ISSN: 2639-0337

Madridge Journal of Diabetes

Research Article

Open Access

Proteomic analyses of Urine Exosomes reveal New Biomarkers of Diabetes in Pregnancy

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Article Info

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Received: January 8, 2016
Accepted: February 1, 2016
Published: February 1, 2016

Citation: Ramachandrarao SP, Hamlin AA, Awdishu L, et al. Proteomic analyses of Urine Exosomes reveal New Biomarkers of Diabetes in Pregnancy. *Madridge J Diabetes.* 2016; 1(1): 11-22.
doi: 10.18689/mjd-1000103

Published by Madridge Publishers

Abstract

Objective: To evaluate 24 hour urine exosome protein content changes among pregnant US subjects with diabetes and obesity during early pregnancy.

Methods: The exosome proteome content from 24 hour urine samples of pregnant subjects with gestational diabetes mellitus (GDM, N=8) and pre-gestational Type 2 diabetes (PGD, N=10) were compared with control samples (CTRL, N=10) obtained at week 20 of pregnancy. Differences in exosome protein load between groups was identified by liquid chromatography/mass spectrometry, analyzed by linear regression in negative binomial distribution, visualized in MetaboAnalyst (version 3.0), and validated by western immunoblotting.

Results: At the 20th week of pregnancy, we identified 646, 734 and 856 proteins in exosomes from 24 hour urine samples of patients from the CTRL, GDM and PGD groups, respectively. S100 calcium binding protein A9, damage associated molecular pattern (DAMP) signal, was found to be significantly increased in both GDM and PGD subjects. In GDM subjects the peptide counts for S100A9 protein independently correlated with maternal obesity and macrosomia of the newborn infants. Early to late pregnancy developmental changes in the GDM group were shown to utilize pathways and protein expression levels differently from those in PGD or CTRL groups.

Conclusions: Urinary exosome proteomic analysis non-invasively provides insights into maternal changes during diabetic pregnancy. Exosome biomarkers early in pregnancy can be potentially used to better understand pathophysiologic mechanisms of diabetes at a cellular level, and to distinguish between gestational and pre-gestational diabetes at the pathway level. This information can aid intervention efforts to improve pregnancy outcomes in women with diabetes.

Keywords: Damage associated molecular pattern; Diabetic pregnancy; Exosome; Gestational diabetes; Urine exosomes; Proteomics; S100A9.

Abbreviations: PGD: Pre-gestational diabetic; MVBs: Multivesicular bodies; PLS-DA: Partial-Least Squares Discriminant Analysis; VIP: Variable Importance in Projection; FDR: False Discovery Rate; PLSD: Partial Least Squares Discriminant; DAMP : Danger-Associated Molecular Pattern.

Introduction

Diabetes during pregnancy increases the risk of poor pregnancy outcomes. Poorly controlled maternal diabetes may increase the risk of malformations involving multiple organ systems, such as fetal cardiac and spinal abnormalities during the first trimester and even fetal loss during the third trimester [1]. Renal glomerular filtration rate and plasma flow increase during normal pregnancy, leading to increases in proteinuria, glucosuria, and lower serum osmolality and sodium levels [2]. In pregnancies complicated by diabetes these changes may be exacerbated causing subclinical renal insufficiency in addition to the other unfavorable outcomes mentioned [3]. Pre-gestational diabetic (PGD) mice develop accelerated renal pathology [4], suggesting that stimuli that elicit benign responses in the non-diabetic pregnancies may lead to renal dysfunction or injury in diabetic pregnancies [5,6]. Further, as demonstrated by the collaborative perinatal project, the PGD mothers carry different risks of giving birth to malformed infants than do the GDM mothers [7]. Mechanisms underlying end organ damage or malformation and the maternal/fetal metabolic programing affecting the kidney function of the newborn still remain incompletely defined [8-11]. New tools that are noninvasive yet specific enough to examine the pathologic alterations in maternal diabetes during pregnancy are required to study clinically relevant sequelae specific to GDM or PGD phenotypes. We believe that urinary exosomes represent a noninvasive paradigm that potentially may be able to address phenotype-specific questions.

Exosomes are membrane bound biologically active nano-vesicles released from every cell type [12,13]. They are formed by inward budding of late endosomes, producing Multivesicular bodies (MVBs). They are then released into the surrounding biofluid by fusion of MVBs with the plasma membrane [14]. The endocytic origin of exosomes and the cellular nature of their contents enable them to be recognized as a rich source of information on the status of the cells producing them. Because their contents significantly change in response to stress or injury [15] they are also potent mediators of intercellular communication [16]. Average human kidneys filter 1500-2000 liters of plasma on a daily basis to produce roughly 1.5 liters of urine. Together with the observation that urinary exosomes contain 3-5% of the total urinary proteins, exosomes represent enriched background information on the subject's systemic status.

Diabetic pregnancy is a stressor on multiple organs, including the kidney. Further, GDM is qualitatively a different type of stress compared to PGD [7]. Therefore, at an early time point during diabetic and normal pregnancy we sought to characterize urine exosome proteins from CTRL (n=10), PGD (n=10) and GDM (n=8) women. We also investigated pathway level differences between pre-gestational and gestational diabetes, as well as developmental changes from early to late pregnancy as reflected in their urine exosome proteomes.

Materials & Methods

Patient Selection

Singleton pregnancies who received prenatal care at the University of California San Diego Medical Center from 2011 to 2013 were consented to participate in the study. The study design and recruitment was reviewed and approved by the institutional review board. Informed consent for participation was obtained from all study participants. Inclusion criteria included pre-existing Type 2 diabetes or gestational diabetes. Diagnosis for gestational diabetes followed the criteria established by the California Sweet Success Program. This criterion includes at least one of the following: a first trimester HbA1c between 5.7-6.4% (39-46 mmol/mol), a fasting plasma glucose between 92-126 mg/dL, or a second trimester two hour glucose tolerance test with a fasting plasma glucose of greater than or equal to 92 mg/dL, one hour greater than or equal to 180 mg/dL, ora two hour plasma glucose greater than or equal to153 mg/dL [17]. Control samples were obtained from consenting women who did not have pre-existing diabetes and screened negative for gestational diabetes. Study participants were required to collect a 24 hour urine sample at 20 and 36 weeks gestation.

Demographics

Data was collected from a chart review. Information regarding diabetes management and control were obtained during first, second, and third trimesters, including HbA1c, fasting plasma glucose levels, diabetes medications (oral hypoglycemics and insulin), and any maternal medical complications. Fetal outcomes including ultrasound, estimated third trimester fetal weight, and birth weight were also collected.

Study design: Urine samples at the 20th week (early time point) of pregnancy from 8 GDM, 10 PGD and 10 CTRL subjects were used for exosome isolation and proteomic analysis in replicates. A subset of these subjects that completed sample collection during 36th week of pregnancy (5 GDM, 3 PGD and 4 CTRL pregnancy subjects) were separately analyzed to evaluate differences between the 20th and 36th week. Proteomic data were analyzed using negative binomial distribution as well as MetaboAnalyst (version 3.0) and validated by Western immunoblotting where applicable.

Urine Sampling and Processing

24 hour urine samples were collected spanning 2 days. The first void of Day 1 was not collected. All voids were collected from the second void on Day 1 to the first void on Day 2, including all nocturnal voids. During collection the samples were kept on ice or refrigerated. After 24 hours collected urine samples were centrifuged at 3000 x *g* for 30 min. The pH of the resulting supernatant was adjusted to 7.0, aliquoted, and frozen at -70 °C until further analysis.

Exosome preparation and proteomic analysis

Exosomes from frozen pregnancy urine samples were prepared using an in-house protocol developed based on the solvent exclusion principle using polyethylene glycol (PEG)-induced precipitation, as described in our recent publication [18]. One-dimensional SDS-PAGE of the exosome proteins prior to in-gel trypsinization was performed.

Data Processing and Statistical Analysis

In all analyses, $p \leq 0.05$ was considered statistically significant. Log transformation of raw data were performed before the tests. Analyses including Student's t-tests, Partial-Least Squares Discriminant Analysis (PLS-DA) and Variable Importance in Projection (VIP) were performed with the MetaboAnalyst 3.0 (www.metaboanalyst.ca) web portal [19]. Negative binomial generalized linear regression models were fit using the statistical software R version 3.1.2.20

PLS-DA and VIP were used both for the classification and significant feature selection [19] with a False Discovery Rate (FDR) of ≤10 % to filter protein candidates for western immunoblotting validation. Negative binomial generalized linear models were fit to determine significant differences in protein counts between CTRL, GDM, and PGD subjects utilizing the raw count data and estimating gene-wide size factors and estimated dispersion-mean relationship via the methods outlined in Anders and Huber in the R package DESeq2 [21]. Significance was reported based on a filtered FDR-adjusted p-value.

