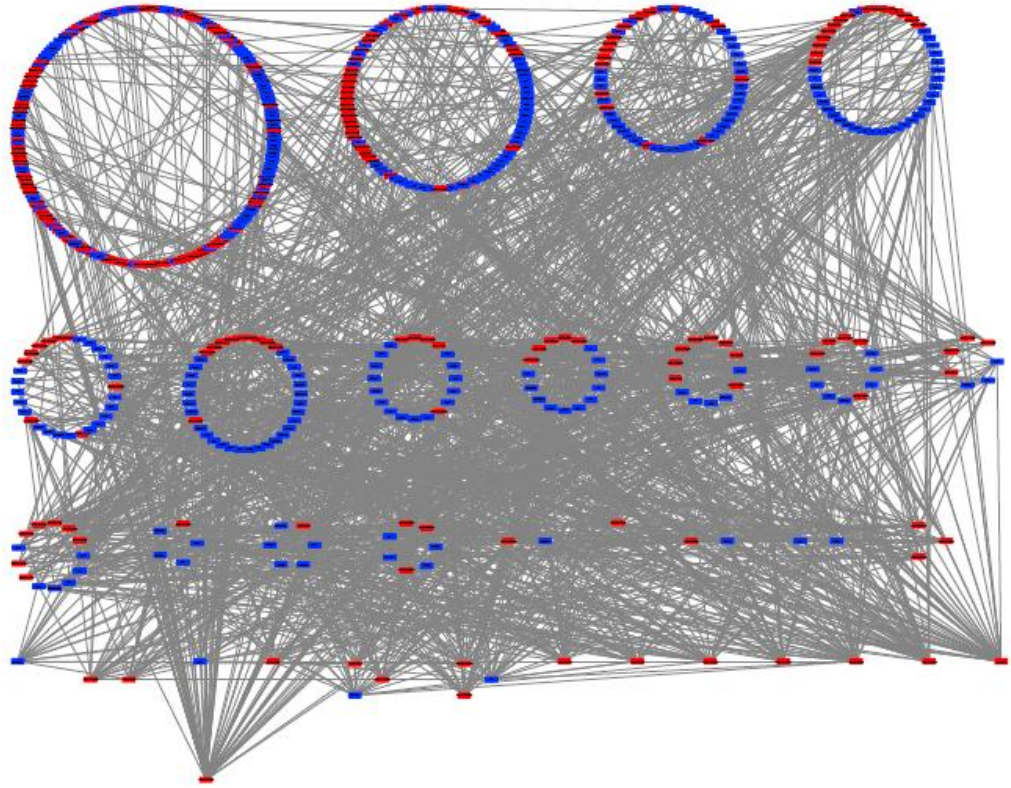

Figure 13 Cytoscape v.3.4.0 analysis gene-miRNA. The bigger circle is all the miRNAs with degree 1.

Table 6 Genes/proteins and the correspondent miRNAs according to mirWalk database. In bold are the proteins with direct relation to the physio-pathology of T2DM.

MiRNAs	Gene	Protein
DBI	hsa-miR-128	-Acyl-CoA-binding protein
IL1RN	**hsa-miR-125b**	**-Interleukin-1 receptor antagonist protein**
TIMP2	**hsa-miR-519c-3p**	**-Metalloproteinase inhibitor 2**
TIMP2	**hsa-miR-519d-3p**	**-Metalloproteinase inhibitor 2**
PON1	hsa-miR-616-3p	-Serum paraoxonase/arylesterase

Conclusion

At the moment, there is no consensus about exclusive miRNAs for Diabetes Mellitus Type II. The bioinformatics analysis performed allowed proposing a group of miRNAs (125b; 519c-3p; 519d-3p; 802; 486-5p; 136-5p, 369-3p, 411-5p, 487a-3p, 487b-3p, 655-3p, 656-3p, 432-5p, 593-3p, 128) that should be investigated as biomarkers for diagnosis and prognosis as well as potential therapeutic targets of T2DM. Some miRNAs are not related to T2DM but may share some common biological pathways with T2DM and therefore deserves further investigation.

