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Circulating microRNAs associated with gestational diabetes mellitus: Useful biomarkers?

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Abbreviations: MicroRNA miRNA, Gestational diabetes mellitus GDM, type 2 diabetes, T2D, oral glucose tolerance test OGTT, International Diabetes Federation IDF, extracellular vesicle EV, normal glucose tolerant NGT, receiver operator curve area under the curve ROC AUC

Abstract

Different types of small non-coding RNAs, especially microRNAs (miRNAs), may be found in the circulation, either protein bound or enclosed in extracellular vesicles. During gestation, and particular during gestational diabetes mellitus (GDM), the levels of several miRNAs are altered. Worldwide the incidence of GDM is increasing, in part driven by the current obesity epidemic. This is a point of public health concern, because offspring of women with GDM frequently suffer from short and long-term complications of maternal GDM. This has prompted the investigation of whether levels of specific miRNA species, detected early in gestation, may be used as diagnostic or prognostic markers for development of GDM. Here, we summarize mechanisms of RNA secretion, and review circulating miRNAs associated with GDM. Several miRNAs are associated with GDM: MiR-29a-3p and miR-29b-3p are generally upregulated in GDM pregnancies, also when measured prior to the development of GDM, while miR-16-5p is consistently upregulated in GDM pregnancies, especially in late gestation. MiR-330-3p in circulation is increased in late gestation GDM women, especially in those with poor insulin secretion. MiR-17-5p, miR-19a/b-3p, miR-223-3p, miR-155-5p, miR-125-a/b-5p, miR-210-3p and miR-132are also associated with GDM, but less so and with more contradictory results reported. There could be a publication bias as miRNAs identified early are investigated the most, suggesting that it is likely that additional, more recently detected miRNAs could also be associated with GDM. Thus, circulating miRNAs show potential as biomarkers of GDM diagnosis or prognosis, especially multiple miRNAs containing prediction algorithms show promise, but further studies are needed.

Introduction

The current worldwide obesity epidemic drives an increased incidence and prevalence of gestational diabetes mellitus (GDM) as well as Type 2 diabetes (T2D) in pregnancy (Sun et al., 2022). In 2019, according to the International Diabetes Federation (IDF) 16% of viable children were affected by hyperglycemia in pregnancy (HIP), corresponding to 20 million live births of which GDM accounts for 75-90% (Sun et al., 2022). GDM is defined as diabetes that is diagnosed for the first-time during pregnancy, at any time during pregnancy. While GDM usually resolves following delivery, women who develop GDM during pregnancy are at high risk of developing T2D later in life or have recurrent GDM in future pregnancies. Of importance, GDM carries a risk of a number of complications for both mother and child (Damm et al., 2016). There is an increased morbidity for both during pregnancy and around birth. For the mother there is an increased risk of preeclampsia, and an increased mortality around the time of birth, while GDM fetuses are also at risk of macrosomia, premature birth, neonatal icterus and perinatal hypoglycemia (Teh et al., 2011). Women developing GDM have a higher degree of insulin resistance compared with women who remain normoglycemic in pregnancy (Kampmann et al., 2019), and late sequelae include a higher risk of developing diabetes, particularly T2DM, and metabolic diseases later in life (Kelstrup et al., 2013).

In efforts to prevent GDM, several risk factors have been identified that may contribute to the onset of GDM in women. These include obesity, smoking, family history of diabetes, birth of a child with macrosomia, ethnicity (all ethnicities except Anglo-European) and maternal age (Teh et al., 2011). Therefore, the IDF and IADPSG recommend screening for GDM (International Association of Diabetes in Pregnancy Study Group Working Group on Outcome et al., 2015), preferably via a measurement of plasma glucose levels (HbA1c, random or fasting plasma glucose values) in all or high-risk women at first antenatal visit, followed by screening using oral glucose tolerance test (OGTT) at gestational week 24-28. This is a very comprehensive screening program and some

77 countries, such as Denmark, rely on screening based on risk factors such as elevated BMI or family
78 history of diabetes or previous birth of a large child. Screening is usually performed from gestational
79 weeks 24-28, as insulin resistance increases in the 2nd trimester and blood glucose levels rise.
80 However, at that time point the unborn child may already have been affected long enough to develop
81 metabolic adaptations that may cause complications later in life, such as increased risk of T2D and
82 cardiovascular disease (Rani and Begum, 2016, Damm et al., 2016). Therefore, there is a search for
83 better biomarkers to allow diagnosis of GDM at an earlier time point or giving more specific
84 prognostic estimates of the risk of GDM. To this end, circulating levels of small non-coding RNA
85 molecules, especially miRNA have been investigated and suggested as a novel category of
86 biomarkers potentially serving to improve diagnosis and prediction of disease development (Condrat
87 et al., 2020). Identifying potential biomarkers for early prediction of GDM before week 20 of
88 pregnancy will help identify and treat incident GDM in pregnant women. The current review aims at
89 describing the current status of circulating small RNAs, primarily miRNAs, associated with
90 gestational diabetes, and further to evaluate the potential of such to act as prognostic biomarkers for
91 GDM.
92
93 ***MicroRNA biogenesis***
94 MiRNAs are small non-coding single-stranded RNA molecules of about 22 nucleotides in length,
95 found in plants, animals, viruses, human tissues, and blood. More than 2,000 different miRNAs have
96 been identified in the human genome (Kozomara et al., 2019). MiRNAs act via RNA silencing to
97 regulate, in a posttranscriptional manner, the degradation of messenger RNA (mRNA) thereby
98 adjusting protein levels and about 30% of protein coding genes are predicted to be targeted by one or
99 more miRNAs.

MiRNAs are transcribed in the nucleus to form a primary transcript, which is cut into a precursor miRNA by the nuclease DROSHA forming a hairpin structure. Next, the pre-miRNA is transported to the cytoplasm by EXPORTIN-5, where it is further cleaved by DICER to form a duplex short RNA with imperfect base-pairing (Fig. 1). The mature miRNA duplex is unwound and the mature single stranded miRNA is assembled into the RNA-induced silencing complex (RISC) (consisting of DICER, TRBP and AGO2) to then induce translational inhibition or transcript degradation of mRNAs to which it can base pair (Fig. 1) (Eichhorn et al., 2014, Guo et al., 2010, Bartel and Chen, 2004, Chendrimada et al., 2005, Chu et al., 2010). The miRNA:RISC complex then identifies possible target mRNAs through sequence complementary of the miRNA seed sequence to the 3' untranslated region (UTR) of the target mRNA. The seed sequence consists of 6-8 nucleotides at the 5' end of the miRNA, where perfect or near perfect complementary base pairing between the miRNA and mRNA results in a rapid degradation of the transcript, while partial complementary between the miRNA:mRNA complex prevents the protein translation process. However, miRNA-dependent repression also results in mRNA decay, which has been shown to account for most miRNA-dependent repression (Filipowicz and Sonenberg, 2015, Mathys et al., 2014).

