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Investigation of Phosphorylated and *O*-Glycosylated Proteins in Gestational Diabetes Mellitus

(Penelitian Protein Terfosforilasi dan Terglikosilasi *O* dalam Kehamilan Diabetes Mellitus)

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ABSTRACT

Gestational Diabetes Mellitus (GDM) is a common but temporary type of diabetes that develops among 10-20% of all pregnant women. It is a major cause of several pre- and post-pregnancy diseases in both mother and offspring. The main causative is altered pregnancy hormones that lead to deficient glucose metabolism. Complications can be preeclampsia, caesarean sections and cardiovascular diseases. This study was designed to determine the phosphorylated and glycosylated proteins present in GDM and to investigate the role of their post-translational modifications (PTMs) in the pathogenesis of GDM. The study was performed on 30 blood samples from pregnant diabetic women (diseased), 30 pregnant non-diabetic women (control) and 30 women without pregnancy and gestational diabetes (normal). Protein extraction was done from blood plasma, and phosphorylated and glycosylated proteins were determined by Enzyme-Linked Immuno-Sorbent Assay (ELISA) by use of specific antibodies. This study showed that proteins found in GDM are highly phosphorylated and *O*-glycosylated ($p<0.01$). Furthermore, a bioinformatic investigation was done, which showed that blood coagulation proteins such as the Fibrinogen alpha chain and lipid metabolism-regulating proteins apolipoprotein E, L1 and C-III showed a high potential for phosphorylation, which suggests that phosphorylation can be used as a therapeutic marker in GDM.

Keywords: Gestational diabetes; glycosylation; phosphorylation; protein modification

ABSTRAK

Kehamilan Diabetes Mellitus (GDM) adalah jenis diabetes biasa tetapi sementara yang berlaku dalam kalangan 10-20% daripada semua wanita hamil. Ia adalah punca utama kepada beberapa penyakit sebelum dan selepas kehamilan pada ibu dan anak. Penyebab utama adalah perubahan hormon kehamilan yang membawa kepada kekurangan metabolisme glukosa. Komplikasi boleh menjadi preeklampsia, pembedahan caesarean dan penyakit kardiovaskular. Kajian ini direka bentuk untuk menentukan protein terfosforilasi dan terglikosilasi yang hadir dalam GDM dan untuk mengkaji peranan pengubahsuaian pasca translasi (PTM) mereka dalam patogenesis GDM. Kajian dilakukan terhadap 30 sampel darah daripada wanita hamil yang menghidap diabetes (berpenyakit), 30 wanita hamil bukan kencing manis (kawalan) dan 30 wanita tidak hamil dan tiada kencing manis (normal). Pengekstrakan protein dilakukan daripada plasma darah dan protein terfosforilasi dan glikosilasi ditentukan oleh Asai Imunosorben Berkait Enzim (ELISA) dengan menggunakan antibodi khusus. Kajian ini menunjukkan bahawa protein yang terdapat dalam GDM adalah sangat terfosforilasi dan O-glikosilasi ($p<0.01$). Tambahan pula, penyelidikan bioinformatik telah dilakukan yang menunjukkan bahawa protein pembekuan darah seperti rantai alfa Fibrinogen dan protein yang mengawal metabolisme lipid apolipoprotein E, L1 dan C-III menunjukkan potensi tinggi untuk fosforilasi yang mencadangkan bahawa fosforilasi boleh digunakan sebagai penanda terapeutik dalam GDM.

Kata kunci: Kehamilan Diabetes Mellitus; pengubahsuaian protein; glikosilasi; fosforilasi

Introduction

Gestational diabetes mellitus (GDM) is defined as a sub-class of disease diabetes mellitus that is only observed during pregnancy (ADA 2019). Typically, enough insulin is produced to handle the excess glucose produced, but once the glucose level exceeds the insulin produced, GDM

may occur (Plows et al. 2018). Several risk factors for GDM comprise advanced maternal age, diabetic family history, high body mass index or obesity. The present sedentary lifestyle and elderly pregnancies have increased the prevalence of GDM in the last few decades (Xu et al. 2017).