The relevance of our study is the translation of saliva-based biomarker assessment to clinical practice, which will allow the development of fast, noninvasive and inexpensive omics-based screening tests for early diagnosis and personalized T2DM management.

Future investigations should determine if the miRNAs circulating in the physiological fluids are related to the activation state of beta cells, for monitoring whether beta-cells are in a compensatory or a failure condition since the pathogenesis of diabetes type II is characterized by beta cell dysfunction (27,34,85). However, many miRNAs are involved in the regulation on beta cells and control molecular signaling pathways. The altered expression of one or multiple miRNAs in diabetes islets can silence genes resulting in the altered expression of some genes that have the importance for beta cell function (13,86). Future studies should ascertain the impact of using free circulating miRNA versus miRNAs in exosomes as biomarker. This is especially relevant for both blood and saliva studies.

The major limitations of our study are the fact that several relevant studies present microarray data but are not validated by qRT-PCR. Our systematic search doesn't compiled experimental studies in saliva. Also the actual nomenclatures of miRNAs it is not follower by all studies which forced a search in supplementary data of which article and use the miRBase database (40) to standardize all the miRNAs with the actual name.

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Annexes

Annex 1 - Summary table of the general characteristics of the studies

Annex 2 - Potential biomarkers proposed

Annex -1

Title	Sample type (Tissue)	Sample size	Country (Ethnicity)	Comparators	Group definition	Method (pre-screening / validation)	Reference for normalization	PubMed ID
Integration of microRNA changes in vivo identifies novel molecular features of muscle insulin resistance in type 2 diabetes	Blood	10+10	Scotland	T2DM vs NGT	WHO	miRCURY LNA™ microRNA qRT-PCR (Exiqon) / Real-Time qRT-PCR, TaqMan® MicroRNA Assay with hydrolysis probes (ThermoFisher Scientific)	RNU48	20353613
				T2DM vs IFG				
Significance of serum microRNAs in pre-diabetes and newly diagnosed type 2 diabetes: a clinical study	Serum	18+19	China (Han Chinese)	T2DM vs NGT	WHO	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR	RNU6B	20857148
		19+19		T2DM vs IFG				
MicroRNA 144 impairs insulin signaling by inhibiting the expression of insulin receptor substrate 1 in type 2 diabetes mellitus.	Blood	21+15	Singapore	T2DM vs NGT	WHO	miRCURY LNA™ microRNA qRT-PCR (Exiqon) / Real-Time qRT-PCR, TaqMan® MicroRNA Assay with hydrolysis probes (ThermoFisher Scientific)	RNU6B	21829658
		21+14		IFG vs NGT				
Downregulation of miR-181a upregulates sirtuin-1 (SIRT1) and improves hepatic insulin sensitivity.	Serum	20+20	China (Xuhui)	T2DM vs NGT	WHO	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR	RNU6B	22476949
Downregulation of microRNA-126 in endothelial progenitor cells from diabetes patients, impairs their functional properties, via target gene Spred-1.	PMBCs	15+15	China (Xuhui)	T2DM vs NGT	WHO	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR	RNU6B	22525256
AngiomiR-126 expression and secretion from circulating CD34(+) and CD14(+) PBMCs: role for proangiogenic effects and alterations in type 2 diabetics.	PBMCs from buffy coats	3+3	Italy	T2DM vs NGT	NS	Qiagen miRNeasy Mini Kit (Qiagen GmbH, Germany) / Real-Time qRT-PCR CFX384TM	RNU6B	23144172
