Investigating mitochondrial dysfunction in gestational diabetes mellitus and elucidating if BMI is a causative mediator

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1 Abstract

Objective: Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance which is diagnosed during pregnancy and poses considerable health risks for mother and child. Maternal body mass index (BMI) correlates with GDM diagnosis and the pathophysiology of this link may be explained through oxidative stress and mitochondrial dysfunction. In this study we investigate if mitochondrial dysfunction is evident in GDM by measuring cell free mitochondrial DNA concentration and determine if a potential relationship exists between maternal mitochondrial function and GDM diagnosis.

Study design: Plasma samples were taken at 20 weeks' gestation from women who subsequently developed GDM (n=44) and matched with women with uncomplicated pregnancies (n=85) as controls. Control group 1 was matched by maternal age and BMI (n=41) to GDM cases, while control group 2 was matched by maternal age alone (n=44). Prediction potential was determined by binary regression analysis. Statistical analysis was performed on SPSS Statistics v25.

Results: Binary regression analysis showed a statistically significant association between mtDNA concentration and GDM diagnosis ($p = 0.032$) in GDM cases versus control group 2, indicating that GDM patients have higher circulating mtDNA concentrations relative to healthy control patients. The lack of statistical significance in control group 1 suggests that BMI may be linked to mitochondrial function in GDM patients.

Conclusion: These results demonstrate a potential pathogenic role for mitochondrial dysfunction in GDM, with BMI presenting as a likely physiological mediator.

2 Introduction

Gestational diabetes mellitus (GDM) can be defined as any degree of glucose intolerance which is diagnosed during pregnancy (1). GDM is one of the most prevalent illnesses to develop during pregnancy, affecting approximately 1- 28% of antenatal mothers (2, 3). GDM is associated with an elevated risk of maternal and neonatal morbidity and mortality, with an increased risk of adverse perinatal complications such as macrosomia, respiratory distress syndrome and fetal hyperinsulinaemia (2). Established risk factors for GDM include ethnicity, obesity, micronutrient deficiencies, advanced maternal age and family history of diabetes (4, 5). GDM itself is an independent risk factor for the development of future health conditions in both the mother and the child. The nature of the relationship between obesity and GDM may result in a vicious cycle of intergenerational obesity and subsequent diabetes diagnosis. However, as a result of the increase in global prevalence of diabetes and obesity, two recognised risk factors for GDM, the numbers of cases of GDM have increased exponentially in the last decade (6).

Currently, the exact pathophysiology of GDM is not fully understood and the molecular mechanisms are yet to be fully elucidated (4). One origin of the insulin resistance associated with GDM is proposed to be that of placental insufficiency and altered placental function (7). Certain physiological changes are at least partly coordinated by hormones and other mediators secreted by placenta including estradiol, progesterone, prolactin, cortisol, human placental lactogen (hPL), and human placental growth hormone (hPGH), which are proposed to facilitate the overall physiological condition of peripheral insulin resistance (7, 8). Maternal hyperglycaemia, whether from pre-existing diabetes or from gestational diabetes, will often lead to fetal hyperglycaemia because glucose is easily transferred across the placenta (9), therefore maintaining optimal blood glucose levels is crucial to reducing these risks for mother and fetus.

During pregnancy, placental development requires an increased mitochondrial capacity to adequately meet the metabolic requirements of the developing fetus. In this way, placental mitochondria may play a vital role in the maintenance of pregnancy and neonatal development through regulation of metabolic activity and ATP production, hormone

