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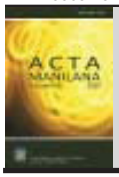

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# Placental immunolocalization of mTOR and downstream signaling targets in Filipino women with gestational diabetes mellitus

**Maria Ruth B. Pineda-Cortel[1,2,3*], Hazel Mae A. Piala[2,3], Brian Akhaphong[4], Therriz P. Mamerto[2,3], Sharisse T. Diaz[1], Jemima G. Martinez[1], Jaynielle S. Quiaoit[1], Gersa May S. Rosales[1], Zianella V. Zamora[1], Rowen T. Yolo[1], Emilyn U. Alejandro[4]**

*[1]Department of Medical Technology, Faculty of Pharmacy, University of Santo Tomas, España Boulevard, 1008 Manila, Philippines.*

*[2]The Graduate School, University of Santo Tomas, España Boulevard, 1008 Manila, Philippines.*

*[3]Research Centerfor the Natural and Applied Sciences, University of Santo Tomas, España Boulevard, 1008 Manila, Philippines.*

*[4]Department of Integrative Biology and Physiology, Medical School, University of Minnesota, Minneapolis, MN 55455, USA.*

## Abstract

The function of mechanistic target of rapamycin (mTOR) during pregnancy involves cellular nutrient transport in placenta from maternal to fetal environment. In pregnancies complicated by gestational diabetes mellitus (GDM), dysregulation of mTOR and its downstream targets were observed that can cause alteration in placental nutrient transport leading to abnormalities in fetal growth and development. This study aimed to determine the protein expression of mTOR, phosphorylation status p-mTOR and its downstream targets: p-4EBPI and p-S6 in syncytiotrophoblast and stromal cells between GDM and non-GDM placenta. Immunohistochemistry was performed with antibodies against mTOR, p-mTOR, p-4EBPI and p-S6 in 20 GDM and 36 non-GDM human term placenta. Results showed an increase in the net placental weight and fetal weight from GDM group compared to non-GDM group. This was associated with strong chromogen intensities of mTOR, p-mTOR, p-S6 in syncytiotrophoblast of GDM placenta and strong chromogen intensities of mTOR, p-mTOR, p-S6 and p-4EBP1 in stromal cells of GDM placenta. Expression levels of p-mTOR, p-4EBP1, and p-S6 in syncytiotrophoblast and stromal cells in GDM placenta were found to be positively correlated with fetal weight. Together, we conclude that the stronger expression of mTOR and its downstream targets in the placenta collected from GDM women suggest its involvement in the pathophysiology of GDM. Further studies to assess the effect of GDM on nutrient transport via mTOR pathway is warranted. Also, functional analysis focusing on molecular mechanisms and metabolomics related with GDM development may be performed.

**Keywords**: Gestational diabetes mellitus; placenta; mTOR; p-mTOR; p-4EBP1; p-S6

Corresponding author: : mbpineda-cortel@ust.edu.ph

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## Introduction

Mechanistic target of rapamycin (mTOR), a 289 kDa serine-threonine protein kinase, serves as the central controller of fetal cellular growth in response to nutrient availability, presence of growth factors, and level of cellular energy [1, 2] a phosphatase that functions antagonistically to PI3K. mTOR regulates cell growth, motility, and metabolism by forming two multiprotein complexes, mTORC1 and mTORC2, which are composed of special binding partners. These complexes are sensitive to distinct stimuli. mTORC1 is sensitive to nutrients and mTORC2 is regulated via PI3K and growth factor signaling. mTORC1 regulates protein synthesis and cell growth through downstream molecules: 4E-BP1 (also called EIF4E-BP1). It is composed of two catalytic complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is known to regulate anabolic processes such as protein synthesis, ribosome biogenesis, transcription, lipid synthesis and nutrient absorption [3].