Global remodeling of the vascular stem cell niche in bone marrow of diabetic patients: implication of the microRNA-155/FOXO3a signaling pathway.	Vascular Stem Cell Niche in Bone Marrow	10+49	United States	T2DM vs NGT	NS	TaqMan® Array Human MicroRNA (vide infra) / Real-Time qRT-PCR	18snRNA or RNU6B	23250986
miRNA-93 inhibits GLUT4 and is overexpressed in adipose tissue of polycystic ovary syndrome patients and women with insulin resistance.	Adipose Tissue	7+5	Georgia	IFG vs NGT	WHO	cDNA synthesis kit for miRNA (Origene) / Real-Time qRT-PCR using an iTag Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc)	ACTB	23493574
miR-135a targets IRS2 and regulates insulin signaling and glucose uptake in the diabetic gastrocnemius skeletal muscle.	Human skeletal muscle	3+3	United States	T2DM vs NGT	WHO	StepOne Plus RT-PCR system	RNU6B	23579070
Elevation of miR-221 and -222 in the internal mammary arteries of diabetic subjects and normalization with metformin.	segment of the internal mammary artery	10+18	United States	T2DM vs NGT	NS	Qiagen miRNeasy Mini Kit (Qiagen GmbH, Germany) / Real-Time qRT-PCR	RNU6B	23648338
Increased MicroRNA-146a Levels in Plasma of Patients with newly diagnosed type 2 Diabetes Mellitus	Plasma	90+90	China (Han Chinese)	T2DM vs NGT	WHO	TaqMan MicroRNA Reverse Transcription Kit (ThermoFisher Scientific) / Real-Time qRT-PCR, TaqMan® MicroRNA Assays (ThermoFisher Scientific)	miRNA-16	24023848
Increased expression of miR-187 in human islets from individuals with type 2 diabetes is associated with reduced glucose-stimulated insulin secretion.	Human islet tissue	9+11	White/African–American/Asian	T2DM vs NGT	NS	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR	RNU6B	24149837
Serum circulating microRNA profiling for identification of potential type 2 diabetes and obesity biomarkers.	Serum	13+20	Spain	T2DM vs NGT	WHO	Qiagen miRNeasy Mini Kit (Qiagen GmbH, Germany) / Real-Time qRT-PCR CFX384TM	miR-30C, miR-103, miR-191 and miR-423-3p	24204780
Argonaute2 mediates compensatory expansion of the pancreatic β cell.	Pancreatic islets	12+15	United States	T2DM vs NGT	NS	Illumina Human WG6v2 microarray / Real-Time qRT-PCR	RNU6B	24361012
Expression and DNA methylation status of microRNA-375 in patients with type 2 diabetes mellitus	Plasma	100+100	China (Chinese Kazak)	T2DM vs NGT	WHO	-- / Real-Time qRT-PCR, TaqMan MicroRNA RT kit with specific primers (ThermoFisher Scientific)	miRNA-16 and RNU6B	24366165
Epigenetic regulation of the DLK1-MEG3 microRNA cluster in human type 2 diabetic islets.	Organ donors	4+3	African American, Caucasian, Hispanic/Latino	T2DM vs NGT	NS	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR	snoRNAs RNU44 and RNU48.	24374217
Plasma miR-126 is a potential biomarker for early prediction of type 2 diabetes mellitus in susceptible individuals.	Plasma	30+30	China (Han Chinese)	T2DM vs NGT	WHO	Real-Time qRT-PCR (ThermoFisher Scientific)	miR-238	24455723
				IFG vs NGT				24455723
Profiling of Circulating	Plasma	30+35	Spain	T2DM vs NGT	WHO	TaqMan® Array Human MicroRNA Card Set (ThermoFisher Scientific) / Real-Time qRT-PCR, TaqMan® MicroRNA Assay with hydrolysis probes (ThermoFisher Scientific)	miR-106a, miR-146a, miR-19b, and miR-223	24478399
The Role of Circulating MicroRNA-126 (miR-126):	Serum	160+138	China	T2DM vs NGT	WHO	-- / Real-Time qRT-PCR, TaqMan MicroRNA RT kit with specific primers (ThermoFisher Scientific)	internal control not used	24927146
		160+157		T2DM vs IFG				