Western Immunoblotting and Quantification

Antibody against S100 A9 was purchased from Proteintech Group, Inc., (Chicago, IL, USA). HRP-conjugated secondary antibody was from GE Life Sciences (Piscataway, NJ, USA). SDS-PAGE gels (with 10% acrylamide) were used to resolve 100 µg of protein from exosomes of normal and diabetic pregnancy urine samples. Immunoblotting and quantification with Image J software (NIH) was done using methods described in our previous publications [22] and plotted using Graph Pad Prism software (San Diego, CA, USA).

Results

Patient Demographics Show Significant Differences between Diabetic and Non-Diabetic Pregnancy Subject Phenotypes

We studied 24 hour urine samples from 28 mothers: 10 non-diabetic subjects that served as controls (CTRL) and 18 with diabetes (8 gestational, GDM and 10 pregestational, PGD) recruited from UC San Diego Reproductive Medicine clinics. Clinical disease data on 6 GDM, 7 PGD and 6 CTRL mothers was collected. Newborn birth weight in PGD averaged 3155.3 gms as compared to 3884.8 gms in GDM group. Medication regimens were different between the two groups: while all 7PGD subjects required medication, only 2 GDM subjects required medication. The level of diabetes control between the PGD and GDM groups was different. For this study, HbA1c ≥7% (53 mmol/mol) was considered a marker for poorly controlled disease. Accordingly, our data indicate that 5 of the 7 PGD patients were poorly controlled, while all of the GDM patients were well controlled. The minimum diagnostic criteria based on HbA1c are 5.7% (39 mmol/mol) for GDM and 6.5% (48 mmol/mol) for PGD. In our cohort, the average HbA1c was 6.0% (42 mmol/mol) for GDM and 7.1% (54 mmol/mol) for PGD subjects, showing an average increase of only 0.053% above the diagnostic criteria for GDM, but an increase of 0.09% above the diagnostic criteria for the PGD group. Thus GDM group subjects had better controlled disease than PGD subjects, possibly due to the shorter time course of GDM as compared to PGD (Table 1).

Variable	PGD, N=7	GDM, N=6	Control, N=6	P value
Age	30.9±8.1	30.7±7.1	33.2±5.8	0.84
Race/Ethnicity				
White	0	0	3	0.04
Black	2	1	0	0.76
Asian	0	1	0	0.56
Hispanic	4	4	2	0.13
Other	2	0	1	0.21
Weight (kg)	87±17.2	89.1±20.4	75.6±13.4	0.48
BMI (m/kg^2)	33.9±4.3	33.6±6.6	27.6±6.8	0.2
HgBA1C (%)				
First Trimester	9.2±1.7	6±0.28	5.3±0.17	0.0003
Second Trimester	7.3±1.2	5.9±0.35	NA	0.09
Third Trimester	6.7±0.8	6	NA	0.48
Fasting Plasma Glucose (mg/dL)	92.4±18.3	92±18.3	74±12.5	0.3
75 mg 2 Hour Oral Glucose Tolerance Test Glucose Level (mg/dL)	NA	170.5±79.9	85.5±21.9	NA
Medications				
Oral	2	2	0	0.13
Insulin	6	0	0	0.13
Pre-eclampsia	1	0	0	0.04
Gestational Hypertension	2	0	0	0.47
Retinopathy	0	0	0	NA
Chronic Hypertension	3	0	0	0.2
Nephropathy	0	0	0	NA

Table 1: Patient Demographics.

Average maternal BMI and neonate head circumference were similar between the GDM and PGD groups (Table 2). None of the PGD neonates in our cohort was macrosomic (average birth weight 3155.3±664.5 gms). Of the 6 GDM neonates, 3 were macrosomic (4485±86.07 gms) and 3 were normal (3285±337 gms). Thus phenotypically, both the mother and the newborn infant in the GDM group were different from that in the PGD group.

Variable	PGD, N=7	GDM, N=6	Control, N=6	P value
Gestational Age (weeks, days)	37 weeks 5 days	38 weeks 3 days	38 weeks 2 days	0.863
Birth Weight (grams)	3155±697	3555±232	3173±285	0.666
Birth Length (cm)	50.1±3	51.1±2.7	49.6±1.6	0.672
Head circumference (cm)	33.6±2.3	34.5±1	34±1.4	0.729

Table 2: Neonatal Outcomes.

The Urinary Exosome Protein Content is Different in Diabetic *versus* Normal Pregnancy Subjects

Urine exosome proteomic data from an early time point of pregnancy (24 hour urine samples from week 20) showed that 1103 proteins were identified with a spread of 645 CTRL proteins, 855 GDM proteins and 733 PGD proteins in each subject of the group.475 proteins were common to all three groups (Figure S1). Tables S1-S5 document the individual protein identity of each of the groups. These protein data show differences in the non-diabetic *versus* diabetic pregnancy urine exosomes' protein signatures, and further differences between GDM and PGD subjects at an early pregnancy time point.

To further delineate semi-quantitative differences between groups we used Negative Binomial Distribution to perform linear regression of the expression data of 1103 proteins. CTRL *vs* GDM analysis showed 70 proteins to be significantly different with S100 calcium-binding protein A9 (S100A9) the most different between the groups. (Table S1, FDR<10%). Similar analysis on CTRL *vs* PGD groups showed 77 proteins to be significantly different, with S100A9 as one of the 7 proteins reaching FDR<10% (Table S2).

We used MetaboAnalyst suite to visualize the proteomic data. Partial Least Squares Discriminant (PLSD) analysis (Figures 1A-1C) showed a clear separation between CTRL and GDM proteins early in pregnancy. To determine which proteins contribute to this discrimination we conducted the Variable Importance in Projection (VIP) analysis (Figure 1D). Table S3 shows that 107 proteins contributed to this discrimination between CTRL and GDM groups, with a VIP score of >1.5, and S100A9 topped this list with a VIP score of 7.7.A similar analysis between CTRL and PGD groups showed clear distinction between protein expressions in the 2 groups and S100A9 with the top VIP score of 4.09 (Table S4).

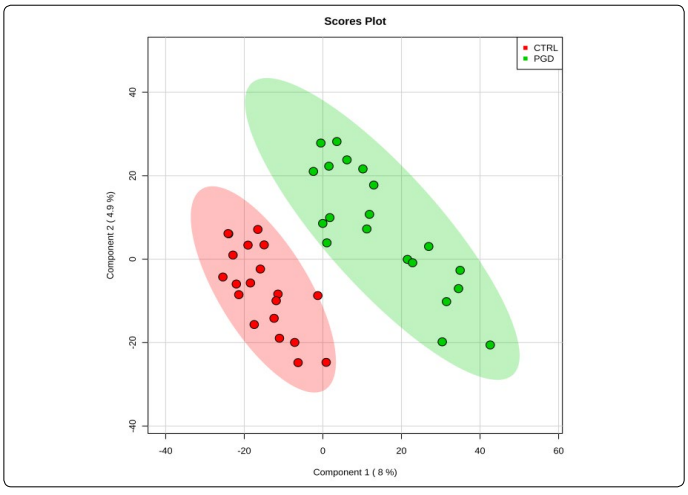

Figure 1A: PGD *vs.* controlconcentration-based proteomic measurements: PLSDA separation using protein NSAF measurements in the urine exosome of Pre-Gestational Diabetes Mellitus (PGD,n=10) vs Control subjects (CTRL,n=10). Lack of overlap between the two groups of exosome proteins signifies clear separation of PGD from CTRL.