mTOR has been reported to be localized in the syncytiotrophoblast cells of the placenta and implicated to have a role in regulating trophoblast proliferation [4]. Trophoblast cells are important in the formation of a functioning placenta and thus, are needed in fetal development [4, 5]. HTR8/SVneo cell, HUVEC cell, the maternal placenta of GDM patients, PE patients and normal pregnancy were detected by qRT-PCR. The cell culture, cell transfection, CCK-8 assay, flow cytometry, wound scratch assay and transwell were carried out to determine the effects of silencing and overexpression of PVT1 on the HTR8/SVneo trophoblast cell line. Nuclear and chromatin RNA fraction assay, RNA-sequencing, Western blot and qRT-PCR were conducted to explore preliminarily possible mechanisms. Results: The relative PVT1 expression level in HTR-8/Svneo cells was higher compared to other cancer cells and HUVEC, and was lower in the GDM and PE placentas than in the normal placentas. The results showed that PVT1 knockdown notably inhibited the proliferation, migration and invasiveness abilities of trophoblast cells, and significantly promoted the apoptosis. Furthermore, overexpression of PVT1 showed the opposite results. We identified 105 differentially expressed genes after PVT1 knockdown, 23 were up-regulated and 82 were down-regulated. GO enrichment analysis and pathway enrichment analysis showed that the DEGs were closely related to the functional changes of trophoblast cells. Because of the enrichment of 7 DEGs and less Q value, PI3K/AKT pathway was prominent and attracted our attention. More importantly, we confirmed that knockdown of PVT1 obviously decreased AKT phosphorylation and decreased the expression of DEGs (GDPD3, ITGAV and ITGB8. Two villous trophoblastic layers, the syncytiotrophoblast (SCT) and cytotrophoblast (CTB), functions to facilitate placental processes such as maternal-fetal nutrient transport, gas exchange, and production of pregnancy-sustaining hormones [6]. With these functions, it is vital that the growth and processes of the placenta are maintained and regulated.

Studies have shown that the expression of mTOR in trophoblast and syncytioptrophoblast of the placenta varies and affects fetal development [7]. Dysregulation of mTOR have been observed and associated with pregnancy post-implantation lethality, gestational diabetes mellitus (GDM), large for gestational age babies and growth retardation of fetus [8–11]. In addition, the link between mTOR and other diseases such as polycystic ovarian syndrome, cancer, cardiovascular complications, endometriosis, premature ovarian follicle and among others has also been provided by previous studies [10, 12].

We recently demonstrated that ablation of placental mTOR in a genetic rodent model causes smaller placenta associated with reduced birthweight and increased metabolic dysfunction in the offspring [31]. A few studies have investigated mTOR signaling in human pregnancies complicated by growth restriction and maternal obesity.

Focusing on GDM, it is characterized by glucose intolerance and insulin resistance with onset during pregnancy. This condition is associated with feto-maternal complications such as fetal overgrowth, fetal malformation, hypoglycemia, and risk of obesity and development of type 2 diabetes mellitus (T2DM) and other metabolic diseases later in life [13–15]. Most often, fetal overgrowth is associated with larger placenta and placental dysfunctions [16]. In the United States, prevalence of GDM impacts 14% of pregnancies or 200,000 pregnant women per year [17]. While in the Philippines, the latest GDM prevalence is 29% based on IADPSG criteria [18].

In a previous study, it was shown that overactivation of mTOR and key signaling downstream targets such as eukaryotic initiation factor 4E-binding protein-1 (p-4EBP1), and ribosomal protein S6 kinase beta-1 (p-p70S6K) were altered in GDM pregnancy leading to fetal complications [9]. Because of the potential association of mTOR regulation in GDM, we hypothesized that high expression of mTOR and increased activity of downstream components will be present in GDM placenta of Filipino women. Thus, this study aimed to determine the expression of mTOR, its phosphorylated form (p-mTOR) and its downstream targets, p-4EBPI and p-S6 in syncytiotrophoblast and stromal cells in GDM and non-GDM placenta of Filipino women. Identification of the expressions of these proteins may shed light to the mechanism of GDM development. In addition, expression levels of mTOR, p-mTOR, p-S6 and p-4EBP1 in syncytiotrophoblast and stromal cells among GDM and non-GDM group will be correlated to net placenta weight and fetal weight.

## Materials and methods

***Study subjects and specimen collection.*** The study was approved by the University of Santo Tomas Graduate School Ethics Review Committee (Protocol Number: E-2016-02-R3). Information about the study were given to potential participants, and those who were interested to join were asked to voluntarily sign a written informed consent. The following inclusion criteria were utilized to screen participants: age above 18 years old, no history of any type of diabetes mellitus and other metabolic disorders, and free of any viral or bacterial infection. A total of 56 pregnant women were included in this study. Participants were grouped into two (GDM = 20; non-GDM = 36) based on their 75-gram oral glucose tolerance test (OGTT) results as interpreted by their attending physician following the International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria, that is, GDM diagnosis is made when at least one of the following cut-off points are met: >7.0 mmol/L (126 mg/dL) for fasting blood sugar, (2) >11.0 mmol/L (200 mg/dL) for 2-hour fasting blood sugar. Placenta were immediately collected upon baby delivery. Processing was within 30 minutes. Important measurements such as weight, depth, length, umbilical cord position were recorded and placental gross appearance was photographed while observing for frank pathology.