		157+138		IFG vs NGT				
Serum miR-23a, a potential biomarker for diagnosis	Serum	24+20	China (Han Chinese)	T2DM vs NGT	WHO	Truseq Small RNA preparation and deep sequencing (Illumina) / Real-Time qRT-PCR, SYBR Green qRT-PCR Gene Expression system (ThermoFisher Scientific)	RNU6B	24981880
		24+20		T2DM vs IFG				
		20+20		IFG vs NGT				
MiR-199a is overexpressed in plasma of type 2 diabetes patients which contributes to type 2 diabetes by targeting GLUT4.	Plasma	64+64	China (Han Chinese)	T2DM vs NGT	WHO	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR	miR-16	25084986
DNA methylation of microRNA-375 in impaired glucose tolerance.	Plasma	44+53	China (Shihezi)	IFG vs NGT	WHO	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR		25120598
		54+53		T2DM vs NGT				
The expression of the miR-25/93/106b family of micro-RNAs in the adipose tissue of women with polycystic ovary syndrome.	Adipose Tissue	9+6	Georgia	IFG vs NGT	WHO	cDNA synthesis kit for miRNA (Origene) / Real-Time qRT-PCR using an iTag Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc)	ACTB and Actb	25243570
MicroRNA-124a is hyperexpressed in type 2 diabetic human pancreatic islets and negatively regulates insulin secretion	Human pancreatic islets	5+6	Italy	T2DM vs NGT	NS	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR	RNU6B, RNU44 and RNU48	25408296
Identification of circulating miR-101, miR-375 and miR-802 as biomarkers for type 2 diabetes.	Human sera	155+49	Japan	T2DM vs NGT	NS	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR	C. elegans spiked-in	25726255
Platelet-derived miR-103b as a novel biomarker for the early diagnosis of type 2 diabetes	MEG-01 megakaryocytic cell line	48+46	China (Han Chinese)	IFG vs NGT	WHO	M-MLV Reverse Transcription Kit / Real-Time qRT-PCR	RNU6B and 18S rRNA	25820527
Profiling peripheral microRNAs in obesity and type 2	Serum	25+25	China	T2DM vs NGT	WHO	miRCURY LNA™ microRNA qRT-PCR (Exiqon): pre-designed PCR panels on pooled samples (pre-screening) / individual microRNA PCR primer sets on individual samples (validation)	miR-30C, miR-103, miR-191 and miR-423-3p	25912229
Circulating miR-126 is a potential biomarker to predict the onset of	Plasma	20+20	China (Han Chinese)	T2DM vs NGT	WHO	-- / Real-Time qRT-PCR, SYBR® Premix Dimer-Eraser kit (TaKaRa)	RNU6B	25986735
Circulating MiRNAs of 'Asian Indian Phenotype' Identified in Subjects with Impaired Glucose Tolerance and Patients with Type 2 Diabetes.	Blood	12+12	China (Chennai)	IFG vs NGT	WHO	miRCURY LNA™ microRNA qRT-PCR (Exiqon) / Real-Time qRT-PCR, TaqMan® MicroRNA Assay with hydrolysis probes (ThermoFisher Scientific)	RNU6B	26020947
				T2DM vs NGT	WHO			
Circulating MiRNAs of 'Asian Indian Phenotype' Identified in Subjects with Impaired Glucose Tolerance and Patients with Type 2 Diabetes.	Blood	12+12	China (Chennai)	T2DM vs IFG		miRCURY LNA™ microRNA qRT-PCR (Exiqon) / Real-Time qRT-PCR, TaqMan® MicroRNA Assay with hydrolysis probes (ThermoFisher Scientific)	RNU6B	26020947
Biomarkers Associated with Ischemic Stroke in Diabetes Mellitus Patients.	Blood	56+30	China (Xiangya)	T2DM vs NGT	WHO	TaqMan® Array Human MicroRNA Card Set / Real-Time qRT-PCR	Spiked-in cel-miR-39	26175178
MicroRNA-223 Expression Is Upregulated in Insulin Resistant Human Adipose Tissue.	Adipose Tissue	8+7	Los Angeles	IFG vs NGT		cDNA synthesis kit for miRNA (Origene) / Real-Time qRT-PCR using an iTag Universal SYBR Green Supermix (Bio-Rad Laboratories, Inc)	ACTB and miR-103	26273679