Circulating miRNA as biomarkers

In addition to playing a major role inside the cell, miRNA also plays an important role outside the cell, in body fluids and elsewhere as circulating miRNAs. Circulating RNAs may be found in blood, saliva, breast milk and urine, as well as other fluids, for example in the case of tissue damage but also as the result of controlled excretion. Approximately 10% of circulating miRNAs are secreted in exosomes, which are a specific type of extracellular vesicles (EVs) (Albanese et al., 2021, Chevillet et al., 2014). The remaining 90% of the miRNAs are encased in other EVs, such as microvesicles or

apoptotic bodies, or forms complexes with proteins such as Ago2, or with HDL particles (Vickers et al., 2011, Boon and Vickers, 2013, Arroyo et al., 2011).

Enclosing the miRNA in vesicles or binding it to protein complexes prevents the miRNA from being digested and thus it remains stable in body fluids protected from ribonucleases. Exosomes derived from multivesicular bodies (MVBs), a specialised subset of endosomes, are loaded with miRNAs at the endoplasmic reticulum (ER) surface and which involves the ER membrane protein VAP-A and the ceramide transfer protein CERT (Barman et al., 2022). This is interesting because it has also been demonstrated that mRNA:miRNA interactions via Ago2 loading take place at the ER membrane (Barman and Bhattacharyya, 2015). The secretion of miRNAs from cells into exosomes depends on the enzyme neutral sphingomyelinase 2 (nSMase2), which is known as a rate-limiting enzyme of ceramide biosynthesis (Kosaka et al., 2010). Exosomal miRNA transfer appears to be a selective process, because the miRNA content within the exosomes is quite dissimilar to the miRNA composition of the parent cell (Guduric-Fuchs et al., 2012, Villarroya-Beltri et al., 2013, Squadrito et al., 2014). Microvesicles, formed by outward budding of the plasma membrane, have miRNAs delivered by the ADP ribosylation factor ARF6, which binds Exportin 5 (Clancy et al., 2019). Thus, the loading of both small and large EVs appear to be under regulatory control. For example, miRNAs, miR-155, miR-210 and miR-23 are selectively loaded into exosomes massively increasing their release from cells during inflammation facilitated by the RNA binding protein FMR1 and a common A-A/U-U/A-GC motif in these miRNAs (Wozniak et al., 2020).

During pregnancy, the number of EVs increase in circulation, especially EVs derived from the placenta (Menon et al., 2019, Salomon et al., 2018). The stability and ease of detection by quantitative reverse transcription PCR (q-RT-PCR) has fostered intense scientific interest in the possible use of miRNAs as circulating biomarkers for a large variety of pathological conditions. Studies measuring miRNAs in circulation make use of different experimental strategies for quantifying miRNA levels,

147 the most commonly used techniques being qRT-PCR, small RNA-seq and arrays. Quantitative RT-
148 PCR is advantageous when few miRNAs are measured in many clinical samples and assays can be
149 made with spike-ins during RNA isolation and during cDNA synthesis, enabling control for variation
150 in RNA isolation and cDNA synthesis efficiency. On the other hand, small RNA-seq has the
151 advantage of measuring all small RNAs in a sample, but is rarely made with spike-ins and the cost
152 usually prohibits measuring large cohorts. Small RNA-arrays can be standardized with spike-ins to
153 enable quantitative measurements, but the suffer from lower sensitivity and therefore require the use
154 of higher sample volumes for RNA extraction (Table 1).
155 Not all circulating miRNA have potential as diagnostic or prognostic biomarkers. MiRNA, as a
156 biomarker, must be able to meet certain criteria to be considered as a biomarker candidate; it must be
157 readably detectable and measurable. Moreover, its specificity and its sensitivity for the condition
158 under investigation are important criteria to be considered and it must have clinical relevance . Since
159 circulating miRNA is a relatively new research area, for most miRNAs in circulation the influence of
160 different parameters such as age, gender and disease/health on their levels, as well as population
161 variability are not known. Moreover, since thrombocytes also contain miRNAs, the levels of specific
162 miRNAs, such as miR-451 and miR-223, are released into the serum following platelet aggregation.
163 Therefore, such miRNAs have different levels in serum and plasma and it is important to make note
164 of the sample material reported in clinical studies of circulating miRNAs.
165
166 ***Literature search methods:***
167 The databases PubMed, Science Direct and Scopus was searched using the string: (Gestational
168 diabetes OR pregnancy-induced diabetes) AND (microRNA OR miRNA OR microribonucl*) AND
169 human. Further literature was identified through searches on bioRxiv.org and medRxiv.org and via
170 the literature references of identified articles.

171

172 ***Circulating RNAs associated with gestational diabetes – what has been found?***

173 Based on our literature searches, we identified 22 original studies (Table 2) investigating circulating
174 levels of more than 130 different miRNA species in relation to GDM (Fig. 2). However, the majority
175 of the different miRNA types were only investigated in one or two studies (Fig. 2): Only 33 different
176 miRNA species were investigated in more than one original study, with miR-29a-3p, miR-16-5p
177 being the most studied miRNAs, investigated in six (Zhao et al., 2011, Wander et al., 2017, Gillet et
178 al., 2019, Martinez-Ibarra et al., 2019, Tagoma et al., 2018, Sorensen et al., 2021, Sorensen et al.,
179 2022) and six (Martinez-Ibarra et al., 2019, Tagoma et al., 2018, Hocaoglu et al., 2019, Cao et al.,
180 2017, Sorensen et al., 2021, Sorensen et al., 2022, Zhu et al., 2015) original studies, respectively.
181 MiR-223-3p (Yoffe et al., 2019, Wander et al., 2017, Tagoma et al., 2018, Sorensen et al., 2021),
182 miR-330-3p (Pfeiffer et al., 2020, Martinez-Ibarra et al., 2019, Xiao et al., 2020, Sorensen et al.,
183 2021), miR-132-3p (Zhou et al., 2019, Zhao et al., 2011, Gillet et al., 2019, Tagoma et al., 2018) were
184 each investigated in four studies, while miR-155-5p (Wander et al., 2017, Hocaoglu et al., 2019,
185 Tagoma et al., 2018), miR-210-3p (Wander et al., 2017, Gillet et al., 2019, Tagoma et al., 2018),
186 miR-19a/b-3p (Stirm et al., 2018, Tagoma et al., 2018, Cao et al., 2017), miR-17-5p (Lamadrid-
187 Romero et al., 2018, Tagoma et al., 2018, Cao et al., 2017) and miR-125a/b-5p (Nair et al., 2018,
188 Zhao et al., 2011, Lamadrid-Romero et al., 2018) were each studied in three original studies (Table
189 1, Fig. 2). Thus, it is clear that the majority of reported circulating miRNAs associated with GDM
190 have only been identified in one study. Hence, we should assume that it is likely that additional
191 miRNAs to those covered below are also associated with GDM, but these have not yet been
192 sufficiently analyzed by the research community to be covered in this review.