Using an imaginary line to divide the placenta into four quadrants, four samples, measuring ~2 $cm^3$, were collected from each quadrant, labelled as A-D. Samples were washed with phosphate buffered saline (PBS) and placed in a tissue cassette. Routine histopathologic tissue processing followed: fixation in 10% neutral formalin for 12 hours; dehydration in ascending grades of ethanol; clearing in xylene; and embedding in paraffin wax.

***Immunohistochemistry and Image Analysis.*** Tissue sections of 5 μm thickness from GDM and non-GDM placental blocks were prepared (Cut 4062 Microtome, SLEE Medical, Mainz, Germany) and collected on SigmaScreen™ poly-L-Lysine-coated slides (Sigma-Aldrich, Singapore, Singapore), dewaxed and rehydrated. The slides were submerged for 10 minutes in a beaker with pre-heated antigen retrieval solution. Permeabilization with PBS with 0.05% TWEEN®20, pH7.4 (Sigma-Aldrich, Singapore, Singapore) followed. After 20 minutes, the previous solutions were removed and 10% normal goat serum (Sigma-Aldrich, Singapore, Singapore) were added on the slides and incubated for 30 minutes at room temperature. The sections were then incubated with primary antibodies depending on the target protein, overnight in 4°C.

The following primary antibodies with their corresponding dilutions were used: 1:100 dilution of rabbit monoclonal mTOR (2983S; Cell Signaling Technology, Beverly Massachusetts); 1:100 dilution of rabbit polyclonal p-mTOR (2971S; Cell Signaling Technology, Beverly Massachusetts); 1:1000 dilution of rabbit monoclonal p-4EBPI (2855L; Cell Signaling Technology, Beverly Massachusetts); and 1:100 dilution of rabbit monoclonal p-S6 (2217L; Cell Signaling Technology, Beverly Massachusetts). The slides were washed with PBS three times and incubated with biotinylated anti-rabbit IgG for 1 hour. The antigen-antibody complexes were detected by using VECTASTAIN® Elite® ABC Kit Peroxidase (HRP) (Vector Laboratories, Burlingame, CA) for 30 minutes followed by three washes of PBS for 5 minutes each. To achieve the desired chromogen signal, the sections were incubated in Pierce™ DAB Substrate Kit for 10 minutes (Thermo Fisher Scientific, Waltham, MA) and rinsed with distilled water. Sections were then counterstained with hematoxylin and mounted on a slide with EUKITT® (Sigma-Aldrich, Singapore, Singapore). A previously prepared human liver tissue block was used as control in each protein target. A negative tissue control was also prepared following the optimized protocol except that primary antibody was not added. Cytokeratin and vimentin were utilized on parallel tissue sections to characterize syncytiotrophoblast and stromal cells. Microscopy images were taken using from BX53 System Microscope (Olympus Corporation, Tokyo, Japan) with DP22 Digital Camera (Olympus Corporation, Tokyo, Japan).

Quantification of protein expression levels through chromogen immunostaining intensity was analyzed using Fiji (http://imagej.nih.gov/ij/). Only intensities seen in syncytiotrophoblast and stromal cells were included. While intensities of non-targeted cellular components in the histology of placenta were removed. Thus, the quantification of localized immunostaining is specifically measured in syncytiotrophoblast and stromal cells. Ten images were randomly captured per sample of GDM and non-GDM groups. A random selection of 10 to 15 stromal cells, and 5 to 10 syncytiotrophoblast cell lines were measured per image. The reciprocal intensity was calculated, divided by the average area of the cells and multiplied by 100. Blind measurement of expression levels was done to eliminate bias.

***Statistical Data Analysis.*** Graphical data are presented as means ± standard error of the mean. Mann-Whitney test was used for comparing physical and biochemical measurements between GDM and non-GDM participants. For comparing expression levels of different target proteins in syncytiotrophoblast and stromal cells, Kruskal-Wallis test was used. In evaluating the correlation of net placental weight and fetal weight to protein expressions, Pairwise Pearson Correlations Analysis was used. All statistical analyses were performed in GraphPad Prism 8 (GraphPad Software, San Diego, CA). A $P$ value of $<0.05$ was considered statistically significant.