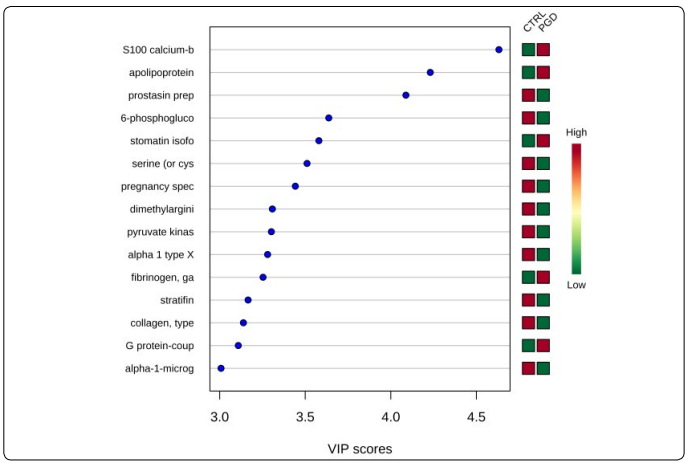

Figure 1B: PGD *vs.* Control variable importance in projection plot: Urinary exosome proteins identified by PLS-DA in a descending order of importance.The graph represents relative contribution of proteins to the variance between the PGD and CTRL urine exosomes. The green and red boxes on the right indicate whether the protein concentration is increased (red) or decreased (green) in the exosome of the PGD urine vs. CTRL urine samples. Similar to analysis in Figure 1b, S100A9 is the top protein (VIP score of >4.5).

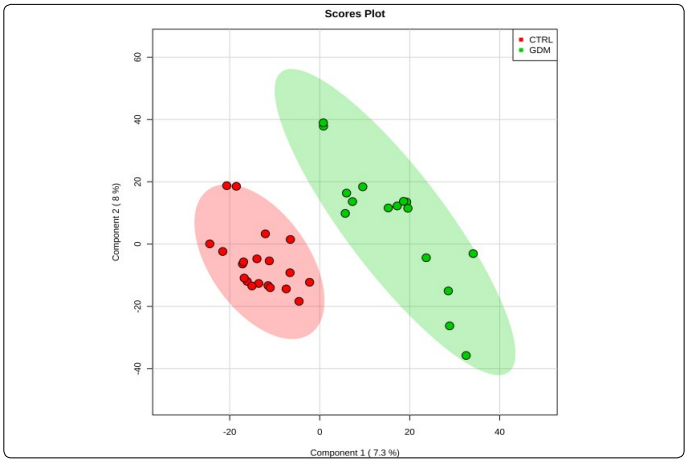

Figure 1C: GDM *vs.* control concentration-based proteomic measurements: Two-dimensional (2D) partial least squares discriminant analysis (PLSDA) separation using protein normalized spectral abundancy factor (NSAF) concentration-based proteomic measurements in the urine exosome of Gestational Diabetes Mellitus (GDM,n=8) vs Control subjects (CTRL,n=10). Clear separation of GDM from CTRL is observed.

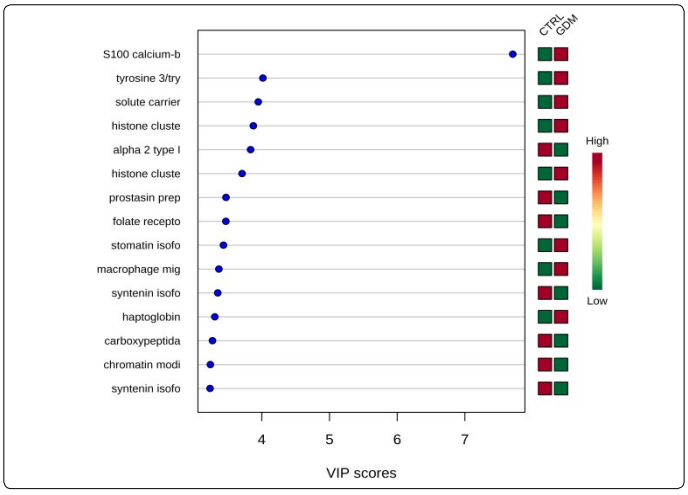

Figure 1D: GDM vs. control variable importance in projection plot: The graph represents relative contribution of proteins to the variance between the GDM and CTRL urine exosomes at week 20 of pregnancy. High value of VIP score for a protein indicates greater contribution of the protein to the separation of groups. The green and red boxes on the right indicate whether the protein concentration is increased (red) or decreased (green) in the exosome of the GDM urine vs. CTRL urine samples. For higher n value, a VIP score of 1.5 is considered to enable discrimination between 2 phenotypes. Even with the low n (=10) per group that is employed in this study, the VIP score of the top 3 proteins is higher than 3. S100 calcium binding protein A9 is the top protein with a VIP score of >7.

S100A9 Protein Upregulation in Early Pregnancy Urine Exosome of Diabetic Subjects is Validated by Western Immunoblotting

We validated the statistically significant upregulation of S100A9 protein in diabetic urine exosomes by western immunoblotting analysis. Equal amounts (50 µg) of protein from each exosome sample was used, (Figure 2A, 2B) showing significant S100A9 upregulation in diabetic exosome. However, all diabetic patients did not uniformly show S100A9 upregulation. We therefore sought to further determine if the demographic feature differences between subjects of the same group correlate with the relative abundance of this protein as denoted by its peptide count.

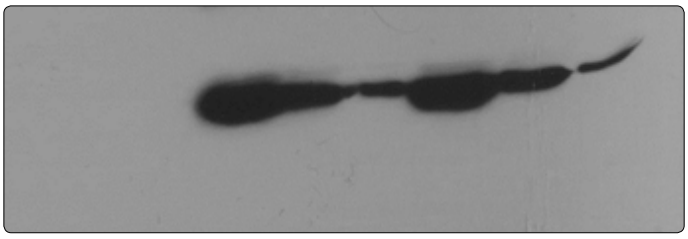

Figure 2A: Immunoblotting of pregnancy urine exosome for S100A9 protein. Lanes 1-3: control; lanes 4-6: GDM; lanes 7-9: PGD.

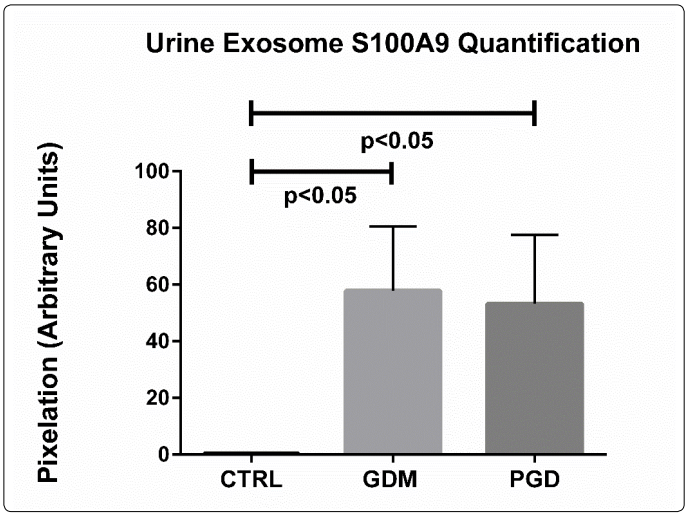

Figure 2B: Quantification of S100A9 from immunoblots in A (control, n=3; GDM, n=3, PGD, n=3). Data are means ± SEM. p<0.05 pregnancy urine exosome S100A9 GDM *versus* CTRL; PGD *versus* CTRL.

S100A9 Protein Peptide Count Data Correlates with Obesity and Macrosomia Differently in GDM *versus* PGD subjects.

The average urinary exosome load of S100A9 peptide count for the PGD group was 22.25±37.89 (N=10) while the GDM had an average peptide count of 23.63±34.7 (N=8). Both were significantly different from the CTRL subject peptide count of 1.15±2.39 (N=10) (p=0.0065, GDM *vs* CTRL and p=0.0174, PGD *vs* CTRL), which also closely tracked with the western blot data.

The average S100A9 peptide count was significantly different in the PGD and GDM subjects (7.4±13.43 *versus* 29.08±38.78, p=0.0209). When level of control of disease within the group was considered, PGD patients with well controlled disease have a lower average peptide count(0.25±0.5) compared with the poorly controlled group of PGD patients. It is noteworthy that both of these averages are less than the GDM group average.

GDM mothers with macrosomicneonates of had an average peptide count of 32.67±54.4 while GDM mothers of non-macrosomic neonates had an average peptide count of 25.5±17.85. Whereas this peptide count difference is likely less significant, it does illustrate a higher maternal peptide count for macrosomic neonates. Interestingly, a significantly higher rate of macrosomia in GDM than in PGD was observed (p=0.0002).