It is estimated that GDM affects around 10-20% of all pregnancies worldwide. The GDM prevalence rate in Pakistan is 15.3% (Badakhsh et al. 2019). Singapore and Vietnam show the highest rates (20.06% and 18.93%, respectively); Korea, Japan, Thailand, and Taiwan have a GDM prevalence of less than 8.0%, while Malaysia and China have a comparable prevalence of GDM 11.91% and 11.83%, respectively (Nguyen et al. 2018).

The primary triggering agent of GDM is the development of insulin resistance in pregnant women. It is a pathophysiological disorder that occurs in tissues which are unable to deliver proper response against flowing insulin levels (Gual, Le Marchand-Brustel & Tanti 2005). At the beginning of GDM, the glucose uptake induced by insulin is reduced in the skeletal muscle and adipose tissues, reducing glucose production by hepatic cells due to hormonal suppression. To cope with the reducing insulin and peripheral insulin resistance levels, pancreatic β-cells secrete higher than normal insulin to maintain normal glucose levels. This hypersecretion of insulin under chronic conditions aggravates β-cell failure and insulin resistance, leading to the progression of glucose intolerance and type 2 diabetes (Saltiel & Kahn 2001). Multiple proteins play a crucial role in GDM, such as the insulin receptor substrate 1 (IRS-1), a critical factor in the insulin phosphatidylinositol 3-kinase (PI3K) signalling pathway that controls the insulin-mediated metabolic effects, including glucose uptake. Post-translational modification (PTM), like phosphorylation, also plays an essential role in developing GDM, as normal glucose tolerance is associated with a decrease Tyr phosphorylation of IRS-1 (Qu et al. 2007). Furthermore, in the placenta trophoblast in GDM patients, IRS-1 Ser phosphorylation (Ser312) has been reported (Feng et al. 2016).

Another critical and abundant PTM is *O*-GlcNAcylation, which is the process in which *O*-linked β-D-N-acetylglucosamine (GlcNAc) covalently attaches to Ser and/or Thr in nuclear and cytoplasmic proteins (Yang & Qian 2017). UDP-GlcNAc is produced in the hexosamine biosynthetic pathway (HBP) by taking 2-3% of glucose produced in the cell (Nagel & Ball 2014). UDP-GlcNAc is produced by HBP and is responsible for the *O*-linked glycosylation of protein (Semba et al. 2014). Within the inconsistent environment of stress and nutrient availability, it is important to control the *O*-GlcNAcylation for the proper cell functions. This process is controlled by the *O*-GlcNAc transferase (OGT), which adds *O*-GlcNAc on Ser and Thr residues (Whelan et al. 2010a). *O*-GlcNAcylation has already been shown to be involved in hyperglycemic pregnancies and its impact on the function of the placenta (Ning & Yang 2021).

Moreover, it has been suggested that *O*-GlcNAcylation is higher in females than males (Ning & Yang 2021) and that male fetuses may lead to an increased risk of GDM (Jaskolka et al. 2015). In some instances, phosphorylation and *O*-GlcNAc modification work together in an inverse relationship on Ser/Thr residues of proteins (Butkinaree, Park & Hart 2010). This phenomenon is called the yin-yang hypothesis, and the sites where this occurs are called yin-yang sites (Kaleem et al. 2021).

Understanding phosphorylation and glycosylation under pathological and physiological conditions is necessary as they work as a switch that turns on and off several pathways and cascades leading to the disease (Ardito et al. 2017). Major research in GDM has developed an effective therapeutic that can reduce its incidence and modify long-term fetal and maternal outcomes. Due to lack of information about underlying mechanisms has hindered the advancement of such therapeutics. The molecular pathways controlling insulin resistance may signify potential candidates, and their interpolation may lead to the development of novel therapeutics that reduce the progress of GDM.