193

The miR-29 family members miR-29a-3p and miR-29b-3p are generally upregulated in GDM pregnancies

The miR-29 family consists of seven miRNAs, of which miR-29a-3p and miR-29b-3p are the two major isoforms. The miR-29a-3p, but also the other miR-29 members, have been found to be increased by obesity or prediabetes in metabolically relevant tissues, such as β-cells, adipose tissue, skeletal muscle and in the liver. Moreover, antisense inhibition of miR-29a-3p improves liver insulin resistance and improves glycemic control, altogether suggesting that the miR-29 family has an essential function in intermediate metabolism (Hung et al., 2019, Dalgaard et al., 2022). Sørensen et al. (Sorensen et al., 2021) examined miR-29a-3p in relation to GDM diagnosed early in pregnancy (before week 20) and late in pregnancy (weeks 24-28) and found significantly increased expression of miR-29a-3p in late-diagnosed GDM compared to the NGT group, which is supported by findings of Martínez-Ibarra et al. (Martinez-Ibarra et al., 2019) also identifying significantly increased miR-29a-3p in 2nd trimester diagnosed GDM cases. Further support of a general upregulation of circulating miR-29a-3p, and possibly also miR-29b-3p in GDM pregnancies can be found in a study by Tagoma et al (Tagoma et al., 2018), which found these two miRNAs upregulated more than 3-fold, although not reaching statistical significance, possibly as a cause of lower power due to a low number of investigated subjects. However, the published literature does not uniformly agree on the association of circulating miR-29a/b with GDM, as Wander et al (Wander et al., 2017) and Zhao et al (Zhao et al., 2011) found no or negative association, respectively, with GDM, although Wander et al. (Wander et al., 2017) observed increased miR-29a-3p in circulation of GDM pregnancies, when carrying a male fetus. Thus, the majority of studies support that miR-29a-3p, and possibly also miR-29b-3p, are upregulated in GDM pregnancies in the 2nd trimester compared with age and gestational age matched control women with NGT. The reason for the different regulation of miR-29 in Zhao et al (Zhao et al., 2011) is not known, but may be due to ethnicity of the sample population as this study was the

only to investigate miR-29a-3p in Chinese women. Alternatively, different sample handling may explain the differing results, as Zhao et al. (Zhao et al., 2011) report blood sample processing within four hours, whereas the studies by Sørensen et al. (Sorensen et al., 2021), Martínez-Ibarra et al. (Martinez-Ibarra et al., 2019), Wander et al. (Wander et al., 2017) and Gillet et al. (Gillet et al., 2019) processed samples within one hour. Although miRNA is regarded as being stable in circulation, the difference in the processing of the samples could possibly result in relatively higher decay of the miRNA.

Altogether, these studies support that circulating miR-29a-3p, and to some degree miR-29b-3p, levels are also increased during GDM and may have biomarker potential as a diagnostic marker. However, prognostic biomarkers for GDM would be much more desirable. While Sørensen et al (Sorensen et al., 2022) demonstrate a significant increase in the level of miR-29a-3p in serum already early in pregnancy (average week 16), which is confirmed by Gillet et al. (Gillet et al., 2019), although measured in EVs, the studies by Wander et al. (Wander et al., 2017) and Martínez-Ibarra et al. (Martinez-Ibarra et al., 2019) do not investigate early pregnancy samples (before week 20). Therefore, it is difficult to fully assess the ability of miR-29a-3p as a prognostic biomarker and to assess the possibility of using miR-29a-3p levels as a prognostic biomarker. Thus, more prospective studies are needed investigating early pregnancies. Interestingly, increased miR-29a-3p was found associated with T2D in a systematic review (Villard et al., 2015), and it is therefore conceivable that pregestational increased levels of miR-29a-3p may be carried forward into gestation.

miR-16-5p is upregulated especially in late GDM pregnancy

MiR-16 is reported to affect insulin sensitivity in human and rodent tissue, but also in models of T2D. Insulin-resistant tissues have lower expression of miR-16 and muscle-specific miR-16 KO mice displayed impaired insulin sensitivity and protein turnover (Lim et al., 2022). Several studies have

examined miR-16-5p and found it significantly increased in late-diagnosed GDM (>24 weeks of gestation) (Cao et al., 2017, Sorensen et al., 2021, Martinez-Ibarra et al., 2019, Zhu et al., 2015). Overall, studies report very similar increases: An approximate 2.5-fold increase in miR-16-5p in 2nd and 3rd trimester diagnosed GDM women compared with NGT pregnant women, with levels increasing throughout gestation (Cao et al., 2017, Sorensen et al., 2022). However, a smaller study was unable to detect any difference in circulating miR-16-5p (Hocaoglu et al., 2019), but this could be due to the low number of included subjects. Moreover, miR-16-5p was also found to be significantly increased in the 1st trimester (Sorensen et al., 2021, Sorensen et al., 2022, Cao et al., 2017), which suggests that miR-16-5p could potentially be used as a diagnostic as well as a prognostic biomarker of GDM. This is emphasized by the finding that miR-16-5p in combination with miR-29a-3p and the also GDM-associated miR-134-5p forms a superior diagnostic measure compared with 2hr plasma glucose following an OGTT (Sorensen et al., 2021), which is one of the recommended diagnostic measures of the IADPSG (International Association of et al., 2010).