Lastly, we considered BMI. 6 of the 7 PGD patients and 4 of the 6 GDM patients were obese. Peptide count average of obese PGD patients was 6.25±12.44, while that of a single non-obese PGD patient was 0. In the GDM group, peptide average of obese patients was 42.5±37.53 and only 2.25±2.87 for non-obese patients. In the non-diabetic group, the peptide average of the obese subjects was 11.5±4.04 and only 1.67±1.51 for non-obese subjects. This strong trend (p=0.0585) demonstrates that peptide count is higher in pregnant obese women than in pregnant non-obese women.

Urine Exosome Protein Content Changes from Early to Late Pregnancy Proceed *via* Different Pathways in CTRL *versus* PGD *versus* GDM

Further, we sought to examine how progression of pregnancy from an early time point to a later time point is reflected in the urine exosome proteins and pathways in these three groups. We performed a nested analysis on urine exosomes from 4 CTRL, 5 GDM and 3 PGD subjects that completed the 36th week collection in addition to the 20th week samples.

We observed considerable differences between exosome proteomes of early *versus* late pregnancy. For the 20th and 36th week samples respectively, 357 and 268 proteins (CTRL), 460 and 396 proteins (GDM) and 369 and 352 proteins (PGD) were identified. Thus the number of proteins identified in urine exosome generally decreased from early to late pregnancy. Groupwise analyses of the 20th and 36th week samples showed that the number of unique proteins identified reduced 5-fold in CTRL, roughly 2-fold in GDM and only slightly decreased in PGD groups (Figures S2A and S2B).

Thus, although the number of total pregnancy proteins identified was the highest in the GDM group (GDM>PGD>CTRL with 530,470 and 377 proteins respectively), within a group, the newer proteins identified during later pregnancy was CTRL < GDM <PGD with 20, 70 and 101 proteins respectively.

In addition, the protein pathways predominantly contributing to changes in progression of pregnancy from week 20 to week 36 were different in each group (Table S5, Figures S3A-S3C). We observed differences even in the extent to which a protein expression changed in one group *versus* the other (Δ_1=Protein1 $NSAF_{Week20}$ – Protein1 $NSAF_{Week36}$). Thus, Δ_1 (for a given protein) for CTRL was different from Δ_1 for GDM, which in turn was different from Δ_1 for PGD group.

Discussion

Urine comprises at least two distinct exosome populations: exosomes that are produced by pre-glomerular organs such as brain and muscle that are filtered from plasma into urine, and those that are produced by the kidney tubular cells distal to glomerular filtration. Urine exosome proteins represent only 3% of the whole urine proteome [23] and their cellular nature allows for the strong likelihood that this 3% contains cell- and cell-state specific markers derived from both renal and pre-renal organs. Given that every living surface is shown to use these bio-active nanoparticles for intercellular communication, [24] urine exosomes potentially have a systemic level representation, and their analysis provides clinically relevant information in describing a disease phenotype such as diabetes during pregnancy. Two recent exosome proteomics analysis from **(a)** Pisitkun, et al. show 295 distinct proteins at least 22 of which have been implicated in various kidney and systemic abnormalities [25] and **(b)** Zhou, et al. show the utility of exosomes as a source of kidney physiology biomarkers in AKI subjects [26].

In this report, we examined the difference in urine exosome protein content of diabetic and non-diabetic pregnancy subjects. In all, we found that 1103 proteins were identifiable from a total of 28 urine exosome samples of non-diabetic and diabetic subjects. Using three independent methods, namely negative binomial distribution for linear regression analysis, partial least squares discriminant analysis, and variable importance in projection analysis of the proteomic data, we have shown that the calcium binding protein S100 A9 is significantly upregulated in both pre-gestational as well as gestational diabetic pregnancies. S100A9 upregulation in diabetics was validated by western immunoblotting. We also showed that the peptide number of this protein independently correlates with macrosomia in the newborn infant as well as maternal obesity, although to a different extent in the GDM *versus* PGD groups. Though the mechanism of S100A9 upregulation in diabetes/obesity and its clinical significance needs to be established, other recent studies [27-29] have demonstrated S100A9 upregulation in diabetes or obesity, further supporting our finding.

S100A9 upregulation as a biomarker of inflammatory processes and immune response is gaining fast ground. S100A9 heterotetramerizes with S100A8 to form calprotectin,which has a plethora of intracellular and extracellular functions.It is predominantly localized in the cytoplasm but is translocated to the cytoskeleton and cell membrane upon elevation of intracellular calcium level, and gets secreted to extracellular space to amplify innate immune response by acting as an endogenous Danger-Associated Molecular Pattern (DAMP) that promotes inflammation [30]. To our knowledge, this report is the first study showing S100A9 elevation in the urinary exosomes of human subjects with obese/diabetic pregnancy. Given its ability to induce neutrophil chemotaxis and adhesion, a role for S100A9 in exosome vesicle trafficking cannot be ruled out. If so, it would be interesting to address whether S100A9 would play a role in increased exosome production in diabetic milieu.

This study has numerous strengths. The 24-hour urine sample collection design lends itself to reduced variation and is more representative of the phenotype. Collection of samples at two time points from the same individuals allows for quantification of intra-subject variability. Additionally, by virtue of their lipid lamellar structure, exosomes serve to transport hydrophobic proteins, so the analysis is not limited to hydrophilic proteins only. One limitation of this study is its small sample sizes. The S100A9 upregulation should be confirmed in a larger population, with subjects that are more tightly grouped together and matched with respect to age, gravidity, and ethnicity. Additionally, the pre-gestational diabetic group did not include any patients with Type 1 diabetes, and therefore the results of this study are limited in their application to Type 1 diabetics and further studies in this population are needed. In our PGD population the date of diabetic diagnosis, and therefore duration of their disease, was not collected. As a result we were unable to analyze the data with regards to duration of disease. Further, the PGD *vs* GDM difference in S100A9 peptide count also needs to be verified in a larger population. It appears that when the data within the PGD group peptide count is considered, higher peptide count is associated with poorer diabetes control. Possibly by extending the time points of sample collection to include the 4^{th}-8^{th} week of pregnancy and 6 weeks after child-birth, this finding can be further validated. By developing a list of plausible risk-stratifiers, non-diabetic and/or non-obese pregnant women at risk for developing diabetes can potentially benefit from these studies.

Finally, we used 1d gel electrophoresis to resolve the exosome proteins prior to LC/MS-MS analysis, which resulted in identification of 1103 proteins overall. If we had conducted direct exosome protein trypsinization instead of following this method, perhaps the number of identified proteins might have increased. In our experience, exosomes cargo contains non-full length peptides as well, and has the potential to confound the analysis. By following the gel electrophoresis method, we ensured that we only compare full length protein differences between groups.

Conclusion

In this study, we summarize our initial findings from diabetic pregnancy urine exosome proteomic analyses. We used exosomes from 24 hour urine samples of diabetic pregnancy subjects obtained during the 20^{th} week of pregnancy and compared them with exosomes from non-

diabetic pregnancy subjects. Our data show major alteration to calcium-handling pathways and cellular-danger signal pathways between groups. The major finding of this study is that the DAMP protein S100A9 sorts into exosomes, and in diabetics this exosomal sorting is upregulated. The exosomal load of this protein correlates with not only maternal obesity but also macrosomia of the new born infant. Further, developmental changes from week 20 to week 36 in GDM and PGD phenotypes employ different protein pathways.

Given that maternal diabetes during human pregnancy alters the quality of life of both the mother and the newborn after childbirth, and considering the non-invasive nature of urinary exosome collection, processing and analysis of 24 hour urine samples is an attractive platform to study diabetes during pregnancy and its consequences.

Work from the Knepper group shows that many important renal proteins (e.g. aquaporins, polycystins and podocyn) are shed in the urine exosome. [25; 31] Our current report adds S100A9 to this group of functionally important proteins to be identified in urine exosomes. Our findings, together with other studies in pancreatic cancer tissue, saliva, and serum suggest that the upregulation of S100A9 DAMP signal is a common and valid biomarker of inflammatory processes and immune response. This report potentially forms the preliminary level basis for future studies wherein the pharmacologic and therapeutic potential of S100A9 and related pathways could be utilized to impart personalized care to a pregnant woman with diabetes.

Acknowledgements: The authors would like to thank Edith Esqueda for her efforts in helping to recruit patients to the study and provide the authors with clinic resources.