## Results and dicussion

***Anthropometric measurements of GDM and non-GDM Filipino pregnant women.*** All participants in GDM and non-GDM groups were of legal age. The pre-pregnancy body mass index of GDM and non-GDM groups were considerably close to each other at 24.2 ± 3.8 and 24.7 ± 6.6, respectively ($P > 0.05$). The results in fasting blood sugar, after 1-hour and 2-hour glucose intake showed a statistically significant increase by 0.8 ± 0.1, 2.5 ± 0.4, and 2.5 ± 0.4, respectively in GDM compared to non-GDM group ($P < 0.05$). Participants were assigned to GDM and non-GDM groups based on their attending physicians diagnosis following the results of OGTT. The rest of the biochemical assays (total cholesterol, triglyceride, high density lipoprotein, very low density lipoprotein and hemoglobin A1c) appeared to have insignificant difference between GDM and non-GDM ($P > 0.05$) (Table 1). These variables were not taken as possible confounding factors that may affect the results of this study.

***Gender offspring frequency between GDM and Non-GDM Filipino pregnant women.*** The gender frequency data of the offspring in each participant of GDM and non-GDM was gathered. In GDM pregnancy, the number of male offspring is greater than the female offspring. While in non-GDM pregnancy, the number of male offspring is less than the female offspring (Fig.1). Similarly, in previous studies, it was shown that women with GDM appear to deliver more male offspring than female offspring, although it is not known if GDM influences sex selection in utero or if having a male fetus influences the later development of GDM [19–21]. Hypothesis of Trivers & Willard explains the leaning towards male offspring as the body's mechanism to identify its ability to sustain offspring resulting to more males in the presence of abundant fuel as observed in GDM cases [19]. On one hand, in a meta-analysis paper, it was observed that there is a 4% higher risk of GDM development in women carrying a male offspring, in which they mentioned that potentially the fetus may have influenced maternal glucose metabolism during pregnancy [20].

In the present study, it appears that GDM pregnant mothers tend to have a male offspring whereas most non-GDM pregnant mothers had a female offspring. Although we did not delve into the details and mechanism of sex variations, it is important to note that Filipino GDM mothers included in our study delivered more male offspring than female offspring. More studies should be done to determine the impact and effect of the gender of offspring in GDM development or on the influence of fetal sex to development of GDM.

**Table 1.** Physical and Biochemical Measurements of participants.

| | GDM (n=20) | Non-GDM (n=36) | P value | Reference Values |
|---|---|---|---|---|
| Age (years) | | | | |
| Mean ± SD | 26.8±4.2 | 28.2±6.0 | 0.5643 | 18> |
| Median (min-max) | 26.0 (19.0-36.0) | 28 (19.0-39.0) | | |
| Pre-pregnancy Body Mass Index | | | | |
| Mean ± SD | 24.2±3.8 | 24.7±6.6 | 0.7117 | 18.5 – 22.9 |
| Median (min-max) | 23.7 (19.9-35.1) | 22.9 (17.0-44.8) | | |
| Fasting Blood Sugar (mmol/L) | | | | |
| Mean ± SD | 5.1±0.6 | 4.3±0.5 | **0.0289** | ≥5.1 |
| Median (min-max) | 5.3 (3.9-5.6) | 4.2 (3.3-5.0) | | |
| After 1-hour of glucose intake (mmol/L) | | | | |
| Mean ± SD | 9.0±1.9 | 6.5±1.5 | **0.0003** | ≥10.0 |
| Median (min-max) | 9.5 (5.3-12.63) | 6.4 (4.2-9.7) | | |
| After 2-hours of glucose intake (mmol/L) | | | | |
| Mean ± SD | 8.9±1.9 | 6.4±1.5 | **<0.0001** | ≥8.5 |
| Median (min-max) | 8.6 (6.1-12.63) | 6.1 (4.2-9.7) | | |
| Total Cholesterol (mmol/L) | | | | |
| Mean ± SD | 4.2±0.3 | 4.3±0.4 | 0.5534 | <5.2 |
| Median (min-max) | 4.2 (4.0-5.0) | 4.3 (3.5-4.9) | | |
| Triglyceride (mmol/L) | | | | |
| Mean ± SD | 1.6±0.4 | 1.6±0.2 | 0.5560 | <1.7 |
| Median (min-max) | 1.4 (1.0-2.3) | 1.6 (1.3-2.1) | | |
| High Density Lipoprotein (mmol/L) | | | | |
| Mean ± SD | 1.4±0.1 | 1.7±0.4 | 0.1796 | >1.0 |
| Median (min-max) | 1.5 (1.1-1.6) | 1.6 (1.1-2.5) | | |
| Very Low Density Lipoprotein (mmol/L) | | | | |
| Mean ± SD | 0.7±0.2 | 0.7±0.1 | 0.6126 | <2.6 |
| Median (min-max) | 0.6 (0.4-1.0) | 0.7 (0.6-0.9) | | |
| Hemoglobin A1c (%) | | | | |
| Mean ± SD | 5.3±0.3 | 5.1±0.3 | 0.5821 | 4.0 – 5.6 |
| Median (min-max) | 5.5 (4.9-5.8) | 5.0 (4.6-5.7) | | |