synthesis and trophoblast oxygen sensing (10). Mitochondria have their own genome with multiple copies of small circular mitochondrial DNA (mtDNA) molecules, whose number is regulated through the processes of biogenesis and mitophagy (11). MtDNA is particularly susceptible to oxidative damage because of its proximity to the electron transport chain (ETC) and its deficiency of protective histones (12) . It has been postulated that mitochondrial dysfunction may activate a feedback loop which results in increased mitochondria biogenesis (13) and previous studies have suggested that cf-mtDNA copy number increases with mtDNA damage or mitochondrial dysfunction (14). Oxidative stress, a potential pathogenic mediator of GDM, is thereby believed to increase the quantity of cell-free circulating mtDNA (cf-mtDNA) by the instigation of mitochondrial dysfunction (15). Although the majority of circulating cf-mtDNA in the maternal circulation is of maternal origin, approximately 5-20% of this cf-mtDNA is of fetal/placental origin, implicating placental dysfunction in the regulation of maternal cf-mtDNA copy number (16). Further to this, recent cohort studies have found significant correlations between mitochondrial DNA heteroplasmy, an important factor in mitochondrial disease, and a higher incidence of pregnancy losses, gestational diabetes, intrauterine growth restriction and post postpartum haemorrhage (17).

Currently, risk prediction for GDM is largely based on the mother's medical history and other clinical risk factors. Risk factor consideration before GDM screening involves the assessment of maternal characteristics, such as family history of diabetes; ethnicity with a high prevalence of diabetes (i.e. non-white ethnicity: including Asian, black Caribbean or Middle Eastern); history of having GDM or a macrosomic infant and/or maternal obesity (18). This study aims to further characterise the role that mitochondrial dysfunction may play in the pathophysiology of GDM and to elucidate if maternal BMI may be a causative mediator in this pathway. We also examined if various lifestyle factors affected cf-mtDNA levels in GDM pregnancies, including exercise, ethnicity, medical history and smoking habits.

3 Materials and Methods

3.1 Study design

Study subjects were recruited from the Screening for Pregnancy Endpoints (SCOPE) study group in Ireland. SCOPE is an international multi-centre prospective cohort study of nulliparous singleton pregnancies aimed to develop a screening test to predict adverse pregnancy outcomes (19). This nested case-control study within SCOPE was conducted to include all GDM cases in SCOPE Cork, Ireland and matched controls with a case-to-control ratio of 1:2. Of the 1774 participants who were recruited and completed follow-up, 44 cases of GDM were diagnosed and are included for the purpose of this study's analysis. A retrospective case-control analysis was designed and performed and the diagnosed cases were matched with 88 participants as controls. These controls included two groups. Control group 1 was matched by maternal age and BMI (n=41), whereas control group 2 was matched by maternal age alone (n=44). GDM cases were diagnosed based on the Oral Glucose Tolerance Test (OGTT) at 24 weeks with locally employed diagnostic criteria.

3.2 Sample collection and DNA extraction

Plasma samples were collected from study participants at the 20 ± 1 weeks' gestation time-point. These samples were collected in BD Citrate Vacutainer tubes, placed on ice and centrifuged at 2400g for 10 minutes at 4˚C. This method was performed according to a standardised lab protocol. These plasma samples were then stored at -80˚C until analysis was performed. Total DNA was extracted from 200µl of plasma with a QIAamp DNA mini kit (Qiagen) as per standardised manufacturer guidelines. DNA was sonicated at 38 kHz ± 10% for 10 minutes to optimise DNA yield. All sample analysis was blinded.

3.3 cf-mtDNA quantification

Mitochondrial DNA was analysed by real-time PCR using a StepOne Plus Detection system. Taqman assays were performed for mitochondrial DNA (hMitoF5, hMitoR5) and nuclear β_2-microglobulin (β2MF2, β2MR2). Absolute quantification of cf-mtDNA concentration was then determined by standard curve analysis and presented as mtDNA copies/ml.

3.4 Statistical analysis

Statistical analysis was performed using SPSS v25. Statistical significance was accepted at $p < 0.05$. The groups were divided into cases (n = 44), controls matched by maternal BMI and age (control group 1) (n = 41) and controls matched by maternal age only (control group 2) (n = 44). The controls matched by age and BMI have missing data for cf-mtDNA concentrations (n=3) due to unavailability of peripheral blood samples for these participants.