Conflicts of Interest: The author(s) report(s) no conflict of interest.

Funding: NIH NIDDK UAB/UCSD O'Brien Core Center for Acute Kidney Injury Research grant (NIH 1P30 DK 079337); the GSK-SSS funding for biomarkers; the UC San Diego CRCHD (NIH/NIMHD-P60 MD000220); and the Proteomics Catalyst grant instituted by UC San Diego Clinical Translational Research Institute.

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Supplementary Data

Sl. #	GI number	p-val	p-val FDR	Protein Identification
1	4506773	0.00003	0.03686	S100 calcium-binding protein A9
2	4502085	0.00074	0.23121	pancreatic amylase alpha 2A precursor
3	20070125	0.00091	0.23121	prolyl 4-hydroxylase, beta subunit precursor
4	21614544	0.00106	0.23121	S100 calcium-binding protein A8
5	77539758	0.00111	0.23121	histone cluster 2, H4b
6	24497480	0.00126	0.23121	solute carrier family 22 member 6 isoform d
7	4504299	0.00193	0.26478	histone cluster 3, H3
8	5803023	0.00222	0.26478	lectin, mannose-binding 2
9	55956899	0.00244	0.26478	keratin 9
10	13162290	0.00262	0.26478	betaine-homocysteine methyltransferase 2
11	21361176	0.00282	0.26478	aldehyde dehydrogenase 1A1
12	119964718	0.00316	0.26478	desmoglein 3 preproprotein
13	38016911	0.00321	0.26478	stomatin isoform a
14	21361392	0.00338	0.26478	pregnancy specific beta-1-glycoprotein 1
15	4506153	0.0036	0.26478	prostasin preproprotein
16	4507033	0.00423	0.28534	solute carrier family 5 (sodium/glucose cotransporter), member 2
17	5031839	0.0044	0.28534	keratin 6A
18	4809279	0.00518	0.28923	annexin VII isoform 2
19	4826762	0.00521	0.28923	Haptoglobin
20	66912162	0.00542	0.28923	histone cluster 2, H2bf
21	31652249	0.00551	0.28923	lipopolysaccharide-binding protein precursor
22	9257217	0.0065	0.32589	folate receptor 1 precursor
23	119395754	0.00718	0.34409	keratin 5
24	44680145	0.00795	0.36555	solute carrier family 23 (nucleobase transporters), member 1 isoform a
25	4557317	0.00871	0.38447	annexin A11
26	37694062	0.0105	0.42637	aquaporin 1
27	4503029	0.01078	0.42637	cellular retinoic acid binding protein 2
28	4557581	0.01082	0.42637	fatty acid binding protein 5 (psoriasis-associated)
29	4502211	0.01197	0.45516	ADP-ribosylation factor 6
30	12408656	0.01304	0.45735	calpain 1, large subunit
31	157805480	0.01339	0.45735	pregnancy specific beta-1-glycoprotein 7 precursor
32	32401453	0.01385	0.45735	solute carrier family 36 (proton/amino acid symporter), member 2
33	153945728	0.01387	0.45735	microtubule-associated protein 1B
34	4757944	0.01417	0.45735	CD81 antigen
35	32189392	0.01474	0.45735	peroxiredoxin 2 isoform a
36	98986445	0.01493	0.45735	carcinoembryonic antigen-related cell adhesion molecule 5 preproprotein
37	48762934	0.01677	0.49797	alpha 2 type I collagen
38	21614513	0.01749	0.01749	aldehyde dehydrogenase 1 family, member L1
39	4557367	0.01767	0.49797	bleomycin hydrolase
40	51468073	0.01806	0.49797	solute carrier family 6, member 19
41	4504517	0.02	0.51727	heat shock 27kDa protein 1
42	54873613	0.02026	0.51727	Agrin
43	109255249	0.02141	0.51727	keratin 4
44	50592994	0.02152	0.51727	Thioredoxin
45	116174789	0.02264	0.51727	centrosome spindle pole associated protein 1 isoform a
46	157671931	0.02268	0.51727	solute carrier family 5 (sodium/glucose cotransporter), member 12
47	87159824	0.0231	0.51727	CD151 antigen
48	116008152	0.02352	0.51727	alpha 1 type XV collagen precursor
49	134254459	0.02413	0.51727	sodium potassium chloride cotransporter 2
50	126012573	0.02441	0.51727	low density lipoprotein-related protein 2
51	116256354	0.02458	0.51727	alpha 2 type IV collagen preproprotein
52	4504349	0.02481	0.51727	beta globin
53	4504763	0.02486	0.51727	integrin alpha-V precursor
54	59850812	0.02623	0.53575	uromodulin precursor
55	7656922	0.02705	0.54245	chromatin modifying protein 2A
56	4758236	0.02946	0.5802	extracellular matrix protein 1 isoform 1 precursor
57	7019485	0.03005	0.03005	programmed cell death 6
58	18765694	0.03076	0.58264	dipeptidylpeptidase IV
59	134244281	0.03117	0.58264	melanoma-associated antigen p97 isoform 1, precursor
60	14150145	0.03211	0.59037	matrix-remodelling associated 8
61	28603818	0.03476	0.62852	hypothetical protein LOC338094
62	29788785	0.03733	0.65087	tubulin, beta
63	119964726	0.03744	0.65087	insulin-like growth factor 2 receptor precursor
64	21361254	0.03777	0.65087	deoxyribonuclease I precursor
65	4502101	0.03859	0.65487	annexin I
66	4503899	0.04423	0.73915	N-acetylgalactosamine-6-sulfatase precursor
67	39995109	0.04539	0.74722	GM2 ganglioside activator precursor
68	87239981	0.0475	0.76713	tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase
69	4826898	0.76713	0.76713	profilin 1
70	42741659	0.04951	0.77688	ATP-binding cassette sub-family B member 1

Table S1: Significantly dysregulated proteins in the GDM group by negative binomial distribution analysis

Sl. #	GI number	p-val	p-val FDR	Protein Identification
1	4502027	0.00006	0.02631	albumin precursor
2	38016911	0.00008	0.02631	stomatin isoform a
3	4506773	0.00009	0.02631	S100 calcium-binding protein A9
4	31652249	0.0001	0.02631	lipopolysaccharide-binding protein precursor
5	122937470	0.00021	0.04555	carboxypeptidase N, polypeptide 2, 83kD
6	115298678	0.00025	0.04555	complement component 3 precursor
7	4504811	0.00061	0.09676	junction plakoglobin
8	4506153	0.00104	0.1336	prostasin preproprotein
9	21264363	0.00109	0.1336	mannan-binding lectin serine protease 2 isoform 1 precursor
10	86793109	0.00156	0.16728	complement receptor 1 isoform S precursor