Circulating miR-17-5p is increased throughout gestation

Downregulation of miR-17-5p was associated with a decrease in size of the pancreatic islets, elevated levels of blood glucose and loss of glucose tolerance. Furthermore, exogenous miR-17-5p inhibited TXNIP and NLRP3 inflammasome activation and decreased streptozotocin-induced β-cell death (Liu et al., 2021). In a longitudinal study, miR-17-5p was measured during each trimester (T1, T2 and T3) corresponding to gestational weeks 16-20, 20-24 and 24-28, and was found to be significantly increased in GDM women at all three measurement periods (Cao et al., 2017). This suggests a general upregulation of miR-17-5p in GDM independently of gestational age. Moreover, this study identified a positive correlation between the level of miR-17-5p and insulin resistance and an interaction with GDM, as the insulin resistance associated with pregnancy may be more pronounced in GDM

pregnancies (Kampmann et al., 2019). Tagoma et al. (Tagoma et al., 2018) measured the level of miR-17-5p at weeks 23-31 of pregnancy and found a 10.9- fold increase in miR-17-5p and a 2.6-fold increase of circulating miR-17-5p levels. However due to large variability these findings were not statistically significant, although the observations were congruent with Cao et al (Cao et al., 2017). Zhu et al. (Zhu et al., 2015) reported a 2-fold increase in miR-17-5p in week 24-18, similarly to a study by Lamadrid-Romero et al. (Lamadrid-Romero et al., 2018), which showed tendencies of increased levels of miR-17-5p in GDM pregnancies, in T1 and T2, while they were unable to show differences in miR-17 levels between GDM and control women in the 3rd trimester. Altogether these findings indicate a generally increased level of miR-17-5p in pregnancy, especially those complicated by GDM, despite the observation that downregulation of miR-17-5p in β-cells is associated with the development of diabetes (Liu et al., 2021).

Circulating miR-19a-3p and miR-19b-3p are only slightly increased in GDM

MiR-19a and miR-19b are both part of the miR-17-92 (miR-17) locus and are excised from the same primary transcript. The direct role of miR-19 has not been thoroughly described, yet it might share some characteristics with miR-17 in supporting a healthy β-cell function. Elevated levels of miR-19 were observed in replicating β-cells compared with non-replicating β-cells (Mandelbaum et al., 2019). Moreover, in skeletal muscle, an inverse relation between miR-19a-3p and citrate synthase, a target of miR-19a-3p, expression was identified, suggesting, that miR-19a-3p might regulate the mitochondrial capacity of skeletal muscles (Pinto et al., 2017). Cao et al. (Cao et al., 2017) examined miR-19a/b-3p and found no significant difference in the expression of either miRNA isoforms in their study in relation to GDM pregnancy. Both isoforms were measured several times during pregnancy at weeks 16-20, 20-24 and 24-28, with no significant difference observed in circulating levels of miR-19a/b-3p at any point. In contrast to this, the studies by Stirm et al. (Stirm et al., 2018) and Zhu et al. (Zhu et al., 2015) demonstrated a significant difference in the expression of both miR-19a-3p and

miR-19b-3p in their screening group during pregnancy in weeks 24-28. MiR-19a-3p displayed a fold change of 2.2 with an associated p-value of 0.002, while miR-19b-3p showed a fold change of 1.6 with an associated p-value of 0.005. Therefore, they chose to include both miRNAs in their validation group. However, in the validation group no significant difference was detected with a p-value of 0.3 for both isoforms. The difference in results between screening and validation group may be due to the population sizes. In the screening group a small population was used causing the result to be non-representative and therefore significant. When the miRNAs were examined in a larger population, the difference was no longer significant. In another study, miR-19a/b-3p were upregulated approximately 9-fold in GDM pregnancies, although this failed to reach statistical significance (Tagoma et al., 2018). Thus, based on these three studies, miR-19a/b-3p is not possible diagnostic biomarker for GDM, as none of the studies found a difference in the expression of miR-19 in relation to GDM, although all three studies consistently showed an increase in circulating miR-19a/b-3p in GDM.

MiR-223-3p could be increased early in gestation in GDM

In β-cells, miR-223 is upregulated by diabetes, and β-cell overexpression of miR-223 enhances proliferation, while deficiency of miR-223 increases apoptosis, ultimately reducing the functional β-cell mass (Li et al., 2019). Moreover, miR-223-3p is associated with impaired insulin sensitivity in adipose tissue (Sanchez-Ceinos et al., 2021). Several studies investigated the correlation between miR-223-3p and GDM: Yoffe et al. (Yoffe et al., 2019) showed a large, 9-fold, significant increase in expression of miR-223-3p between control and GDM groups with an adjusted p-value of 1.4×10^{-7}, performed on samples from the 1st trimester i.e., up to and including week 12. However, the study by Sørensen et al. (Sorensen et al., 2021) did not identify any difference between GDM and NGT in

early gestation, and thus it was concluded that miR-223-3p could not be used as a prognostic biomarker. However, it may be difficult to compare these two studies, as the early GDM group in Sørensen et al. (Sorensen et al., 2021) was defined as less than gestational week 20 with an average of gestational week 15.3. Furthermore, it is unknown whether the upregulated miR-223-3p in the 1st trimester, in the study by Yoffe et al. (Yoffe et al., 2019), was maintained or decreased again in the 2nd trimester. In another study, levels of miR-223-3p in circulation decreased through gestation (Sorensen et al., 2022). In samples measured during the 2nd trimester, Wander et al. (Wander et al., 2017) found miR-223-3p significantly increased in GDM women, if the data were corrected for the sex of the fetus. The study concluded that the miRNA was upregulated only in women, who were pregnant with boys. But based on the lack of significant difference without correction for fetal sex, the results from this study agrees well with the conclusion of Sørensen et al. (Sorensen et al., 2021, Sorensen et al., 2022). Tagoma et al. (Tagoma et al., 2018) examined the expression of miR-223-3p during pregnancy in weeks 23-31, and although the study showed a fold increase of 11 for miR-223-3p, this was not significant ($p=0.24$). The studies that examined miR-223-3p therefore do not uniformly agree on the potential for use as a diagnostic biomarker for GDM. The studies are also not comparable due to different periods of examination, however, there is a general trend that miR-223-3p is increased in early gestation of GDM women, although larger studies early in pregnancy are needed to conclusively determine this.