The aim of this study was to investigate the function of PTMs, particularly *O*-glycosylation and phosphorylation, in the etiology of GDM. This is an important and urgent research as GDM is a transient kind of diabetes that affects both the health of the mother and child. This work will in future be helpful in the development of therapeutic markers and inhibitors.

Materials and Methods

Protein Extraction

Samples were collected from local hospitals in Lahore (Ethical approval code: 10395). 90 venous blood samples were collected, 30 GDM pregnant women, 30 non-diabetic pregnant women and 30 normal women (non-diabetic, not pregnant) to use as a control. The following

inclusion criteria was used: Confirmed diagnosis of GDM and pregnant at time of sample collection. For controls women without GDM, but pregnant at time of sample collection were selected. For normal samples, women without GDM and pregnancy were selected. The following exclusion Criteria was used: Individuals with pre-existing medical conditions other than GDM (e.g., cardiovascular diseases) were excluded. Samples that were not stored at the recommended temperature (4 ℃) were excluded from the study to ensure sample integrity. Samples were collected within age range of 40 years, and individuals outside this range were excluded.

Blood was directly obtained from individuals into EDTA vacutainers for extraction of protein. About 27 mL of 0.17M of ammonium chloride (NH_4Cl) solution was added to 3 mL of blood taken from each individual in 50 mL falcon tubes. The falcon tubes were then placed at agitator with moderate agitation for 5 min at room temperature. The samples were then centrifuged in a refrigerated centrifuge for 10 min at 3500 rpm at 4 °C. The supernatant was removed, and the pellet was resuspended in 300 µL of 1X PBS (phosphate buffer saline) solution. Again, centrifugation was performed at 3500 rpm for 8 min at 4 °C. The pellet was again suspended in 300 µL of chilled PBS and centrifuged at 3500 rpm for 8 min at 4 °C. The washing step was repeated 5-6 times until a whitish color pellet was obtained with very little amount of red blood cells. After washing steps all the PBS was removed carefully without disturbing the pellet and dissolved in 100µl of SDS loading buffer, (100 µL of protease inhibitor and 20 µL Sodium Orthovanadate or Thiamet G). Sodium orthovanadate (Sigma) is a phosphatase inhibitor, and was added to the samples to determine phosphorylation; Thiamet G (Sigma) is an *O*-GlcNAcase inhibitor, and was added to determine *O*-glycosylation; and both sodium orthovanadate and thiamit G were added to some samples to determine the interplay between phosphorylation and *O*-glycosylation. After adding inhibitors, syringing was performed of all samples to separate out the protein. Finally, boiling of samples was performed at 100 °C for 10 min. All the samples were stored at -20 °C, and were used within one month of storing.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The samples were diluted 10 times in 0.05M PBS buffer. The samples, with an initial protein concentration of at least 10 mg/mL, were diluted 10 times in a 0.05M PBS buffer. 100 µL of diluted samples were added into the wells of the ELISA plate. The plate was kept on a shaking incubator overnight at 4 °C. The following day, the plate was washed thrice with 100 µL of PBS on slight agitation for 5 min. 100 µL of freshly prepared blocking solution (1% gelatin in PBS) was then added into the wells and incubated for two and half hours at room temperature. Again, the plate was inverted to remove any excess solution and was washed with 100 µL of 0.1% PBST (PBS+Tween 20) for three times on slight agitation for 5 min. The primary antibody solution was prepared by diluting anti-phospho and anti-*O*-GlcNAc primary antibodies (anti-Phospho antibody (ab17464, Abcam) and anti-*O*-GlcNAc antibody (sc59624, Santa Cruz Biotechnology)) (1:1000) with 1% Bovine serum albumin. 100 µL of the primary antibody was loaded into the wells of the plate. The plate was then incubated for 2 h at 37 °C. The washing step was repeated three times, and 100 µL of anti-phosphorylation and/or anti-*O*-GlcNAc secondary antibodies (catalogue numbers: ab97051, sc516102) (1:1000) were transferred into wells of the plate. The plate was incubated for 1 h at room temperature. After washing 3 times, 100 µL of TMB (3,3′,5,5′-Tetramethylbenzidine) substrate and 100 µL 3% peroxidase solution were added to the wells and the plate was incubated for 30 min at room temperature. The washing step was performed 3 times and was added into wells for enzymatic reaction for 30 min at 37 °C and the reaction was then stopped by adding 100 µL of stop solution (2M H_2SO_4). The plate was then placed on an ELISA reader, and absorbance was measured at 450 nm (Weber et al. 2013). All samples were analyzed as triplicates, and the one-way ANOVA test was also performed to check the significance ($p<0.01$) of the results.