***Increased net placental and fetal weight in Filipino pregnant women with GDM.*** The net placental weight and fetal weight is significantly higher in GDM group compared to non-GDM placenta group ($P < 0.05$; $P < 0.05$) as shown in Table 2. Also shown in Table 2 is the placental efficiency, which was obtained by dividing the fetal weight with placental weight. When placental efficiency between GDM and non-GDM was compared, no significant difference between the two groups was observed, although GDM group has lower placental efficiency than non-GDM group (P value > 0.05). A decreased placental efficiency may indicate reduced nutrient transport or a failure to adapt and is said to be observed in pregnancies complicated with fetal growth restriction, GDM, small for gestational age, and pre-eclampsia [22].

But there is no significant difference on the placental efficiency between GDM and non-GDM groups may indicate that growth was maintained as it responds to the increased demand of nutrients for fetal development [23]. Although increased placental weight and fetal weight were observed among GDM women compared to non-GDM pregnancy, placental efficiency was maintained within their environmental conditions suggesting a compensation effect. A maintained placental efficiency indicates how placental development and function reacted well despite environmental alterations to provide fetal nutritional requirements [23].

***Increased expression levels of mTOR, p-mTOR, p-S6 and p-4EBPI in syncytiotrophoblast and stromal cells of GDM placenta.*** An appearance of very strong chromogen intensities was notably observed in syncytiotrophoblast and stromal cells of GDM placenta incubated in primary antibodies of mTOR, p-mTOR, p-S6 and p-4EBPI (Fig. 2 - A3, B3, C3, D3). Whereas in non-GDM placenta, strong to moderate chromogen intensities were observed in syncytiotrophoblast and stromal cells. (Fig. 2 – A4, B4, C4, D4). Some vascular wall in GDM and non-GDM placenta were also found to have immunostaining in mTOR (Fig. 2 - A3) and p-4EBPI (Fig. 2 - D4). There are also moderate chromogen staining observed in stem villi (Fig. 2 - A3, B3), terminal villi (Fig. 2 - A4, B4, D3) and mature intermediate villi (Fig. 2 - A4). Each of the four protein targets showed a significant increase in expression levels located in syncytiotrophoblast and stromal cells of GDM compared to non-GDM placenta. The statistically significant difference in increase of mTOR, p-mTOR, p-S6 and p-4EBP1 expression levels in syncytiotrophoblast of GDM group compared to non-GDM group were 40.8 ± 0.1, 32.0 ± 0.2, 77.9±0.2, and 9.1 ± 0.1, respectively ($P < 0.0001$) (Fig. 3). The statistically significant difference in increase of mTOR, p-mTOR, p-S6 and p-4EBP1 expression levels in stromal cells of GDM group compared to non-GDM group were 52.0 ± 0.3, 106.8 ±1.4, 79.9 ± 0.6, and 69.2 ± 0.7, respectively ($P < 0.0001$) (Fig 4). The negative control showed a very weak chromogen staining (Fig. 2 – A2, B2, C2, D2) while the positive control in human liver tissue showed strong chromogen intensities in all protein targets (Fig. 2 – A1, B1, C1, D1). Cytokeratin and vimentin were used as specific markers to syncytiotrophoblast and stromal cells, respectively (Fig. 5).

In Table 3, correlation of net placental weight with protein expression levels in syncytiotrophoblast and stromal cells of GDM and non-GDM groups is presented. The p-4EBP1 expression in stromal cells of non-GDM placenta was found to be significantly correlated with net placental weight ($P < 0.05$). All other correlations were statistically not significant. Also, correlation of fetal weight with protein expression levels in syncytiotrophoblast and stromal cells of GDM and non-GDM group is presented in Table 4. In GDM placenta group, expression levels of p-mTOR, p-4EBP1, and p-S6 in syncytiotrophoblast and stromal cells were found to be positively correlated to fetal weight ($P < 0.05$).