Circulating miR-330-3p levels are associated with GDM in late gestation

The miR-330-3p gene is located within an intron of a longer non-coding RNA named EML2 (EMAP like 2 (EML2) (Genome Browser: genome.ucsc.edu), with wide-spread expression pattern (Ludwig et al., 2016). It was reported upregulated in 2nd trimester plasma samples from GDM women and high levels of miR-330-3p was associated with increased risk of cesarian section and decreased β-cell

function (Sebastiani et al., 2017). Similarly, two other studies found miR-330-3p increased in GDM pregnancies in the 3rd trimester (Pfeiffer et al., 2020, Martinez-Ibarra et al., 2019), while early GDM had lower levels of miR-330-3p compared with matched glucose-tolerant control women (Sorensen et al., 2022). Interestingly, increased levels of miR-330-3p led to decreased levels of glucokinase in insulin secreting cells (INS-1 cells) (Xiao et al., 2020), in concordance with findings of lower insulin secretion among GDM women with high levels of miR-330-3p in circulation (Sebastiani et al., 2017). In correlation to this, miR-330-5p might also play a role in the function of adipose tissue through actions on the macrophages, as an inverse correlation between the miR-330-5p and Tim-3 (T cell immunoglobulin domain, mucin domain) protein levels was observed in macrophages of mice, resulting in insulin resistance (Sun et al., 2018).

MiR-155-5p in circulation is not consistently associated with GDM

MiR-155-5p is an inflammation-regulated miRNA, involved in multiple inflammatory diseases (Worm et al., 2009, Moura et al., 2019, Ma et al., 2011). As hyperglycemia induces inflammatory signaling (Kelstrup et al., 2012), it is a credible hypothesis that miR-155-5p should be upregulated in GDM. MiR-155 participates in the regulation of insulin sensitivity in adipose tissue, liver and skeletal muscle. Impaired glucose tolerance and decreased insulin sensitivity has been found in mice lacking miR-155, although these mice had an unaltered insulin production capacity. Dysregulated expression of miR-155 has also been shown to predict the development of some late complications of diabetes mellitus - such as retinopathy, neuropathy and nephropathy (Jankauskas et al., 2021).

We identified three studies, in which circulating levels of miR-155-5p were examined in relation to GDM, all of them in mid to late pregnancy. Wander et al. (Wander et al., 2017) examined miR-155-5p during the 2nd trimester and detected an increased level in GDM women, when corrected for

gestational age and fetal sex. The 2nd study, by Tagoma et al (Tagoma et al., 2018), found miR-155-5p increased 4.6 fold but this did not reach statistical significance. The third study by Hocaoglu et al. (Hocaoglu et al., 2019) examined miR-155-5p in the 3rd trimester, but did not find any significant difference in their GDM group compared to their control group, although miR-155-5p was found to be decreased in pre-eclampsia. However, the study examined the level of the miRNA in leukocytes and not whole blood or serum. This creates a bias when comparing with other studies, which examined plasma or serum. Thus, it appears that there is little to no biomarker potential of measuring miR-155-5p in GDM.

MiR-125a-5p and miR-125b-5p appear differentially regulated during gestation

MiR-125a/b are up-regulated by obesity in adipose tissue (Herrera et al., 2009). Overexpression of miR-125a in mice increased insulin sensitivity, while knock-down reduced insulin sensitivity. Furthermore, miR-125a knock-down has been shown capable to attenuate the lipid accumulation in hepatocytes (Liu et al., 2020). For the miR-125b-2 isomer, congruent observations were made, as deletion of miR-125b-2 increase liver and adipose tissue, and causes an accumulation of fat, reduced glucose utilization and decreased insulin sensitivity (Wei et al., 2020). These findings suggest that miR-125a and miR-125b play an important role in the regulation of insulin resistance and lipogenesis. Moreover, miR-125b is a negative regulator of insulin secretion, possibly via control intracellular lysosomes (Cheung et al., 2022), altogether suggesting that upregulation of miR-125 species may contribute to development of T2D.

Lamadrid-Romero et al. (Lamadrid-Romero et al., 2018) investigated the expression of miR-125b-5p during all three trimesters of pregnancy (T1, T2 and T3) and showed increased circulating levels of miR-125b-5p in T2 and T3 in women with NGT during pregnancy, while levels in GDM

complicated pregnant women were regulated differently showing increased levels of miR-125b-5p during T1, but decreased levels in during T2 and T3. These observations indicate that the timing of sampling could be important. Zhao et al. (Zhao et al., 2011) examined the amount of miR-125b-5p at weeks 16-19, but showed no significant changes in the expression of miR-125b-5p, while Nair et al. (Nair et al., 2018) showed a significant increase (p=0.05) in the expression of the miR-125a-5p isoform, when measuring on circulating exosomes in plasma. However, as these exosome measurements were performed after birth, it is of cause uncertain, when the rise in miR-125a-5p containing plasma exosomes occurred. However, Zhang et al. (Zhang et al., 2021) found significantly decreased levels of miR-125b-5p in isolated exosomes from week 24-28. In the 2nd and 3rd trimesters, Tagoma et al (Tagoma et al., 2018) observed a 3-fold increase in circulating miR-125b-5p, however, this did not reach statistical significance (p=0.14). Thus, studies regarding miR-125a/b-5p are inconsistent, as studies report increase in early gestation (Lamadrid-Romero et al., 2018) or no difference (Zhao et al., 2011), while in mid-to-late gestation levels were either insignificantly increased (Tagoma et al., 2018) or significantly decreased (Lamadrid-Romero et al., 2018). Based on these observations, miR-125a/b-5p cannot be used as a biomarker for GDM, but studies investigating miR-125 species are generally small and have low power. Therefore, new studies for this miRNA should focus on investigating a larger number of subjects.

The hypoxamiR, miR-210-3p, in relation to GDM

MiR-210-3p is known as a hypoxamiR, because it is upregulated by hypoxia in most cell types and participates in the coordinated response to hypoxia (Zaccagnini et al., 2022). Decreased levels of miR-210-3p in β-cells results in β-cell functional impairment and apoptosis, which could contribute to the development of β-cell dysfunction and death during T2D (Nesca et al., 2013). Three studies studied circulating levels of miR-210-3p in GDM; Gillet et al. (Gillet et al., 2019) observed a

significant increase of 1.3-fold in extracellular vesicles from GDM women, examined between 6-15 weeks of gestation, while Wander et al. (Wander et al., 2017) observed a 1.5-fold increase measured in the 2nd trimester, but this was not significant. In another study, by Tagoma et al. (Tagoma et al., 2018), no significant difference between NGT and GDM was detected at gestational weeks 23-31, although average levels were increased in GDM. Thus, miR-210-3p was upregulated in extracellular vesicles in early gestation, but when measured in plasma in 2nd trimester, no significant changes were observed. No studies examined extracellular vesicles at 2nd trimester. Thus, although increases in miR-210-3p are reported, the magnitude of the increases is small and data appear variable. Based on this, miR-210-3p is not suitable as a diagnostic or prognostic marker of GDM. Possibly, miR-210-3p as a hypoxamiR would be a more relevant marker of intrauterine growth retardation due to insufficient placental function.