BIOINFORMATICS INVESTIGATION

Serum proteins in GDM were investigated. Proteins were selected from the research by Li et al. (2021), who investigated serum proteins in GDM by parallel reaction monitoring quantification (PRM). The protein sequences were retrieved from UniprotKB and their phosphorylation was predicted by Netphos 3.0 (Blom, Gammeltoft & Brunak 1999). The following steps were performed: Step 1. Download all FASTA sequences of selected proteins from the Uniprot database, Step 2. Open the Netphos 3.1 server and paste all the downloaded FASTA sequences on the webpage, and Step 3. Click on predict Ser/Thr phosphorylation and download all the potential predicted phosphorylation sites.

Results and Discussion

Determination of Proteins in GDM by ELISA

The ELISA was performed following standard protocol and after the antigen-antibody reaction, absorbance was measured at 450 nm. ANOVA statistical test was performed on the ELISA reading with a p-value <0.01, showing significant results (Figures 1 & 2). In both figures, the A bars show that no inhibitor was added, whereas in B, C, and D phosphatase, *O*-GlcNAc and both inhibitors were added. In Figure 1, the anti-phospho antibody was added, which showed that highly phosphorylated proteins were present in GDM patients, and in Figure 2, where the anti-*O*-GlcNAcase antibody was added the proteins were found to be highly glycosylated as expected.

Finally, the interplay between the two modifications was investigated, as shown in Figures 1(D) and 2(D), which showed that phosphorylated proteins were highest in normal non-diabetic patients, but *O*-GlcNAc modified proteins were highest in GDM patients. These results suggest that in GDM patients, the OGT shows higher activity than kinases, but in non-diabetic, its activity is lowered, suggesting that glycosylated proteins play an essential role in GDM.

Bioinformatics Investigations

Serum proteins in GDM patients were investigated by selecting different proteins using different publications showing the differential expression of proteins in GDM (Lapolla & Trandi 2022; Li et al. 2021). The selected protein Ser/Thr phosphorylation was predicted using Netphos 3.0 (Table 1) (Blom, Gammeltoft & Brunak 1999).

The % phosphorylated Ser/Thr residues in the different proteins were calculated, which showed that P02656, followed by P02671, had the most phosphorylated sites (Figure 3(a)). When the phosphorylated status was investigated, it showed that out of the total number of Ser/Thr residues present in the protein P02656 had more than 90% phosphorylated Ser/Thr residues, suggesting that this protein becomes highly phosphorylated in GDM (Figure 3(b)). Furthermore, it was also found that most proteins had more than 50% phosphorylated Ser/Thr residues in GDM, which suggests that besides *O*-GlcNAc modification, phosphorylation also plays an important essential role in GDM.