11	6912586	0.00169	0.16728	6-phosphogluconolactonase
12	58530840	0.00182	0.16728	desmoplakin isoform I
13	65506891	0.00216	0.17759	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2 isoform c
14	4503491	0.00225	0.17759	epidermal growth factor (beta-urogastrone)
15	126012573	0.00294	0.20392	low density lipoprotein-related protein 2
16	4502179	0.00319	0.20392	aquaporin 2
17	24497480	0.00325	0.20392	solute carrier family 22 member 6 isoform d
18	115298674	0.00345	0.20392	nidogen 1 precursor
19	21361181	0.00351	0.20392	Na+/K+ -ATPase alpha 1 subunit isoform a proprotein
20	126091152	0.0037	0.20392	cubilin
21	6382064	0.0042	0.21566	0.21566
22	154146262	0.0044	0.21566	Fc fragment of IgG binding protein
23	116256354	0.00456	0.21566	alpha 2 type IV collagen preproprotein
24	7524354	0.00469	0.21566	dimethylarginine dimethylaminohydrolase 2
25	73858570	0.00498	0.21982	complement component 1 inhibitor precursor
26	116008152	0.00579	0.23976	alpha 1 type XV collagen precursor
27	4502067	0.00604	0.23976	alpha-1-microglobulin/bikunin precursor
28	4503635	0.00609	0.23976	coagulation factor II precursor
29	31542984	0.00632	0.24053	inter-alpha (globulin) inhibitor H4
30	4504965	0.00765	0.28112	L-plastin
31	18765694	0.00829	0.29513	dipeptidylpeptidase IV
32	37694062	0.00944	0.31977	aquaporin 1
33	4758950	0.00957	0.31977	peptidylprolyl isomerase B precursor
34	153945728	0.01071	0.34747	microtubule-associated protein 1B
35	88853069	0.01115	0.35145	vitronectin precursor
36	48762934	0.01237	0.37128	alpha 2 type I collagen
37	51468073	0.01279	0.37128	solute carrier family 6, member 19
38	4502511	0.01284	0.37128	complement component 9
39	27894376	0.01313	0.37128	cell adhesion molecule with homology to L1CAM precursor
40	21264578	0.01375	0.37905	tetraspan 1
41	126012571	0.0148	0.3981	heparan sulfate proteoglycan 2
42	4506403	0.01587	0.40814	G protein-coupled receptor, family C, group 5, member A
43	11321561	0.01591	0.40814	hemopexin
44	7706451	0.01685	0.42238	G protein-coupled receptor, family C, group 5, member B precursor
45	58761548	0.01747	0.42812	tau tubulin kinase 1
46	21071030	0.01872	0.44894	alpha 1B-glycoprotein precursor
47	4507033	0.02107	0.48572	solute carrier family 5 (sodium/glucose cotransporter), member 2
48	91105159	0.02114	0.48572	CD14 antigen precursor
49	4502503	0.02162	0.48677	complement component 4 binding protein, alpha chain precursor
50	10863877	0.02247	0.48742	phospholipid scramblase 1
51	59850812	0.02297	0.48742	uromodulin precursor
52	4507377	0.02298	0.48742	serine (or cysteine) proteinase inhibitor, clade A, member 7
53	134254459	0.02377	0.49073	sodium potassium chloride cotransporter 2
54	116256333	0.02414	0.49073	membrane metallo-endopeptidase
55	7706635	0.02461	0.49073	cornulin
56	134244281	0.02491	0.49073	melanoma-associated antigen p97 isoform 1, precursor
57	124256496	0.0268	0.51852	heat shock 70kDa protein 1-like
58	53729346	0.02772	0.5246	plakophilin 1 isoform 1b
59	4503689	0.02806	0.5246	fibrinogen, alpha polypeptide isoform alpha-E preproprotein
60	4502085	0.0292	0.53519	pancreatic amylase alpha 2A precursor
61	21361176	0.0296	0.53519	aldehyde dehydrogenase 1A1
62	4502105	0.03044	0.54091	annexin IV
63	4557759	0.0309	0.54091	myeloperoxidase
64	4503549	0.03192	0.55011	elastase 2, neutrophil preproprotein
65	40217833	0.03353	0.55862	G protein-coupled receptor family C, group 5, member C isoform b
66	87196339	0.03359	0.55862	collagen, type VI, alpha 1 precursor
67	49574489	0.03434	0.55862	Na+/K+ -ATPase beta 1 subunit isoform b
68	4504763	0.03482	0.55862	integrin alpha-V precursor
69	112380628	0.03495	0.55862	lysosomal-associated membrane protein 1
70	5031857	0.03669	0.57818	lactate dehydrogenase A
71	5123454	0.03775	0.58646	heat shock 70kDa protein 1A
72	45439327	0.04147	0.04147	periplakin
73	109255249	0.04235	0.63491	keratin 4
74	4503143	0.0426	0.63491	cathepsin D preproprotein
75	4504061	0.04344	0.63892	glucosamine (N-acetyl)-6-sulfatase precursor
76	5174387	0.04407	0.63957	prominin 1
77	9665262	0.04894	0.7011	EGF-containing fibulin-like extracellular matrix protein 1 precursor

Table S2: Significantly dysregulated proteins in the PGD group by negative binomial distribution analysis

Sl. #	GI #	VIP scores	Annotations
1	4506773	7.7075	S100 calcium-binding protein A9
2	5803227	4.0166	tyrosine 3/tryptophan 5 -monooxygenase activation protein, theta polypeptide
3	65506891	3.9476	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2 isoform c
4	4504299	3.8755	histone cluster 3, H3
5	116256354	3.8346	alpha 2 type IV collagen preproprotein
6	77539758	3.709	histone cluster 2, H4b
7	4506153	3.4717	prostasin preproprotein
8	9257217	3.4696	folate receptor 1 precursor
9	38016911	3.434	stomatin isoform a
10	4505185	3.3661	macrophage migration inhibitory factor (glycosylation-inhibiting factor)
11	56243522	3.3497	syntenin isoform 1
12	4826762	3.3065	haptoglobin
13	122937470	3.2718	carboxypeptidase N, polypeptide 2, 83kD
14	7656922	3.2405	chromatin modifying protein 2A
15	55749523	3.2358	syntenin isoform 3
16	77539055	3.2276	ubiquitin and ribosomal protein L40 precursor
17	31652249	3.214	lipopolysaccharide-binding protein precursor
18	4809279	3.0934	annexin VII isoform 2
19	32189392	2.9747	peroxiredoxin 2 isoform a
20	21361181	2.948	Na+/K+ -ATPase alpha 1 subunit isoform a proprotein
21	24234699	2.9417	keratin 19
22	4504253	2.8854	H2A histone family, member X
23	21614544	2.8484	S100 calcium-binding protein A8
24	4503117	2.8454	cystatin B
25	66912162	2.7888	histone cluster 2, H2bf
26	4826898	2.7639	profilin 1
27	116008152	2.6908	alpha 1 type XV collagen precursor
28	4502211	2.6618	ADP-ribosylation factor 6
29	7019485	2.6024	programmed cell death 6
30	9966777	2.5502	resistin
31	5031857	2.5453	lactate dehydrogenase A
32	4557287	2.536	angiotensinogen preproprotein
33	21361176	2.4817	aldehyde dehydrogenase 1A1
34	7705885	2.4528	vacuolar protein sorting 28 isoform 1
35	4504349	2.4504	beta globin
36	40354205	2.4308	aldolase B
37	14150145	2.402	matrix-remodelling associated 8
38	20070125	2.3701	prolyl 4-hydroxylase, beta subunit precursor
39	155969697	2.3701	keratin 6C
40	164663836	2.3322	pregnancy specific beta-1-glycoprotein 11 isoform 1
41	119964718	2.2922	desmoglein 3 preproprotein
42	11386147	2.2884	prosaposin isoform a preproprotein
43	4504347	2.2462	alpha 1 globin
44	17921989	2.1848	tubulin, alpha 4a
45	24308440	2.1839	family with sequence similarity 125, member A
46	4885179	2.1769	defensin, alpha 3 preproprotein