MiR-132-3p is decreased in circulation in 2nd trimester GDM patients

MiR-132 downregulation correlates with a minor, but significant, inhibition of cell proliferation whereas upregulated miR-132 expression was detected in different T2D models. Inhibition of the miR-132 expression in pancreatic β-cells has been associated with an increase in the cleavage of caspase-9, while upregulation of miR-132 resulted in reduced levels of caspase-9, indicating a necessary role of miR-132 in the protection against apoptosis (Mziaut et al., 2020). Moreover, *in vivo* whole-body silencing of miR-132 using an antisense oligonucleotide reduces blood glucose levels and increase insulin secretion (Bijkerk et al., 2019). Therefore, miR-132 might have a role in protection against development of diabetes by promoting β-cell expansion and decreasing β-cell death (Eliasson and Esguerra, 2020).

We identified four studies investigating miR-132-3p in relation to GDM. In early gestation extracellular vesicles miR-132-3p was increased 1.65-fold (Gillet et al., 2019), and, insignificantly, in plasma form 2nd trimester GDM patients (2.3-fold, P=0.14) (Tagoma et al., 2018). However, two other studies found miR-132-3p decreased in GDM pregnancies (Zhou et al., 2019, Zhao et al., 2011); Zhao et al. (Zhao et al., 2011) observed a 32% reduction of miR-132-3p plasma of 2nd trimester GDM patients, and Zhou et al. (Zhou et al., 2019) observed a highly significant 46% reduction of serum miR-132-3p, which gave rise to a receiver operator curve area of under the curve (ROC AUC) of 0.89. The number of studied subjects in the studies by Zhao et al. (Zhao et al., 2011) and Zhou et al. (Zhou et al., 2019) is more than twice the numbers studies by Gillet et al. (Gillet et al., 2019) and by Tagoma et al. (Tagoma et al., 2018), and it seems most likely that miR-132-3p is downregulated in circulation in GDM. The downregulation of miR-132-3p in GDM is interesting given the observation that silencing miR-132-3p using antagomir injections in mice increased β-cell insulin secretion and lowered blood glucose (Bijkerk et al., 2019).

Placenta-derived extracellular vesicle associated miRNAs as biomarkers of GDM

The amount of extracellular vesicles in circulation increase during gestation, as well as the number of exosomes derived from the placenta, carrying the placental alkaline phosphatase marker PLAP (Salomon et al., 2016), and numbers of PLAP+ exosomes from GDM patients were more proinflammatory and increased significantly compared with control women during gestation (Liu et al., 2018, Salomon et al., 2016). Physiologically, small (s) EVs from pregnant women play a role in regulating insulin sensitivity, as mice infused with sEVs isolated from pregnant women in 2nd trimester displayed decreased insulin sensitivity, whereas sEVs from GDM patients induced overt glucose intolerance in mice (James-Allan et al., 2020). Moreover, placental exosomes control insulin sensitivity, possibly via their content of miRNAs (Nair et al., 2018). MiR-92a-3p was suggested as a

mediator of these responses (Nair et al., 2021). Of note, miR-92a-5p is part of the same miRNA cluster (miR17HG) also containing miR-17-5p and miR-19a/b-3p (https://genome.ucsc.edu), and share regulatory sequences. It is possible that the entire miRNA cluster could be relevant for GDM diagnosis or prognosis either measured in plasma or in isolated extracellular vesicles from the placenta.

Conclusions and perspectives

In this review, we aimed to collect and discuss the published literature for circulating miRNAs being associated with GDM, to determine if miRNAs, by currently available information, could potentially be biomarkers for early detection of GDM. Although we initially searched for studies investigating all types of small RNAs, identified original studies were generally focused on miRNAs, which therefore became the focus of this review, however, other types of small RNAs, such as piwi-associated RNAs (piRNAs) and tRNA fragments are also present in circulation and could also be possible biomarker candidates. There are relatively few miRNAs for which levels in circulation are consistently associated with GDM: miR-29a/b-3p and miR-16-5p, whereas other reported miRNAs are not consistently associated with GDM. MiR-29a/b-3p and miR-16-5p generally have good discriminatory power to detect GDM, and they may also have predictive capabilities for GDM when measured early in gestation.

It is clear from the literature that there is large heterogeneity between studies with regard to miRNAs being associated with GDM, with only few miRNAs being convincingly and reliably associated with GDM. Both methodological as well as clinical factors are likely to contribute to the diversity in findings. Methodological factors can be divided into pre- and post-analytical factors. Differences between studies with regard to pre-analytical factors such as sample type (serum, plasma or full blood), sample collection and processing (i.e. centrifugation times), RNA-extraction methods and

quality control, as well as the qRT-PCR-assay can all result in variability between studies. Moreover, a main challenge of post-analytical level is data normalization as qRT-PCR relative quantification is often used (de Gonzalo-Calvo et al., 2022). For measurements of circulating miRNAs no standardized protocols exist. Consensus guidelines for development of assays for circulating RNAs underscore the importance of standardized assays, and emphasize the necessity of validation at all steps of an RNA measurement from how the sample is treated, the RNA extracted and measured (i.e. using qRT-PCR) as well as to how the data are analyzed and reported (Acuna-Alonzo et al., 2010).

Clinical factors also contribute to disparity between studies, because cohorts may be recruited from different underlying patient populations, through different inclusion and exclusion criteria, and specifically for GDM diagnostic criteria differ between countries creating lack of comparability between studies. Furthermore, as miRNA levels change during gestation, the gestational sampling time point also offers a source of heterogeneity between studies (Sorensen et al., 2022). Furthermore, due to the discovery nature of many biomarker studies, a general flaw is the risk of them being underpowered, which promotes publication bias and lack of reproducibility (Ioannidis et al., 2014). Thus, to improve the likelihood of circulating miRNAs to be used in a clinically validated test in relation to GDM, increased transparency of study reporting, standardized workflows, assays and rigorous data analytical pipelines are needed (de Gonzalo-Calvo et al., 2022). Moreover, there is a need for systematic reviews and meta-analyses on circulating miRNAs in GDM in order to extract data from the existing literature in a systematic and unbiased manner.