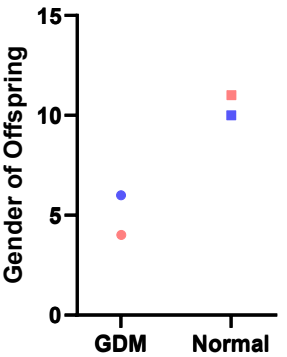


**Figure 1.** The frequency of male and female offspring among GDM and non-GDM. Blue color represents male offspring. Pink color represents female offspring.

**Table 2.** Net placental weight and fetal weight in GDM and non-GDM groups.

| | GDM (n=20) | non-GDM (n=36) | P value |
|---|---|---|---|
| Net Placental Weight, g | | | |
| Mean ± SD | 507.2±56.0 | 409.4±84.0 | 0.0005 |
| Median (min-max) | 520 (413.0-583.0) | 400 (269.0-574.0) | |
| Fetal Weight, g | | | |
| Mean ± SD | 3227.0±265.9 | 2970.0±291.4 | 0.0473 |
| Median (min-max) | 3173.0 (2800.0-3690.0) | 3075.0 (2420.0-3370.0) | |
| Placental Efficiency | | | |
| Mean ± SD | 6.93±1.39 | 7.72±1.27 | 0.2171 |
| Median (min-max) | 7.06 (5.01-9.06) | 7.52 (6.03-10.2) | |

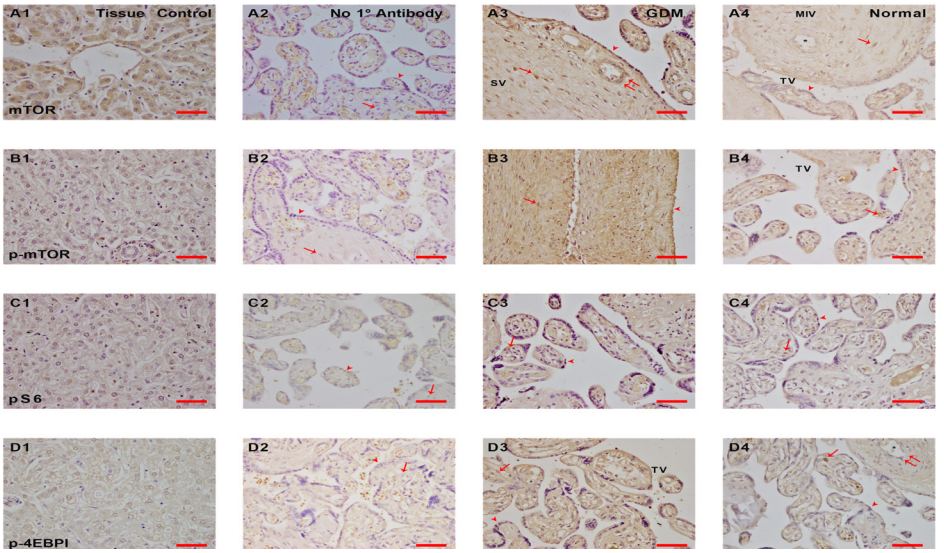


**Figure 2.** The immunolocalization of (A) mTOR, (B) p-mTOR, (C) p-S6, and (D) p-4EBP1 in syncytiotrophoblast and stromal cells of GDM and non-GDM placenta. Tissue control: Human liver; scale bar: 50µm applies to all panels. Red single arrow: stromal cells, arrow head; syncytiotrophoblast; double arrow: vascular wall; asterisk: endothelium of blood vessels; TV: terminal villi, SV: stem villi; MIV: mature intermediate villi; magnification: 40x.

In non-GDM placenta group, p-mTOR expression levels in syncytiotrophoblast ($P < 0.05$) and stromal cells ($P < 0.05$) were found to be inversely correlated with fetal weight. The increase in fetal weight observed in correlation with expressions of mTOR and its downstream components may be attributed to the increased placental nutrient transport, and is said to be a mechanism of adapting to the environment in pregnancies complicated with GDM [9].

The appearance of very strong chromogen staining in syncytiotrophoblast and stromal cells of mTOR, p-mTOR, p-S6 and p-4EBP1 in GDM placenta indicates the overactivation of mTOR signaling network in mTORC1 pathway. Our findings are consistent with previous studies identifying the association of increased expression of mTOR with GDM [9, 24, 25].