[1] NGT: Normal glucose tolerance; IFG: Impaired glucose tolerance; T2DM: Type 2 diabetes mellitus; NS: Not stated; WOS: T2DM= (fasting plasma glucose≥7 mmol/l (126 mg/dl). IFG = (FPG 5.6–6.9 mmol/l (100–125 mg/dl) and impaired glucose tolerance (IGT) [(FPG<5.6 mmol/l (100 mg/dl) and IFG + IGT [FPG 5.6–6.9 mmol/l (100–125 mg/dl). (NGT) = fasting glucose <110 mg/dL.

Annex - 2

MiRNAs=59	Present in Saliva	Expression change (IFG vs NGT)	Expression change (T2D vs NGT)	Expression change (T2D vs IFG)	Ref (PubMed ID)		T2D biomarker	Differentiating T2D biomarker
hsa-let-7i-5p	+		decrease			24981880	YES	NO
hsa-miR-101-3p	+		increase			25726255	YES	NO
hsa-miR-103b	-	decrease				25820527	NO	NO
hsa-miR-106b-5p	+	increase				25243570	NO	NO
hsa-miR-124-3p	-		**increase**		20857148, 25408296		YES	NO
hsa-miR-125b-5p	+		decrease			24478399	YES	NO
hsa-miR-126-3p	+	**decrease**	**decrease**	decrease	IFGvsNGT: 24927146, 24455723; T2DvsIFG: 24927146; T2DvsNGT: 25986735, 24927146, 24455723, 23144172, 22525256, 24478399		YES	YES
hsa-miR-128-3p	+	increase				26020947	NO	NO
hsa-miR-130a-3p	+		decrease			22525256	YES	NO
hsa-miR-130b-3p	+		**increase/decrease**	increase	T2DvsNGT: 26020947, 24478399; T2DvsIFG: 26020947		NO	NO
hsa-miR-133a-3p	+		decrease			20353613	YES	NO
hsa-miR-135a-5p	+		increase			23579070	YES	NO
hsa-miR-136-5p	+		decrease			24374217	YES	NO
hsa-miR-140-5p	+		increase			24478399	YES	NO
hsa-miR-142-3p	+		increase			24478399	YES	NO
hsa-miR-144-3p	+	increase	**increase**		IFGvsNGT: 21829658; T2DvsNGT: 21829658, 26175178		YES	NO
hsa-miR-146a-5p	+	decrease	**decrease (4)/increase (2)**	increase	IFGvsNGT: 21829658; T2DvsNGT: 24981880 (D), 21829658 (D), 21249428 (D), 25500583 (D), 24023848 (U), 20857148 (U); T2DvsIFG: 20857148		NO	NO
hsa-miR-150-5p	+	decrease	increase		IFGvsNGT: 21829658; T2DvsNGT: 21829658		YES	YES
hsa-miR-155-5p	+		decrease			23250986	YES	NO
hsa-miR-181a-5p	+		increase			22476949	YES	NO
hsa-miR-182-5p	+	increase	decrease		IFGvsNGT: 21829658; T2DvsNGT: 21829658		YES	YES
hsa-miR-184	+		decrease			24361012	YES	NO
hsa-miR-186-5p	+		decrease	decrease	T2DvsNGT: 24981880; T2DvsIFG: 24981880		YES	YES
hsa-miR-187-3p	+		increase			24149837	YES	NO
hsa-miR-191-5p	+		decrease	decrease	T2DvsNGT: 24981880; T2DvsIFG: 24981880		YES	YES