47	157671931	2.1546	solute carrier family 5 (sodium/glucose cotransporter), member 12
48	37694062	2.1335	aquaporin 1
49	19913424	2.1262	ATPase, H+ transporting, lysosomal 70kD, V1 subunit A, isoform 1
50	4503029	2.1207	cellular retinoic acid binding protein 2
51	6598323	2.1154	GDP dissociation inhibitor 2
52	4885607	2.0963	small proline-rich protein 3
53	67191208	2.0877	ubiquitin C
54	89357932	2.0783	keratin 5b
55	50592994	2.0491	thioredoxin
56	4758984	2.0242	Ras-related protein Rab-11A
57	5803225	2.0225	tyrosine 3/tryptophan 5 -monooxygenase activation protein, epsilon polypeptide
58	13162290	2.0047	betaine-homocysteine methyltransferase 2
59	4757944	2.0019	CD81 antigen
60	6382064	1.9736	prostatic acid phosphatase precursor
61	21361392	1.957	pregnancy specific beta-1-glycoprotein 1
62	24497480	1.9544	solute carrier family 22 member 6 isoform d
63	32189394	1.9416	ATP synthase, H+ transporting, mitochondrial F1 complex, beta subunit precursor
64	4502101	1.939	annexin I
65	88853069	1.9077	vitronectin precursor
66	4503471	1.8901	eukaryotic translation elongation factor 1 alpha 1
67	24430144	1.8457	glutathione S-transferase A3
68	32401453	1.8451	solute carrier family 36 (proton/amino acid symporter), member 2
69	4557581	1.8396	fatty acid binding protein 5 (psoriasis-associated)
70	118582275	1.8343	superoxide dismutase 3, extracellular precursor
71	119703753	1.8246	1.8246
72	18765694	1.8066	dipeptidylpeptidase IV
73	12667788	1.7874	myosin, heavy polypeptide 9, non-muscle
74	5031809	1.7777	immunoglobulin superfamily containing leucine-rich repeat
75	49574489	1.7717	Na+/K+ -ATPase beta 1 subunit isoform b
76	8393956	1.7516	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 13
77	4507033	1.7399	solute carrier family 5 (sodium/glucose cotransporter), member 2
78	10280622	1.7383	amylase, pancreatic, alpha-2B precursor
79	4507377	1.7349	serine (or cysteine) proteinase inhibitor, clade A, member 7
80	4506979	1.7337	solute carrier family 13 (sodium-dependent dicarboxylate transporter), member 2
81	47419930	1.7058	chondroitin sulfate proteoglycan 4
82	44680145	1.6934	solute carrier family 23 (nucleobase transporters), member 1 isoform a
83	115298674	1.6917	nidogen 1 precursor
84	40068518	1.6912	phosphogluconate dehydrogenase
85	87159824	1.691	CD151 antigen
86	4885111	1.6836	calmodulin-like 3
87	4758236	1.6805	extracellular matrix protein 1 isoform 1 precursor
88	4557321	1.6767	apolipoprotein A-I preproprotein
89	4504517	1.6746	heat shock 27kDa protein 1
90	6912582	1.6657	penta-EF-hand domain containing 1
91	30089930	1.6653	N-acylsphingosine amidohydrolase (acid ceramidase) 1 isoform b
92	4502067	1.6635	alpha-1-microglobulin/bikunin precursor
93	17865802	1.6598	vacuolar protein sorting factor 4B
94	7524354	1.6502	dimethylarginine dimethylaminohydrolase 2
95	5031635	1.6369	cofilin 1 (non-muscle)
96	5803185	1.6322	synaptophysin-like 1 isoform a
97	20357529	1.6111	guanine nucleotide-binding protein, beta-2 subunit
98	4507025	1.5925	solute carrier family 4, sodium bicarbonate cotransporter, member 4 isoform 2
99	31542306	1.5884	chromatin modifying protein 1B
100	87196339	1.586	collagen, type VI, alpha 1 precursor
101	148536848	1.5748	carboxyl ester lipase precursor
102	23943854	1.5601	pepsinogen 5, group I
103	98986445	1.5597	carcinoembryonic antigen-related cell adhesion molecule 5 preproprotein
104	12408656	1.5565	calpain 1, large subunit
105	63252913	1.5509	gelsolin-like capping protein
106	29788785	1.5331	ubulin, beta
107	4506403	1.5005	G protein-coupled receptor, family C, group 5, member A

Table S3: Variable Importance in Projection Scores for proteins by spectral count, NL vs GDM

Sl. #	GI #	VIP scores	Annotations
1	4506773	4.632	S100 calcium-binding protein A9
2	4557321	4.2308	apolipoprotein A-I preproprotein
3	4506153	4.0883	prostasin preproprotein
4	6912586	3.6375	6-phosphogluconolactonase
5	38016911	3.5797	stomatin isoform a
6	4507377	3.5105	serine (or cysteine) proteinase inhibitor, clade A, member 7
7	164663836	3.4421	pregnancy specific beta-1-glycoprotein 11 isoform 1
8	7524354	3.3078	dimethylarginine dimethylaminohydrolase 2
9	33286418	3.302	pyruvate kinase, muscle isoform 1
10	116008152	3.2799	alpha 1 type XV collagen precursor
11	70906439	3.2533	fibrinogen, gamma chain isoform gamma-B precursor
12	5454052	3.1659	stratifin
13	87196339	3.1386	collagen, type VI, alpha 1 precursor
14	40217833	3.1087	G protein-coupled receptor family C, group 5, member C isoform b
15	4502067	3.0079	alpha-1-microglobulin/bikunin precursor
16	4757944	3.0035	CD81 antigen
17	5802984	2.9308	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 1
18	4504811	2.9082	junction plakoglobin
19	122937470	2.8826	carboxypeptidase N, polypeptide 2, 83kD
20	21361181	2.8526	Na+/K+ -ATPase alpha 1 subunit isoform a proprotein
21	73858570	2.8491	complement component 1 inhibitor precursor
22	115298674	2.7204	nidogen 1 precursor
23	10863877	2.6684	phospholipid scramblase 1
24	5123454	2.6459	heat shock 70kDa protein 1A
25	116256354	2.6122	alpha 2 type IV collagen preproprotein
26	18765694	2.5936	dipeptidylpeptidase IV
27	4557871	2.5471	transferrin
28	7706451	2.5324	G protein-coupled receptor, family C, group 5, member B precursor
29	24497480	2.4617	solute carrier family 22 member 6 isoform d
30	31377727	2.4543	solute carrier family 44, member 2
31	42716297	2.4472	clusterin isoform 1
32	4502179	2.4194	aquaporin 2
33	115298678	2.4178	complement component 3 precursor
34	31652249	2.4169	lipopolysaccharide-binding protein precursor
35	7706635	2.3959	cornulin
36	71773110	2.3568	apolipoprotein A-IV precursor
37	6382064	2.3309	prostatic acid phosphatase precursor
38	58530840	2.2471	desmoplakin isoform I
39	21071030	2.2143	alpha 1B-glycoprotein precursor
40	16418467	2.2057	leucine-rich alpha-2-glycoprotein 1
41	157671931	2.1969	solute carrier family 5 (sodium/glucose cotransporter), member 12
42	4507953	2.1733	tyrosine 3/tryptophan 5 -monooxygenase activation protein, zeta polypeptide
43	4503635	2.1671	coagulation factor II precursor
44	4505881	2.164	plasminogen
45	4826772	2.1637	insulin-like growth factor binding protein, acid labile subunit
46	21361392	2.1522	pregnancy specific beta-1-glycoprotein 1
47	4758950	2.1491	peptidylprolyl isomerase B precursor

48	24430192	2.1243	keratin 16
49	50659080	2.1066	serpin peptidase inhibitor, clade A, member 3 precursor
50	67782351	2.0669	complement component 4B preproprotein
51	4505591	2.0643	peroxiredoxin 1
52	4502205	2.0557	ADP-ribosylation factor 4
53	9257232	2.055	orosomucoid 1 precursor
54	13929462	2.0409	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase 1, membrane-bound form
55	5174387	2.0403	prominin 1
56	prominin 1	2.0343	vacuolar protein sorting 37D
57	19913424	2.0339	ATPase, H+ transporting, lysosomal 70kD, V1 subunit A, isoform 1
58	21264363	2.0322	mannan-binding lectin serine protease 2 isoform 1 precursor
59	21361302	2.0251	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 4
60	42740907	2.0186	clusterin isoform 2
61	21361176	1.9999	aldehyde dehydrogenase 1A1
62	34335272	1.9927	endothelial protein C receptor precursor
63	65506891	1.9858	solute carrier family 3 (activators of dibasic and neutral amino acid transport), member 2 isoform c
64	89903012	1.9843	cell division cycle 42 isoform 1
65	70906435	1.9481	fibrinogen, beta chain preproprotein
66	23943854	1.932	pepsinogen 5, group I
67	28395033	1.9184	ras homolog gene family, member C precursor
68	145701025	1.9094	multiple EGF-like-domains 8
69	5803023	1.9071	lectin, mannose-binding 2
70	61835217	1.8962	3-mercaptopyruvate sulfurtransferase
71	4504965	1.8932	L-plastin
72	16418397	1.8861	MAL2 proteolipid protein
73	4503491	1.8649	epidermal growth factor (beta-urogastrone)
74	4506403	1.8364	G protein-coupled receptor, family C, group 5, member A
75	4502403	1.8286	biglycan preproprotein
76	21389623	1.8208	prominin 2
77	51468073	1.8142	solute carrier family 6, member 19
78	7661678	1.8103	RAP1B, member of RAS oncogene family
79	5803185	1.7818	synaptophysin-like 1 isoform a
80	119703753	1.7729	keratin 6B
81	24111250	1.7602	guanine nucleotide binding protein (G protein), alpha 13
82	4502085	1.7546	pancreatic amylase alpha 2A precursor
83	5902072	1.7323	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 3
84	4502511	1.719	complement component 9
85	91105159	1.7171	CD14 antigen precursor
86	42761474	1.6983	CD59 antigen p18-20
87	40217831	1.6803	G protein-coupled receptor family C, group 5, member C isoform a
88	37694062	1.6679	aquaporin 1
89	148612887	1.6665	choline transporter-like protein 4
90	4503143	1.6648	cathepsin D preproprotein
91	31542984	1.661	inter-alpha (globulin) inhibitor H4
92	12667788	1.6575	myosin, heavy polypeptide 9, non-muscle
93	11321561	1.642	hemopexin
94	6912236	1.6322	angiopoietin-like 2 precursor
95	89357932	1.6119	keratin 5b
96	21361195	1.5958	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5
97	41152114	1.5949	aldo-keto reductase family 7, member A3
98	24308440	1.5879	family with sequence similarity 125, member A
99	4507033	1.5843	solute carrier family 5 (sodium/glucose cotransporter), member 2
100	63252913	1.5821	gelsolin-like capping protein
101	67190748	1.5632	complement component 4A preproprotein
102	4502599	1.5364	carbonyl reductase 1
103	73858564	1.5172	corticosteroid binding globulin precursor