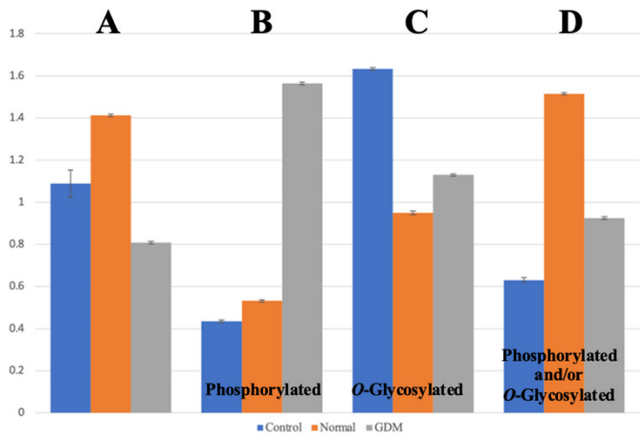

FIGURE 1. Detection of proteins by ELISA using anti-Phospho antibody in blood samples from pregnant non-diabetic women (control), not pregnant women (normal) and pregnant diabetic women (GDM). A: No phosphatase or *O*-GlcNAcase inhibitors; B: Phosphatase inhibitor; C: *O*-GlcNAcase inhibitor; D: Both Phosphatase and *O*-GlcNAcase inhibitors

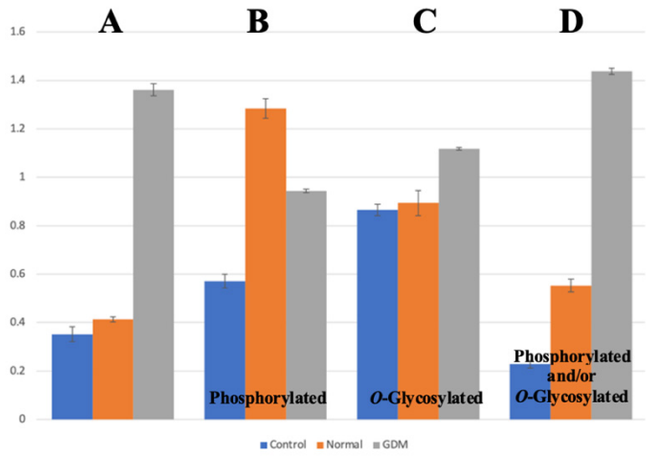

FIGURE 2. Detection of proteins by ELISA using anti-*O*-GlcNAc antibody in blood samples from pregnant non-diabetic women (control), not pregnant women and pregnant diabetic women (GDM). A: No phosphatase or *O*-GlcNAcase inhibitors; B: Phosphatase inhibitor; C: *O*-GlcNAcase inhibitor; D: Both Phosphatase and *O*-GlcNAcase inhibitors

TABLE 1. Potential phosphorylation sites in serum proteins in GDM

	Protein Accession No.	Protein Name	Total no. of AA	Total no. of Ser/ Thr	Phosphorylated Ser/Thr sites
1.	Q13822	Ectonucleotidepyro phosphatase/ phosphodiesterase family member 2	863	116	64
2.	Q9UIQ6	Leucyl-cystinyl aminopeptidase	1025	149	97
3.	O43184	Disintegrin and metalloproteinase domain-containing protein 12	909	102	48
4.	P02671	Fibrinogen alpha chain	866	166	127
5.	P01034	Cystatin-C	146	16	10
6.	P02649	Apolipoprotein E	317	26	18
7.	P02656	Apolipoprotein C-III	99	17	16
8.	O14791	Apolipoprotein L1	398	48	28
9.	P25311	Zinc-alpha-2-glycoprotein	298	28	16
10.	P01023	Alpha-2-macroglobulin	1474	224	142
11.	P35542	Serum amyloid A-4 protein	130	21	11
12.	P11597	Cholesteryl ester transfer protein	493	79	48

