SHORT COMMUNICATION

MicroRNA profiling in Human Immunodeficiency Virus (HIV)-infected South African women with Gestational diabetes mellitus

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Abstract

Background

Recently we reported that microRNAs (miRNAs) miR-20a-5p and to a lesser extent miR-222-3p, hold potential as biomarkers for Gestational diabetes mellitus (GDM) in human immunodeficiency virus (HIV)-negative South African women.

Methods

In this preliminary study we measured the expression of these miRNAs in HIV-positive women (GDM=15, non-GDM=52; median 26.0 weeks; range 16-30 weeks).

Results

Although the same trend of decreased expression of miR-20a-5p (↓1.5-fold) and miR-222-3p (↓1.4-fold) was observed in sera of women with GDM compared to women without GDM, these differences were not statistically significant. Stratification according to antiretroviral treatment (ART) confirmed decreased expression of miR-20a-5p and miR-222-3p in ART naïve and ART treated women with GDM, although this was again not statistically significant.

Conclusion

Our results demonstrate that HIV-infection modifies the expression of miR-20a-5p and miR-222-3p in women with GDM. Importantly, this study highlights the complexities of miRNA profiling and the need for GDM biomarker discovery in both HIV-infected and uninfected individuals, particularly in South Africa where approximately 30% of pregnancies are complicated by HIV. Further studies to elucidate the mechanisms that underlie these miRNA differences are needed.

Key findings

- MicroRNAs (miRNAs) miR-20a-5p and to a lesser extent miR-222-3p, hold potential as biomarkers for Gestational diabetes mellitus (GDM) in human immunodeficiency virus (HIV)-negative South African women.
- South Africa has the highest prevalence of HIV infection globally, particularly in women of reproductive age. An estimated 30% of all pregnancies in South Africa are complicated by HIV infection.
- This study shows that HIV-infection may modify miR-20a-5p and miR-222-3p expression, highlighting the complexities of miRNA profiling and supporting the need for GDM biomarker discovery in both HIV-infected and uninfected individuals, particularly in countries with a high prevalence of HIV.

1. Introduction

Gestational diabetes mellitus (GDM) is defined as glucose intolerance that is first diagnosed during pregnancy and usually resolves after delivery [1]. Progressive insulin resistance due to a combination of factors including inflammation, placental hormones, decreased adiponectin secretion and excess lipolysis have been implicated in the development of GDM [2]. In many pregnant women, pancreatic beta cells are able to compensate for insulin resistance by increasing insulin secretion, however, in some women pancreatic function is unable to adapt to the increasing insulin demand, leading to glucose intolerance and GDM. In 2017, the International Diabetes Federation estimated that globally 14% of pregnancies are complicated by GDM [3], although these rates vary according to diagnostic criteria and population differences. Recently, a GDM prevalence of 25.8% was reported in an urban setting in South Africa [4], a rate considerably higher than previously reported in this country. GDM is associated with perinatal complications and an increased risk for future metabolic disease in both mothers and their offspring [5, 6]. Bellamy et al. reported that women with GDM are approximately seven-fold more likely to develop Type 2 diabetes (T2D) in later life compared to women who did not have GDM [7]. Glucose management during GDM improves health outcomes [8], thus the identification of biomarkers to improve detection and management of GDM is a health priority.

MicroRNAs (miRNAs) are short, non-coding RNA molecules that regulate diverse biological processes including development, proliferation, differentiation and apoptosis through post-transcriptional mechanisms [9]. They bind to the 3'untranslated region of messenger RNA (mRNA) inducing either silencing or degradation of the mRNA transcript. MiRNAs are widely regarded as master regulators of biological function, with studies suggesting that miRNAs regulate about 30% of the genome [10, 11]. Altered miRNA expression in metabolic pathways such as lipid metabolism, insulin signaling, pancreatic beta cell function, glucose homeostasis and inflammation have been implicated in the pathophysiology of conditions including obesity, T2D and cardiovascular disease, to name a few [12]. In recent years, circulating miRNAs in biological fluids such as serum and plasma have attracted interest as biomarkers for disease [13]. These miRNAs are speculated to play a role in cell-to-cell communication acting in either an autocrine or paracrine manner, and their dysregulation have been demonstrated in various metabolic disorders including GDM [14].