hsa-miR-192-5p	+		**decrease (2)/increase (1)**	decrease	T2DvsNGT: 24478399 (D), 24981880 (D), 21829658 (U); T2DvsIFG: 24981880		NO	NO
hsa-miR-195-5p	+		decrease			24478399	YES	NO
hsa-miR-199a-5p	+		increase			25084986	YES	NO
hsa-miR-206	+			decrease		20353613	NO	NO
hsa-miR-21-5p	+		decrease		T2DvsNGT: 22525256		YES	NO
hsa-miR-221-3p	+		increase			23648338	YES	NO
hsa-miR-222-3p	+		**increase**		24478399, 23648338		YES	NO
hsa-miR-223-3p	+	increase	decrease		IFGvsNGT: 26273679; T2DvsNGT: 26175178		YES	YES
hsa-miR-23a-3p	+	decrease	decrease	decrease	IFGvsNGT: 24981880; T2DvsNGT: 24981880; T2DvsIFG: 24981880		YES	YES
hsa-miR-25-3p	+	increase			IFGvsNGT: 25243570		NO	NO
hsa-miR-27a-3p	+		decrease			22525256	YES	NO
hsa-miR-27b-3p	+		decrease			22525256	YES	NO
hsa-miR-29a-3p	+	increase	**increase**	increase	IFGvsNGT: 21829658; T2DvsNGT: 20857148, 21829658; T2DvsIFG: 20857148		YES	YES
hsa-miR-30d-5p	+	increase	**decrease/increase**		IFGvsNGT: 21829658; T2DvsNGT: 21829658 (D), 20857148 (U)		NO	NO
hsa-miR-320a	+	decrease	increase		IFGvsNGT: 21829658; T2DvsNGT: 21829658		YES	YES
hsa-miR-34a-5p	+		increase	increase	T2DvsNGT: 20857148; T2DvsIFG: 20857148		YES	YES
hsa-miR-369-3p	+		decrease			24374217	YES	NO
hsa-miR-374a-5p	+		increase			26020947	YES	NO
hsa-miR-375	+	decrease	**increase**	increase	IFGvsNGT: 25120598; T2DvsNGT: 24366165, 20857148, 25726255, 25120598; T2DvsIFG: 20857148		YES	YES
hsa-miR-411-5p	+		decrease			24374217	YES	NO
hsa-miR-423-5p	+	decrease	decrease		IFGvsNGT: 26020947; T2DvsNGT: 24478399		YES	NO
hsa-miR-432-5p	+		decrease			24374217	YES	NO
hsa-miR-486-5p	+		decrease			24981880	YES	NO
hsa-miR-487a-3p	-		decrease			24374217	YES	NO
hsa-miR-487b-3p	+		decrease			24374217	YES	NO
hsa-miR-503-5p	+		decrease			24204780	YES	NO
hsa-miR-532-5p	+		decrease			24478399	YES	NO
hsa-miR-593-3p	-		decrease			25912229	YES	NO
hsa-miR-655-3p	+		decrease			24374217	YES	NO
hsa-miR-656-3p	+		decrease			24374217	YES	NO
hsa-miR-802	-		increase			25726255	YES	NO
hsa-miR-9-5p	+		increase	increase	T2DvsNGT: 20857148; T2DvsIFG: 20857148		YES	YES
hsa-miR-93-5p	+	**increase**			IFGvsNGT: 25243570, 23493574		NO	NO
hsa-miR-96-5p	+		decrease	decrease	T2DvsNGT: 24981880; T2DvsIFG: 24981880		YES	YES