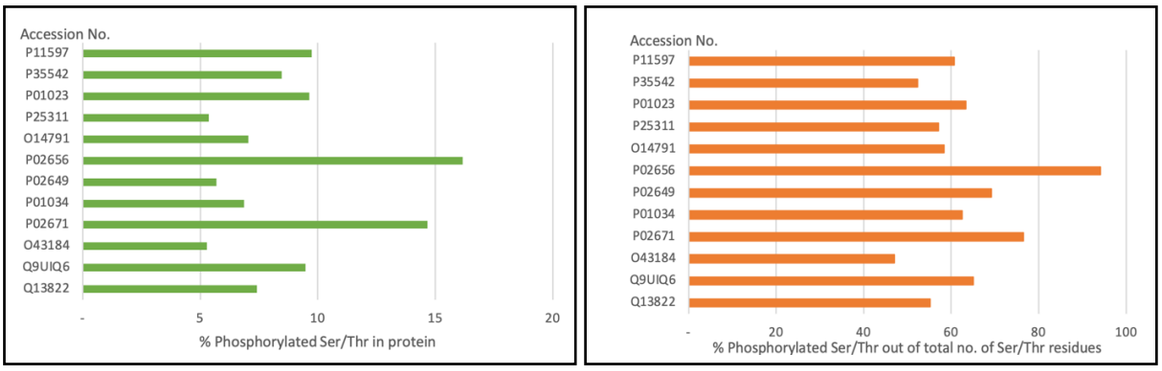

FIGURE 3. (a) Percentage of phosphorylated Ser/Thr sites in selected proteins in GDM, (b) Percentage of phosphorylated Ser/Thr sites out of total number of Ser/Thr residues in proteins in GDM

DISCUSSION

GDM is a high-risk pregnancy disease posing significant risks to mother and offspring and affecting 10-20% of all pregnant women. Although the severity of the disorder is high, less is known about the underlying mechanisms and pathways that cause GDM. This study was performed as an effort to add to the knowledge already available about the proteomics of GDM.

O-GlcNAcylation is a PTM occurring on Ser/Thr amino acid of various proteins by transferring a single sugar molecule from the substrate UDP-GlcNAc. Increased phosphorylation or glycosylation disturbs the glucose uptake pathway, leading to glucose intolerance in pregnant women that causes GDM. Numerous proteins take part in the pathology of GDM. Lapolla and Traldi (2022) reported that placental proteins like Galectin-1 and Vimentin are upregulated, and proteins like ApolipoproteinA-I and Caspase-1 are downregulated in GDM. Furthermore, Li et al. (2021) also showed multiple proteins to be differentially expressed in GDM.

In this work, the ELISA results showed that proteins expressed in GDM can be either phosphorylated and/or *O*-glycosylated (Figures 1 & 2). Specifically, when using the anti-phospho antibody, it was found that phosphorylated proteins were abundant (Figure 1), and when using the anti-*O*-GlcNAc antibody, the interplay between phosphorylation and *O*-GlcNAc modified proteins was high (Figure 2) in GDM as compared to control. These results suggest that proteins can be highly phosphorylated in GDM. When OGT becomes available due to high glucose levels, the proteins also become susceptible for *O*-GlcNAc modification. These results agree with Zhang et al. (2017), who showed that the inhibition of pyruvate dehydrogenase activity by its phosphorylation may be involved in GDM. Next, the phosphorylated proteins in GDM were investigated by using bioinformatic tools. Different proteins were selected using different publications showing the differential expression of proteins in GDM (Lapolla & Trandi 2022; Li et al. 2021). The result of these showed that proteins have the potential to be highly phosphorylated (Figure 3).

Both the blood coagulation and lipid metabolism system becomes affected by the poor blood glucose control in GDM (Teliga-Czajkowska et al. 2019). The blood coagulation protein such as Fibrinogen and plasminogen, which play a major role in homeostasis and thrombosis, is elevated in GDM, suggesting the tendency of developing thrombosis in GDM (Gorar et al. 2016). Previous studies have shown increased levels of Fibrinogen in GDM patients (Mahjabeen et al. 2022), and it has been reported that increased phosphorylation of Fibrinogen after surgery occurs to prevent bleeding (de Vries et al. 2020). The Fibrinogen alpha chain (P02671) has been shown to have a high potential for phosphorylation, with more than 75% of all the Ser/Thr residues being phosphorylated (Figure 3). These results suggest that the protein Fibrinogen has a high potential for phosphorylation, and further work is needed to determine its role in GDM.