South Africa has the highest prevalence of human immunodeficiency virus (HIV) infection worldwide, with an estimated five million people infected, accounting for approximately 10% of the population [15, 16]. Since 2004, all HIV-infected individuals receive antiretroviral therapy (ART) in South Africa, although the criteria for treatment has changed over the years [17]. Treatment has significantly increased life expectancy, however, reports that ART, particularly the first generation protease inhibitors, increases the risk for metabolic disease soon emerged [18]. Although reverse transcriptase inhibitors rather than protease inhibitors are used in newer generation ART, and have decreased the adverse effects of ART, they are still associated with metabolic dysregulation [19, 20]. In South Africa, the incidence of HIV is higher amongst women than men, particularly in women of reproductive age [15]. A national survey conducted in South Africa in 2015 estimated that 30% of pregnant women attending antenatal care are HIV positive [17].

Recently we reported that miRNA-20a-5p and to a lesser extent miRNA-222-3p, hold potential as biomarkers for GDM in HIV-negative South African women [21]. These miRNAs were identified after screening eight miRNAs with potential as biomarkers for GDM in other populations [22, 23] in South African women with GDM. A limitation of this study was the analysis of only HIV-negative women. Accumulating evidence suggest that HIV modulates miRNA expression to promote its own replication or that the host regulates miRNA expression to control viral infection [24]. Furthermore, HIV infection alters the expression of miR-20a [25] and miR-222 [26] in CD4+ T cells, supporting the need for miRNA quantification in both HIV-negative and HIV-positive pregnant women. In this preliminary study we measured the expression of miRNAs, previously shown to hold potential as biomarkers of GDM [21], in HIV-positive women.

2. Methods

2.1 Participants

The study was approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Pretoria (Protocol 180/2012). All women gave written informed consent. A total of 1000 pregnant women of black ethnicity who were between the ages of 18 and 40 years were enrolled at their first clinic visit and excluded if they had pre-existing diabetes, had a twin or multiple pregnancy and were acutely ill [4]. Pre-existing diabetes included women with known either Type 1 diabetes or T2D, or who were diagnosed with overt diabetes for the first time in pregnancy according to criteria recommended by the International Association of Diabetes and Pregnancy Study Group (IADPSG) [27]. Women who were included were requested to return for GDM testing and blood collection within two weeks. GDM was diagnosed using the 75 g 2-hr oral glucose tolerance test (OGTT) at 24–28 weeks of pregnancy according to IADPSG criteria and managed according to International Federation of Gynecology and Obstetrics (FIGO) recommendations with diet and lifestyle modifications, metformin or insulin [28]. Pregnant women were offered HIV testing with rapid HIV kits, and results were confirmed with a different HIV kit according to guidelines of the South African Department of Health [29]. HIV-positive women were treated with Atripla™, the fixed-dose co-formulation of three anti-HIV drugs Efavirenz, Emtricidabine and Tenofovir, which is taken once daily [29]. Women in this cross-sectional study were selected from the prospective cohort study on GDM screening [4]. The sample consisted for 81 HIV-negative pregnant women, whose characteristics were described previously [21] and 67 HIV-positive women. Demographic information was collected using a standardized questionnaire [4]. Anthropometric measurements were obtained according to standard procedures. Blood samples were collected after an overnight fast, and fasting plasma glucose and C-reactive protein concentrations were measured in an accredited laboratory (Vermaak and partners (Pretoria)/PathCare (Cape Town) Laboratories, South Africa). Serum adiponectin concentrations were quantified using the human adiponectin enzyme-linked immunosorbent assay (ELISA) (Merck, Darmstadt, Germany). Serum for miRNA analysis was stored at -80 °C until required.

2.2 MicroRNA isolation

MiRNAs were isolated as previously described [21]. Briefly, miRNAs were extracted from 200 µL of serum using the miRNeasy® Serum/Plasma kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To correct for technical variation during miRNA isolation, 3.5 µl of spike-in control (*Caenorhabditis elegans* miR-39) (1.6 × 108 copies/µl working solution) was added to the RNA during the extraction process. The quantity and quality of RNA was assessed using the NanoDrop® ND-1000 instrument (Nanodrop Technologies, Wilmington, DE, USA) and on an Agilent® 2100 Bioanalyser (Agilent Technologies, Palo Alto, CA, USA) using the Agilent® small RNA kits, respectively.