Potentially, because of the increased blood glucose in the maternal blood, mTOR pathway, which responds to environmental changes, is activated and led to the very strong expression seen in the GDM placenta. mTOR signaling pathway plays a role in regulating fetal growth and development, and with the alterations in the GDM placenta, as reported by previous studies, including overgrowth and immaturity, mTOR may be implicated affecting placental structures and functions [9, 23]. The placenta plays an essential role in maternal-fetal nutrient exchange required for fetal growth and development, which includes glucose, amino acids, fatty acids and other vitamins and minerals [23, 26]. An interplay between fetal and maternal factors has been described to control and regulate the placenta [23, 27]. With these vital functions of placenta, proper regulation of the maternal-fetal nutrient exchange is necessary to ensure normal fetal growth and maturation.

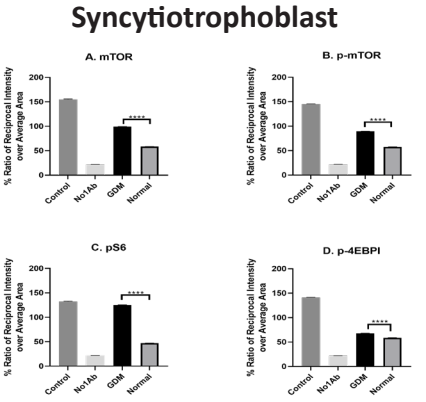


**Figure 3.** Comparison of expression levels of mTOR, p-mTOR, p-S6, and p-4EBP1 in syncytiotrophoblast of GDM and non-GDM placenta. There is a significant increase of expression levels in mTOR and its downstream signaling targets in GDM placenta. Data represents mean ± SEM. Statistical significance denoted by: ****P<0.0001.

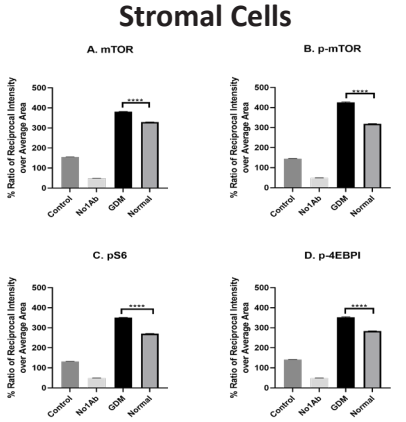


**Figure 4.** Comparison of expression levels of mTOR, p-mTOR, p-S6, and p-4EBP1 in stromal cells of GDM and non-GDM placenta. There is a significant increase of expression levels in mTOR and its downstream signaling targets in GDM placenta. Data represents mean ± SEM. Statistical significance denoted by: ****P<0.0001.

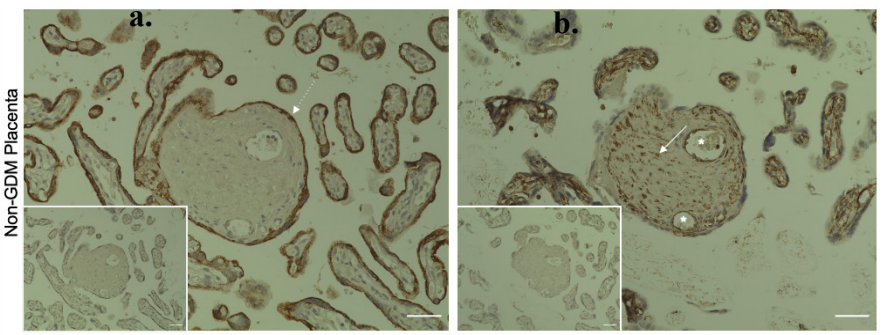


**Figure 5.** Cytokeratin (a.) and stromal cells (b.) were the reference markers used for syncytiotrophoblast and stromal cells, respectively. Tissue used was a non-GDM placenta. Scale bars represent 50 µm. Inset image magnification: 4x.