4 Results

4.1 Patient characteristics in the study cohort

Of the total SCOPE study cohort that were recruited and completed follow-up, 44 women (2.5%) were diagnosed with GDM at 24 weeks' gestation. Our results were grouped and analysed as defined by the three study groups. The variables we included for analysis were maternal age, maternal BMI, recent exercise exertion (vigorous, moderate and recreational), ethnicity, family history of diabetes diagnosis and recent smoking habits. We compared study groups for each variable by using one-way ANOVA for continuous outcomes and McNemar's test for dichotomous outcomes. There were no significant differences found between groups except for that of maternal BMI, where GDM cases and control group 1 had a significantly higher BMI mean when compared to the control group 2 ($p=0.002$ and $p=0.004$ respectively) and family history of type 2 diabetes, where the cases had a significantly higher incidence of a family history of type 2 diabetes relative to both control group 1 and control group 2 ($p=0.0004$ and $p= 0.0022$ respectively) (see Table 1).

4.2 Significantly higher BMI in GDM cases relative to control participants

An initial descriptive statistical analysis was performed to define the distributions of maternal BMI values across the study groups (Figure 1). A one-way ANOVA followed by Tukey's multiple comparison analysis was applied to look for any difference in mean maternal BMI value between the study groups. This analysis established that the GDM cases and the control group 1 had a significantly higher mean BMI compared to control group 2 ($p=0.002$ and $p=0.004$ respectively). It was expected that there would be no significant difference in maternal BMI values between cases and control group 1 as BMI was a matching criteria for these groups.

Figure 1 - Distributions of maternal BMI across study groups. GDM cases and control group 1 had a significantly higher BMI mean when compared to the control group 2 using one-way

ANOVA followed by Tukey's multiple comparison analysis (p=0.002 and p=0.004 respectively).

4.3 Significantly higher cf-mtDNA concentration in GDM cases relative to healthy uncomplicated pregnancy controls

We then performed descriptive analysis to compare cf-mtDNA concentration (copies/ml) in maternal circulation across the study groups (see Figure 2). A one-way ANOVA followed by Tukey's multiple comparison analysis was applied to look for any significant differences in cf-mtDNA concentrations between the study groups. This analysis established that GDM case participants had a significantly higher mean cf-mtDNA concentration compared to control group 2 but not when compared to control group 1 (p=0.037 and p=0.149 respectively).

Figure 2 - Distribution of cf-mtDNA concentration (copies/ml) across study groups. GDM case participants had a significantly higher mean cf-mtDNA concentration compared to control group 2 but not to control group 1 using one-way ANOVA followed by Tukey's multiple comparison analysis (p=0.037 and p=0.149 respectively).

4.4 Increased cf-mtDNA significantly predicts an increased incidence of GDM diagnosis

Binary regression analysis was performed to further investigate the relationship between variables of BMI and cf-mtDNA concentration in identifying a GDM diagnosis. Initially, GDM diagnosis was analysed using data from the cases and the control group 2 to investigate the relationship between BMI and GDM diagnosis. This analysis showed a statistically significant increase GDM diagnosis with increasing maternal cf-mtDNA concentration (p = 0.032) in the analysis of GDM cases against control group 2. These results showed that as cf-mtDNA concentration increases, the likelihood of GDM diagnosis increases (Figure 3).

Figure 3 - Increased cf- mtDNA concentrations significantly predicts an increase in GDM diagnosis when cases were compared to control group 2 (p = 0.032).

4.5 Matching of GDM cases with controls for maternal BMI eliminates any evidence of elevated cf-mtDNA

Binary regression analysis was then performed on cf-mtDNA concentration in the cases against the control group 1. These results did not detect a significant difference in cf-mtDNA concentrations between the two groups (p = 0.697) (Figure 4).

Figure 4 - No significant prediction was found between an increase in cf-mtDNA concentrations with an increase in GDM diagnosis when cases were matched bases on both maternal age and BMI (p = 0.697).