2.3 Quantitative real time PCR

Forty nanogram of miRNA-enriched total RNA was reverse-transcribed into complimentary DNA (cDNA) using the miScript II RT kit (Qiagen). The cDNA was diluted with 200 µl of RNAse-free water and then used as a template in

quantitative real-time PCR (qRT-PCR). Each reaction was performed in a final volume of 10 µl containing 1 µl of diluted cDNA, 5 µl of miScript 2x SYBR Green, 1 µl of the specific miScript 10x primer assay (hsa-miR-20a-5p (MS00003199), hsa-miR-222-3p (MS00007609) and cel-miR-39-3p (MS00019789)), 1 µl of miScript 10x universal primer as the reverse primer and double-distilled H_2O according to the manufacturer's instructions (Qiagen). The thermal cycler (ABI 7500 Instrument, Life Technologies) was set at 95 °C for 5 min, followed by 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 34 s, according to the manufacturer's instructions. The spike-in-control, *Caenorhabditis elegans* miR-39, was used as an exogenous control. The relative expression of miRNAs was calculated using the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt was calculated by subtracting the mean ΔCt value of the control group from the ΔCt of the experimental samples; ΔCt was calculated as Ct_{sample} - $Ct_{cel\text{-}39}$ (Ct, threshold cycle). All qRT-PCR experiments included a no template control and reactions were conducted in duplicate.

2.4 Statistical analysis

Numerical data are expressed as the median and interquartile range, mean (standard deviation) for normally distributed data or count (%) for categorical variables. Testing for normality was conducted using the Shapiro–Wilk test. The unpaired Student t test was used to compare data that were normally distributed, the Mann-Whitney test for data that were not normally distributed, and the Fisher's exact test for categorical variables. A p value ≤ 0.05 was considered statistically significant. Statistical analysis was performed using STATA® version 14.0 (StataCorp, College Station, TX, USA).

3. Results

3.1 Participants

Of the 1000 potentially eligible participants [4], HIV status was available for 989 participants, of whom 295 (29.8%) were HIV-positive. C-reactive protein concentrations were measured in 318 (214 HIV-negative and 77 HIV-positive) women and were higher in HIV-infected women compared to uninfected women (8.4 (4.8-16.3) vs. 5.7 (3.3-8.9) mg/L, $p<0.001$). Of the 77 HIV-positive women, C-reactive protein concentrations were not different between women who were ART naïve compared to women on ART (8.4 (5.5-17.5) vs. 8.7 (3.3-15.9) mg/L, $p=0.666$). Surprisingly, adiponectin concentrations were higher in HIV-infected compared to uninfected women (14.2 (9.2-20.7) vs. 9.1 (6.4-13.1) ng/mL, $p<0.001$, n=348 (210 HIV-negative and 138 HIV-positive)). Although GDM was more prevalent in HIV-positive women compared to uninfected women, the difference was not statistically significant (30.6% vs. 24.1%, $p=0.113$). Due to sample availability, serum from 81 HIV-negative [21] and 67 HIV-positive women were profiled for miRNAs. The characteristics of HIV-positive women according to GDM and ART status are illustrated in Table 1.

3.2 MicroRNA profiling

MiRNA levels were normalized to the exogenous control *C. elegans* miR-39 and relative expression calculated using the ΔΔCt method. The expression of miR-20a-5p and miR-222-3p were decreased by 1.5-fold ($p=0.223$) and 1.4-fold ($p=0.475$) in sera of HIV-positive women with GDM compared to women without GDM. Stratification according to ART confirmed decreased expression in pregnancies complicated by GDM, although again differences were not significant. For comparative purposes, results from our previously published study in HIV-negative women [21] are also demonstrated in Figure 1.