**Table 3.** Correlation of net placental weight and protein expression level in GDM and non-GDM placenta.

| | **GDM** | | | **NON-GDM** | | |
|---|---|---|---|---|---|---|
| | Correlation | 95% CI for p | *P* value | Correlation | 95% CI for p | *P* value |
| Syncytiotrophoblast | | | | | | |
| mTOR | 0.236 | (-0.294, 0.655) | 0.379 | -0.202 | (-0.611, 0.293) | 0.422 |
| p-mTOR | -0.011 | (-0.452, 0.433) | 0.962 | 0.278 | (-0.427, 0.772) | 0.437 |
| p-4EBP1 | 0.347 | (-0.160, 0.709) | 0.172 | 0.269 | (-0.282, 0.687) | 0.332 |
| p-S6 | -0.519 | (-0.832, 0.045) | 0.069 | 0.113 | (-0.279, 0.473) | 0.576 |
| Stromal Cells | | | | | | |
| mTOR | 0.266 | (-0.265, 0.673) | 0.320 | 0.031 | (-0.472, 0.519) | 0.908 |
| p-mTOR | -0.171 | (-0.571, 0.293) | 0.470 | 0.190 | (-0.430, 0.689) | 0.553 |
| p-4EBP1 | 0.323 | (-0.170, 0.686) | 0.192 | 0.527 | (0.043, 0.811) | **0.036** |
| p-S6 | -0.423 | (-0.790, 0.167) | 0.150 | -0.098 | (-0.461, 0.293) | 0.627 |

**Table 4.** Correlation of fetal weight and protein expression levels in GDM and non-GDM placenta.

| | **GDM** | | | **NON-GDM** | | |
|---|---|---|---|---|---|---|
| | Correlation | 95% CI for p | *P* value | Correlation | 95% CI for p | *P* value |
| Syncytiotrophoblast | | | | | | |
| mTOR | 0.556 | (-0.027, 0.856) | 0.061 | -0.593 | (-0.880, 0.011) | 0.055 |
| p-mTOR | 0.823 | (0.473, 0.949) | **0.001** | -0.821 | (-0.980, -0.027) | **0.045** |
| p-4EBP1 | 0.675 | (0.125, 0.907) | **0.023** | -0.278 | (-0.692, 0.273) | 0.316 |
| p-S6 | 0.697 | (-0.016, 0.940) | 0.055 | 0.276 | (-0.275, 0.691) | 0.319 |
| Stromal Cells | | | | | | |
| mTOR | 0.540 | (-0.049, 0.850) | 0.070 | -0.634 | (-0.878, -0.128) | **0.020** |
| p-mTOR | 0.836 | (0.503, 0.953) | **0.001** | -0.872 | (-0.981, -0.346) | **0.011** |
| p-4EBP1 | 0.659 | (0.098, 0.902) | **0.027** | 0.383 | (-0.244, 0.219) | 0.219 |
| p-S6 | 0.734 | (0.136, 0.940) | **0.024** | 0.249 | (-0.302, 0.675) | 0.372 |

Our previous study in rodents have described that changing the maternal-fetal nutrient exchange via placental knockout of mTOR predisposes offspring to metabolic dysfunction by reducing both placental and fetal weight [31]. Thus, disrupting the maternal-fetal nutrient exchange have been implicated to predispose offspring to metabolic dysfunction. There are numerous factors affecting placental function and overgrowth of placenta that had been observed in certain conditions such as obesity and GDM, which indicates placental dysfunction [9, 11, 28]. GDM conditions have been shown to change circulating fetal insulin, glucose, amino acid transporter expression, and others also affect placental functions [16, 29]. In our previous study, we have also described that maternal weight, nutrition, lifestyle, hormones, and among others influence placental regulation [30]. In another study using a rat model, they observed that regulating mTOR pathway via administering a therapeutic substance that suppresses the pathway may attenuate the impact of GDM [32, 33]. Moreover, in another study, rapamycin was utilized in mice to inhibit mTOR complex, and its use improved the mice model's insulin sensitivity and reduced the weight gain, which are potential ways to counteract the effect of mTOR dysregulation [33]. These studies, although done in animal models, were able to show the important role of mTOR pathway in influencing placental function in GDM [31–33]. These studies examined the changes of placental function and morphology via maternal nutrition and mTOR regulation in GDM, but the actual role of mTOR expression and activity have not been fully described and elucidated yet.

## Conclusion

This study was able to observe stronger expression of mTOR and downstream components of mTOR signaling, namely, p-mTOR, p-S6 and p-4EBP1, in GDM placenta. The mTOR signaling network might have contributed to the overgrowth of placenta and increased offspring weight observed in GDM women. Further experimentation is needed to confirm the results of this study, as well as functional and mechanistic approaches underlying mTOR regulation in GDM placenta may be performed.

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## Conflict of Interest

The authors declare no conflict of interest.

## Author Contribution

HMAP, TPM, BA, ST, JM, JQ, GR, ZZ and MRBPC conducted experiments and wrote the paper. TPM, HMAP, RTY, MRBPC and EUA interpreted the data and edited the manuscript. TPM and MRBPC designed the study protocols. MRBPC conceived the study, acquired funding and is in charge of the overall direction and guarantor of this work.

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