5 Discussion

With increasing visceral adipose tissue mass, adipocyte dysfunction increases which results in an upregulation of reactive oxyen species (ROS) production. This deleterious ROS generation has been correlated with an increase in insulin resistance in both the adipose and other peripheral tissues (20). Mitochondrial activity is critical for maintenance of glucose homeostasis and alteration in mitochondrial content or function may further lead to the development of systemic insulin resistance (21). The impact of this ROS cascade on mitochondrial dysfunction has been linked to that of mtDNA alteration and damage, leading to diminished oxidative phosphorylation capacity, mtDNA fragment release and further ROS production, all components of metabolic disease states (22). Mitochondrial dysfunction is also hypothesized to play a pivotal role in a compensatory increase in mitochondrial biogenesis (23). Hence, we initially examined the causative relationship between maternal BMI and GDM. Both GDM cases and control group 1 respectively had a significantly higher BMI distribution compared to the control group 2. The significantly lower concentration of cf-mtDNA in the maternal circulation in the control group 2 relative to the case participants, which is not evident in control group 1 participants, supports the suggestion that an individual's BMI may be a key component in modifying cf-mtDNA concentration.

Previous research has already shown links between visceral obesity and cf-mtDNA copy number, specifically that the deleterious state of obesity is linked to increased cf-mtDNA concentrations (24) and our results complement this understanding of the physiology of adipose dysregulation. In obesity, intracellular lipid overload can induce an oxidative stress response. This response is in part due to the impact of high levels of free fatty acids on the mitochondrial membrane structure, instigating a release of reactive oxygen species (ROS). In addition to being a major producer of ROS, mitochondria are equally a target for cellular ROS which may lead to further oxidative damage to the mitochondrial membrane and mtDNA (25). This increase in ROS production can instigate a homeostatic increase in mitochondrial biogenesis and this described cycle of mitochondrial damage and ROS production may play a key role in cellular dysfunction and disease conditions (26).

There is emerging evidence that the described dysfunctional mitochondrial activity in the adipocytes, itself, has a detrimental effect on the metabolism of fatty acids, altering their

storage in the cell in the form of triacylglycerol and determining their release into the blood stream. Upon reaching the circulation, these fatty acids may deposit in ectopic sites such skeletal muscles and liver and thereby progressively display their potent capacity to induce insulin resistance (21). In addition, research investigating the physiology of human subcutaneous adipocytes has established that an increase in BMI is inversely related to mitochondrial oxidative phosphorylation capacity (27). This evidence strongly suggests that BMI is a clinical variable which can certainly be used to counsel regarding the risk of developing GDM, but alone it is not a sufficient screening tool (28).

To further define the predictive nature of cf-mtDNA on GDM diagnosis, we used binary regression analysis to investigate the relationship between the shift in maternal cf-mtDNA concentration and GDM diagnosis in the GDM cases. By selectively analysing our cases against control groups 1 and 2, matched and unmatched by maternal BMI respectively, we investigated the confounding effect that BMI may have on the relationship between maternal cf-mtDNA and GDM diagnosis. Here we found that cf-mtDNA concentration was a significant predictor for GDM diagnosis in the cases and the control group 2, with the GDM diagnosed participants having a higher cf-mtDNA blood concentration relative to the control group 2. Repeated regression analysis on cases against control group 1 did not elude to any clinical predictive nature of cf-mtDNA in these participants.

We also compared other demographic characteristics in our study cohort, to elucidate any other possible confounding variables. From this data, the only other finding of interest was that of a significantly higher incidence in a family history of type 2 diabetes in GDM case participants relative to their control counterparts. This is unsurprising considering the established pathological link between GDM and type 2 diabetes, as women diagnosed with GDM have a 3-7 fold increased risk of developing type 2 diabetes within 10 years and their child from this pregnancy also has a higher risk of diabetes diagnosis (29, 30). Other studies have similarly established a family history of type 2 diabetes as a significant risk factor for GDM (31-33). Considering our cohort characteristics, it is unlikely that we would have been able to capture any significant effects of ethnicity or smoking habits on GDM diagnosis, as both characteristics had very low variation within the cohort.