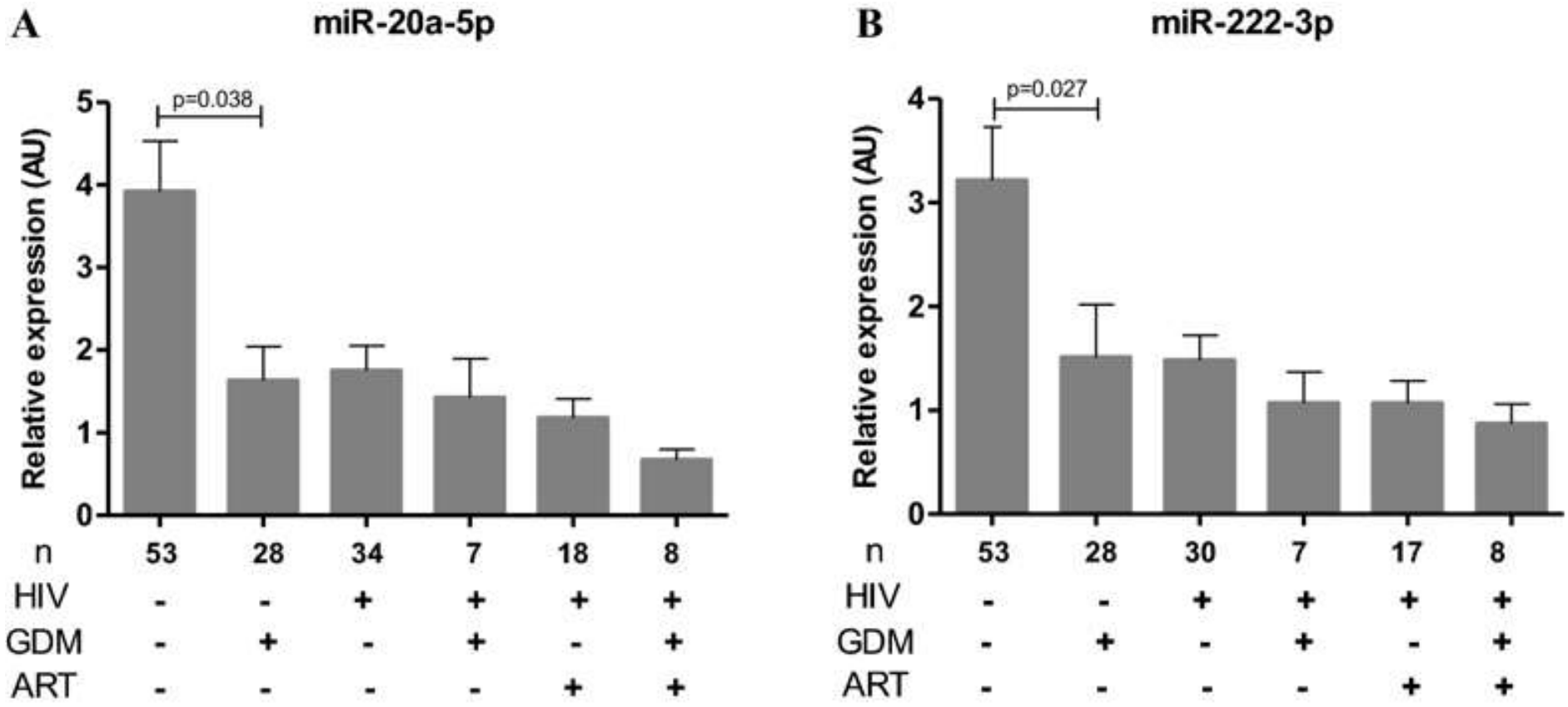

Figure 1. HIV infection and antiretroviral therapy affects the expression of **a** miR-20a-5p and **b** miR-222-3p in women with or without gestational diabetes mellitus. Data are presented as mean ± standard error of the mean. *ART* antiretroviral therapy, *GDM* gestational diabetes mellitus

4. Discussion

In 2017 it was estimated that 36.9 million people globally are infected with HIV [30]. Sub-Saharan Africa accounts for almost 70% of the global HIV burden, however, many other high-, middle-and low-countries worldwide are also affected by HIV [16, 30]. This preliminary study shows that HIV infection may modulate miR-20a-5p and miR-222-3p expression and affect miRNA differences between woman with or without GDM. MiRNAs are increasingly being studied for their potential as biomarkers, and are often studied in HIV-negative individuals or without consideration of HIV status [13, 31, 32]. For example, many studies profiling miRNAs during GDM thus far, were conducted in populations with a low prevalence of HIV thus do not report the HIV or ART status of women [22, 23, 33–35]. Reports show that the incidence of HIV in low prevalence countries such as in China has increased over the last decade, with the general population rather than only high-risk intravenous drug users being increasingly affected [36]. South Africa has the highest prevalence of HIV infection globally [15, 16]. In this study it was estimated that approximately 30% of pregnant women were HIV-positive, which is consistent with national estimates in South Africa [17]. Our results suggest that HIV infection may affect the molecular mechanism of GDM pathophysiology.