In order to proceed with these miRNAs as diagnostic or prognostic markers for GDM further studies are necessary, in larger cohorts and in patient with other pregnancy associated morbidities such as

pre-eclampsia. In order for a miRNA to be developed into a biomarker, it is also necessary to identify a population base-line to establish if the miRNA is specific for GDM. Moreover, it seems likely that miRNAs may be used as GDM biomarkers in combination with each other, because the combination of several miRNAs will yield a more specific algorithm with a higher ROC AUC.

There are indications that circulating RNA markers are entering the clinic, i.e. there is a marketed prediction algorithm (NIS4, offered by Genfit) for development of NASH or NASH related fibrosis that involves miRNA-34a in combination with alpha-2 macroglobulin, YKL-40, and glycated hemoglobin (Harrison et al., 2020). Moreover, within the cancer field, the Thyramir miRNA 11 miRNA based classifier for thyroid nodules is offered by Interpace Diagnostics and shows superior performance (Finkelstein et al., 2022). However, there are no clinically validated and marketed commercial tests based on miRNAs for predicting future GDM, based on an early sample, although such a test would be very valuable in clinical management of women at risk of GDM.

As GDM share many features with T2D it would also be of interest to investigate levels of the GDM-associated miRNAs in subjects with impaired glucose tolerance to determine if these would be able to predict later progression to overt T2D. Moreover, by detecting the risk of GDM earlier in pregnancy, it will be possible to initiate preventive measures and treatment earlier to prevent exposure of the fetus to extended periods of hyperglycemia. The use of miRNA as a diagnostic biomarker from a patient perspective could be beneficial, because miRNA is easy to measure, as it can be done with an ordinary blood test, which for most would be preferable to an OGTT.

Another interesting question is from which tissue differentially regulated RNAs originate. If we could pinpoint the tissue or cell type of origin of the RNAs differentially regulated in GDM would provide important pathophysiological and novel information about the RNA secretion patterns in pregnancy and in GDM.

Declaration of interest

There are no declarations.

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Author contributions

LTD conceived the study, researched the data and wrote the manuscript. SD and AE-F researched the data and wrote the manuscript. All authors approved the final version.

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Legends:

Fig. 1: MicroRNA and EV biogenesis. The canonical miRNA biogenesis starts as a primary miRNA transcript is formed in the nucleus by RNA polymerase II. Primary miRNA transcripts contain partially complementary hairpin structures. MiRNA genes may be located as isolated transcriptional units or be located in exons or introns of protein-coding genes. DROSHA, a nuclease, removes the sequences outside the hairpin to form the precursor miRNA, in combination with the partner DGCR8. Subsequently, the miRNA precursor is exported to the cytoplasm by EXPORTIN 5 using RAN-GTP. Then, the nuclease DICER with TRBP removes the hairpin turn and the resulting duplex mature miRNA is unwound and inserts itself into the RISC complex. The miRNA-RISC complex then degrades or halts transcription of mRNAs which are recognized by the miRNA. The RISC-complex may dock to the ER-membrane to load miRNAs into exosomes and multivesicular bodies. Extracellular RNA may also be released from cells in microvesicles that bud from the plasma membrane or may be bound by RNA-binding proteins, both of which will protect the miRNA in circulation from degradation. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

Fig. 2: Distribution of miRNAs examined in relation to GDM. The majority of original studies examining miRNAs for association with GDM were only examined in one published study, while miR-16-5p and miR-29a-3p were investigated in six studies each. Thus, the majority of reported circulating miRNAs associated with GDM were only identified in one study.

822 **Table 1 Commonly used methods for miRNA biomarker studies**

Measurement method	**Principle**	**Advantages**	**Limitations**
Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)	In qRT-PCR, cDNA is synthesized using a reverse transcriptase enzyme and the target amplified by PCR. The quantification is done using fluorescent detection during the PCR amplification, and relies on the first detectable cycle of product formation (named Cq or Ct). Often performed on one miRNA target at a time, although qPCR arrays can measure up to 384 wells at a time.	Highly quantitative Very sensitive Spike-ins can be added during RNA-isolation and cDNA synthesis, which are then quantified during the qPCR to enable absolute quantification. Low cost enables the measurement of larger cohorts.	Is a targeted approach, hence only candidate miRNAs are investigated. No general and consensus data analysis strategy exists.
Small RNA-sequencing (smRNA-seq)	In smRNA-seq, RNA linkers are ligated to the RNA and used to generate a smRNA library, which is amplified, size-selected and sequenced using massive parallel sequencing methods (i.e. using Illumina or Ion Torrent sequencing).	Allows the detection of all small RNAs in a sample. Partially quantitative: Although spike-ins can be added during library synthesis, this is rarely implemented. Very sensitive.	Per sample higher cost. Highly abundant miRNAs constitute a large fraction of the reads, requires higher sequence depth to increase costs. No general and consensus data analysis strategy exists.
Small RNA-arrays	All small RNAs in a sample are labeled and hybridized to an oligo nucleotide array. To increase hybridization strengths to the short miRNAs, oligos are often modified using locked nucleic acid analogues.	Allows the detection of all known miRNAs. RNAs in a given sample. Not sensitive Per sample cost is low, given the number of miRNAs measured.	Has a limited dynamic range. Data analysis strategies are more uniform.

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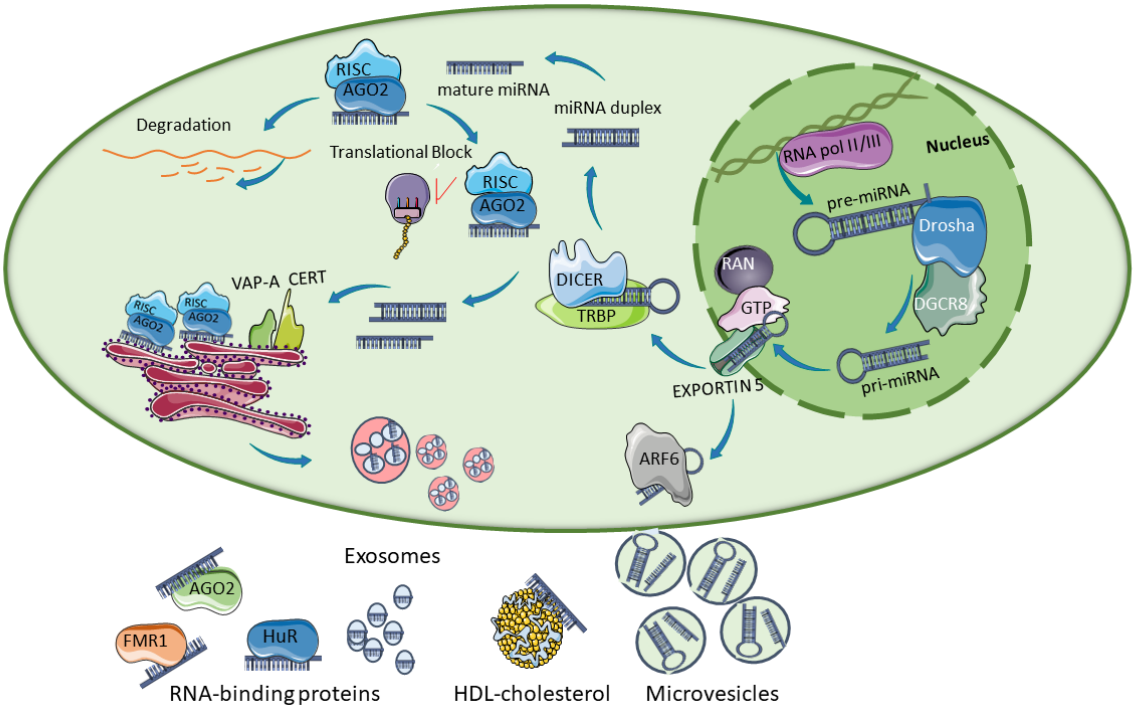
825 **Table 2 Studies of circulating miRNAs associated with GDM included in the narrative review**