Our results suggest that cf-mtDNA concentration may be a potential clinical predictor of GDM development, independent of maternal age but dependent on maternal BMI. Recent

research that suggested that not only is mitochondrial dysfunction related to increased ROS production, but that this ROS production, which may be further induced by the hyperglycaemic state associated with GDM, could contribute to the development and progression of diabetes-related complications (34). This theory suggests the presence of a feed-forward relationship between ROS production stimulated by hyperglycaemia and concurrent GDM disease progression.

6 Conclusion

In this study, we provide evidence that an increased maternal peripheral concentration of cf-mtDNA strongly predicts GDM diagnosis, with maternal BMI suspected to regulate this pathophysiological link. BMI is therefore a useful guide as a GDM risk factor but is not sufficient as a stand-alone biomarker for the condition. Our findings support a pathological link between mitochondrial dysfunction and insulin resistance in pregnancy, and suggest that targeting oxidative stress responses may ameliorate the deleterious effects of increased visceral adiposity on GDM diagnosis risk. Longitudinal studies are, however, needed to further define the potential cause–effect relationship between changes in cf-mtDNA and GDM pathophysiology in order to elucidate its potential as a clinical biomarker of GDM.

7 List of abbreviations

BMI – body mass index

GDM – gestational diabetes mellitus

DNA - deoxyribonucleic acid

mtDNA – mitochondrial DNA

cf-mtDNA – cell-free mtDNA

hPL – human placental lactogen

hPGH – human placental growth hormone

ATP – adenosine triphosphate

OGTT – oral glucose tolerance test

ROS – reactive oxygen species

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9 Tables and figures

		Cases (n=44)	*Control group 1 (n=41)*	*Control group 2 (n=44)*
Maternal age (years) (mean ±SD)		31.14 ±5.1	30.55 ±4.53	31.3 ±3.22
Maternal BMI (mean ±SD)		28.33 ±5.32	28.07 ±5.32	24.64 ±3.94
Vigorous exercise in the last month	Never	38 (86%)	27 (66%)	33 (75%)
	Once/week	3 (7%)	6 (15%)	8 (18%)
	2-3x/week	2 (5%)	6 (15%)	2 (5%)
	4-6x/week	1 (2%)	0	0
	Daily	0	1 (2%)	0
	>once/day	0	1 (2%)	1 (2%)
Moderate exercise in the last month	Never	12 (27%)	9 (22%)	15 (34%)
	Once/week	13 (30%)	10 (25%)	8 (18%)
	2-3x/week	10 (23%)	16 (39%)	7 (16%)
	4-6x/week	3 (7%)	1 (2%)	7 (16%)
	Daily	5 (11%)	4 (10%)	7 (16%)
	>once/day	1 (2%)	1 (2%)	0
Recreational walk in the last month	Never	6 (14%)	6 (15%)	10 (23%)
	Once/week	21 (48%)	15 (36%)	10 (23%)
	2-3x/week	12 (26%)	14 (34%)	18 (40%)
	4-6x/week	2 (5%)	2 (5%)	3 (7%)
	Daily	3 (7%)	4 (10%)	3 (7%)
	>once/day	0	0	0
Ethnicity	Caucasian	42 (95%)	41 (100%)	44 (100%)
	Other (Indian)	2 (5%)	0	0

Family history of type 1 diabetes	Yes No	4 (9%) 40 (91%)	0 41 (100%)	1 (2%) 43 (98%)
Family history of type 2 diabetes	Yes No	18 (41%) 26 (59%)	2 (5%) 39 (95%)	4 (9%) 40 (91%)
Family history of GDM	Yes No	4 (9%) 40 (91%)	1 (2%) 40 (98%)	2 (5%) 42 (95%)
Smoking during the first trimester	Yes No	12 (27%) 32 (73%)	16 (39%) 25 (61%)	9 (20%) 35 (80%)

Table 1 – Participant characteristics in the study cohort, distributed by cases (patients diagnosed with GDM), control group 1 (control patients matched by age and BMI) and control group 2 (control patients matched by age only). Data is represented as mean ± standard deviation or percentage of the group cohort.