MiRNAs play an important role as metabolic and developmental regulators during pregnancy [37]. Both miR-20a-5p and miR-222-3p have been shown to be involved in placental development and pregnancy complications associated with GDM [37–40]. Furthermore, several studies have reported that circulating expression of miR-20a-5p and miR-222-3p have potential as biomarkers for GDM. Recently, we reported that the expression of miR-20a-5p and miR-222-3p are decreased in women with GDM compared to controls [21], while others reported that women with GDM had higher levels of miR-20a-5p [22, 33] and miR-222-3p [35] compared to controls. Discrepancies in miRNA expression are often ascribed to differences in population, biological source, measurement platform and normalization strategies [21, 31]. Our data suggests that HIV infection presents another source of miRNA variation, which should be considered in biomarker discovery. Thus, in addition to the pre-analytical and analytical challenges of miRNA analysis [41], this study further highlights the complexities of miRNA profiling that need to be addressed before they can become clinically applicable. HIV-infected women presented to the clinic earlier than HIV-negative women, thus pregnancy duration could have contributed to the heterogeneous miRNA responses. Longitudinal studies are required to investigate the effect of pregnancy duration on miRNA expression. Due to inadequate health systems in South Africa, women seek antenatal care late and at various times during their pregnancies, which could potentially contribute to heterogeneous miRNA responses and hindering their candidacy as biomarkers. Furthermore, studies should integrate miRNA expression with target gene expression in placenta to support the plausibility of miRNAs as biomarkers of GDM and aim to elucidate the mechanisms that underlie miRNA differences.

When looking at all HIV-positive women, adiponectin concentrations were surprisingly higher in HIV-infected pregnant women compared to HIV-negative women. Decreased adiponectin concentrations are observed with pregnancy duration [45], thus lower adiponectin concentrations in HIV-negative women may be due to the time of blood collection since these women presented to the clinic later than HIV-positive women. Adiponectin is an adipose-derived peptide cytokine that is postulated to play an important role in glucose and lipid metabolism [42]. Low levels

of adiponectin is associated with inflammation, dyslipidemia and insulin resistance, and have been reported during HIV-induced lipodystrophy with first generation ART, particularly the protease inhibitors [18]. Although Atripla™, the newer generation of ART used in this study has been shown to induce fewer metabolic derangements compared to older regimens consisting of protease inhibitors, they have nevertheless been shown to increase dyslipidemia [19]. Recently, Pepin et al. demonstrated that Atripla™ exacerbates diet-induced inflammation in mice, impairing glucose metabolism, energy balance, and metabolic dysfunction [20]. A limitation of the study was that total adiponectin, rather than the isoforms of adiponectin were measured. High molecular weight (HMW) adiponectin is considered the most active form associated with insulin sensitivity [42], although low molecular weight adiponectin, but not the HMW form has been shown to activate 5' adenosine monophosphate-activated protein kinase (AMPK), an enzyme that plays an important role in glucose and fat homeostasis in rat skeletal muscle [43], and to inhibit NFκβ activity and proinflammatory cytokine secretion from monocytes [42]. Further studies are required to measure different adiponectin isoforms, which may aid in elucidating the mechanisms underlying increased adiponectin concentrations in HIV-infected women.

HIV infection has been reported to affect insulin sensitivity [19], however, studies on the association between GDM and HIV are limited in Africa. Although GDM was slightly more prevalent in HIV-infected women compared to non-infected women, the difference was not statistically significant. These results are in agreement with Jao et al. who reported that HIV infection was not associated with GDM in Cameroonian women [44]. Recently, a meta-analysis on HIV-positivity and GDM reported no significant association between HIV-positivity and GDM, however, the authors acknowledged that the studies included in their review had high risk of bias and called for further studies to delineate the relationship between HIV and GDM [45]. A limitation of the studies on the association between GDM and HIV, including ours, are that they are cross-sectional in nature. In future, longitudinal studies to monitor disease progression and correlate these with clinical outcomes are required to more clearly define the association between GDM and HIV.

To our knowledge, this preliminary study is the first to investigate the effect of HIV infection and ART on miRNA expression during GDM. Although the study has several limitations, which include the small sample size, the lack of measuring insulin concentrations, viral load, CD4+ count, HIV and ART duration, it highlights the complexities of miRNA profiling and the need for GDM biomarker discovery in both HIV-infected and uninfected individuals.

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Compliance with Ethical Standards

Conflict of interest

Carmen Pheiffer, Stephanie Dias, Paul Rheeder, and Sumaiya Adam declare that they have no conflicts of interest.

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Ethical approval and informed consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

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