Reference	*n* GDM	*n* NGT	Trimester investigated	Sample material	Quantification methods
(Cao et al., 2017)	85	72	Not given	Plasma	RT-qPCR
(Filardi et al., 2022)	12	12	T3	Plasma	RT-qPCR
(Gillet et al., 2019)	23	46	T1 + T2	Exosomes	RT-qPCR
(Hocaoglu et al., 2019)	19	28	T3	Serum	RT-qPCR
(Lamadrid-Romero et al., 2018)	12/24/16	13/24/20	T1/T2/T3	Serum	RT-qPCR
(Martinez-Ibarra et al., 2019)	18	22	T2	Serum	RT-qPCR
(Nair et al., 2018)	12	12	At birth (T3)	Exosomes	RT-qPCR
(Peng et al., 2018)	11	12	T3	Plasma	RT-qPCR
(Pfeiffer et al., 2020)	31	29	T2 + T3	Serum	RT-qPCR
(Qi and Wang, 2019)	108/48	100	T3	Serum	RT-qPCR
(Sebastiani et al., 2017)	21	10	T2/T3	Plasma	Taqman array → RT-qPCR
(Stirm et al., 2018)	38	38	T2 + T3	Whole blood	qPCR
(Sorensen et al., 2021)	82	41	T1 and T2/3 GDM	Serum	qPCR
(Sorensen et al., 2022)	82	41	T1 + T2 +T3 GDM	Serum	RT-qPCR
(Tagoma et al., 2018)	13	9	T2 + T3	Plasma	RT.qPCR
(Wander et al., 2017)	36	80	T2	Plasma	RT-qPCR
(Xiao et al., 2020)	30	10	T2 + T3	Serum	qPCR
(Yoffe et al., 2019)	23	20	T1	Plasma	qPCR
(Zhang and Chen, 2020)	30	30	Not given	Serum	RT-qPCR
(Zhang et al., 2021)	61	57	T2	Exosomes	RT-qPCR
(Zhao et al., 2011)	24	24	T2 + T3	Serum	RT-qPCR
(Zhou et al., 2019)	108	50	T2 + T3	Serum	RT-qPCR
(Zhu et al., 2015)	10	10	T2	Plasma	RT-qPCR

826 ***Table 2:*** *Gives an overview of the studies included in the review. T1: Trimester 1. T2: Trimester 2. T3: Trimester 3.*

Figure 1


RISC
AGO2
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TRBP
RAN
GTP
DGCR8
VAP-A
CERT
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ARF6
Exosomes
AGO2
FMR1
HuR
RNA-binding proteins
HDL-cholesterol
Microvesicles

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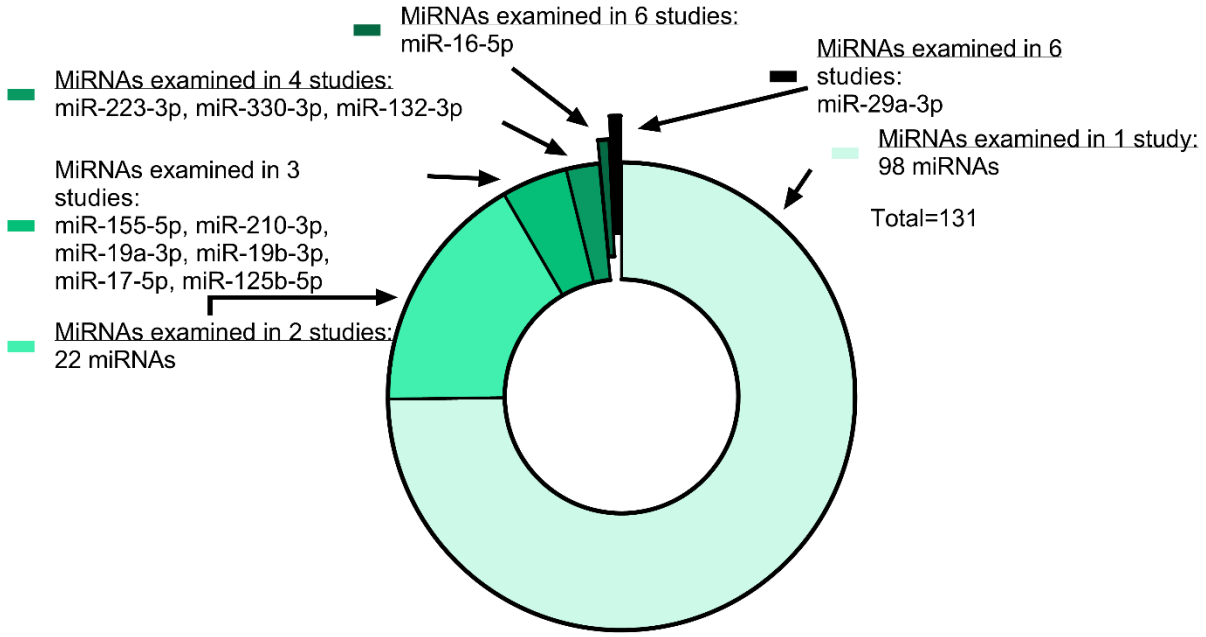
Figure 2



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