In adipose tissue, which plays a major role in reduced insulin sensitivity, IRS-1 was found to become phosphorylated on Ser307 in late pregnancy (Sevillano

et al. 2007), and previous studies have shown the role of Tyr phosphorylation in insulin receptors and measured expression of protein and insulin resistance in skeletal muscles of GDM patients (Chu et al. 2014). Another study studied the increased expression of IRS-1 and TLR4 expression in the placenta of GDM women, which was positively related to increased maternal glucose tolerance (Feng et al. 2016). Interestingly, it has been reported that almost all of the major players of the insulin signalling pathway, such as IRS1, PI3K, PDK1, AKT, and FOXO1 are also glycosylated, often reciprocal to the phosphorylation sites on these proteins and thereby regulate insulin signalling through positive/negative feedback. Therefore, chronic elevation of *O*-GlcNAc could be considered a mechanism in the development of insulin resistance at least in part through *O*-GlcNAcylation of PI3K or AKT on stimulatory Ser residues (Ma & Hart 2013; Whelan et al. 2010).

Increased phosphorylation in GDM is associated with increased p70 S6K activation. As p70 S6K is activated by elevated amounts of glucose and amino acids, there is an excess of glucose in GDM, which may cause its activation, activating IRS-1 phosphorylation. According to Kaleem et al. (2021), Ser and Thr glycosylation and phosphorylation on IRS-1 have an important role in diabetes mellitus type ll.

The different apolipoproteins also play a crucial role in the development of GDM. The apolipoprotein C-III has been shown to increased in women who subsequently developed GDM (Kim et al. 2012). ApoA2 is correlated with GDM and may be used as a biomarker of premature delivery (Ramanjaneya et al. 2021). Li et al. (2021) found increased levels of apolipoprotein C-III, L1 and E, and decreased levels of cholesteryl ester transfer protein, supporting the vital role of lipid metabolism and transport in GDM. Both apolipoprotein E (P02649) and L1 (O14791) showed potential for phosphorylation (around 50% out of total no. of Ser/Thr residues present in the protein (Figure 3). In contrast, apolipoprotein C-III (P02656) showed highest potential for phosphorylation (more than 90%). The family of Galectin proteins has been found to be involved in different pathological pregnancies such as GDM, preeclampsia, fetal growth restriction and preterm birth (Chen et al. 2022), and Galectin-1, which has been found to be upregulated in GDM (Lapolla & Traldi 2022), has been suggested to act as a prognostic marker in epithelial ovarian cancer (Mielczarek-Palacz et al. 2022). These findings indicate the importance of understanding the role of the different proteins involved in GDM.

The bioinformatics analysis suggests that the occurrence and development of GDM involve numeral pathological processes, including clot formation, lipid metabolism, and blood coagulation. This study contributes to the importance of PTMs of the different protein's effect on GDM. Further study in future is needed to experimentally determine the phosphorylation of proteins, which eventually can be used as a biomarker for the progression of GDM.

CONCLUSIONS

The research focuses on gestational diabetes mellitus (GDM), a high-risk pregnancy syndrome that affects 10% to 20% of pregnant women. The study looks into the involvement of protein phosphorylation and *O*-GlcNAcylation in the development of GDM. The study discovered a complicated interplay between these PTMs, influencing crucial processes such as insulin signalling, blood coagulation, and lipid metabolism. Fibrinogen, IRS-1, apolipoproteins, and galectins are the proteins implicated and identified as possible biomarkers. The findings highlight the importance of further investigating these PTMs and their consequences in GDM care and diagnosis in order to enhance maternal and newborn health outcomes.

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