Table S4: Variable Importance in Projection Scores for proteins by spectral count, NL vs PGD

GI #	VIP Scores	Top discriminating Proteins in the CTRL group
4885049	8.093	cardiac muscle alpha actin 1 proprotein
4501889	6.1709	actin, gamma 2 propeptide
42716297	5.9226	clusterin isoform 1
42740907	4.6241	clusterin isoform 2
14389309	3.7985	tubulin alpha 6
67782351	3.4398	complement component 4B preproprotein
153792590	3.2261	heat shock protein 90kDa alpha (cytosolic), class A member 1 isoform 1
33286422	3.1219	pyruvate kinase, muscle isoform 2
19923483	3.0449	GTPase Rab14
33695095	2.967	ras-related GTP-binding protein RAB10
GI #	**VIP Scores**	**Top discriminating Proteins in the GDM group**
4503143	4.0378	cathepsin D preproprotein
17986283	3.9943	tubulin, alpha 1a
4502317	3.8459	vacuolar H+ ATPase E1 isoform a
7706683	3.5649	Rh family, C glycoprotein
19913428	3.5516	vacuolar H+ATPase B2
4885049	3.5127	cardiac muscle alpha actin 1 proprotein
14389309	3.4618	tubulin alpha 6
21361181	3.4455	Na+/K+ -ATPase alpha 1 subunit isoform a proprotein
19913426	3.4341	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B1
11545731	3.3769	gigaxonin
GI #	**VIP Scores**	**Top discriminating Proteins in the PGD group**
21361181	4.1922	Na+/K+ -ATPase alpha 1 subunit isoform a proprotein
4885049	4.136	cardiac muscle alpha actin 1 proprotein
169171979	3.9129	PREDICTED: similar to hCG1811191
4502111	3.7935	annexin VII isoform 1
7706683	3.7318	Rh family, C glycoprotein
42741659	3.636	ATP-binding cassette sub-family B member 1
7706451	3.5819	G protein-coupled receptor, family C, group 5, member B precursor
31377727	3.4821	solute carrier family 44, member 2
21389623	3.3071	prominin 2
42740907	3.1718	clusterin isoform 2

Table S5: CTRL, GDM and PGD VIP protein differences between week 20 and week 36

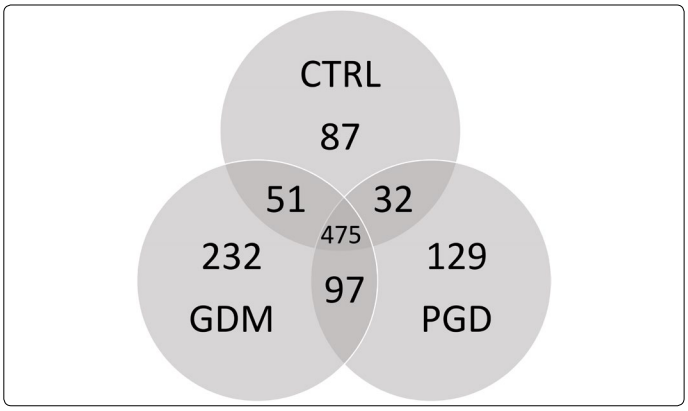

Figure S1: Venn diagram depicting the relationship of 20th week proteins for control (CTRL), gestational diabetic (GDM), and Pregestational diabetic (PGD) proteins. Only proteins present in at least 1 of the 2 replicates of every subject in the group are shown.

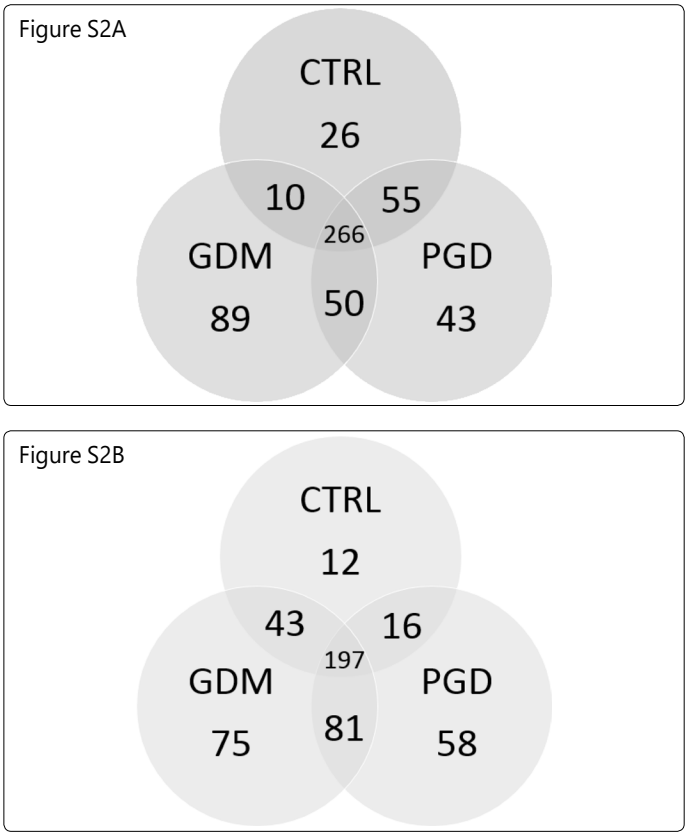

Figure S2: Venn diagram depicting the relationship of 20th week (A) and 36th week (B) proteins for control (CTRL), gestational diabetic (GDM), and Pregestational (PGD) groups.

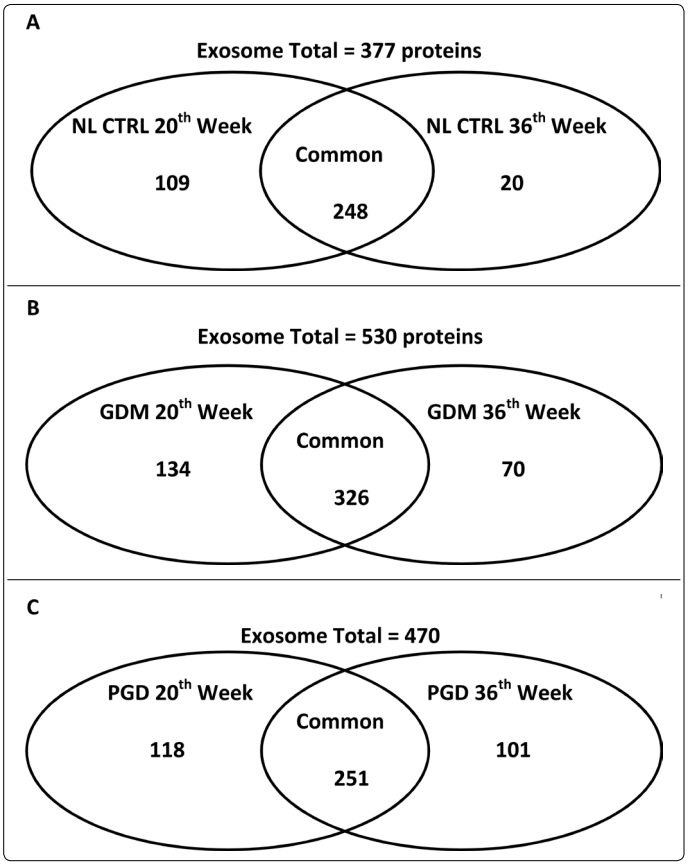

Figure S3: Groupwise 2-way split Venn diagram describing the spread of proteins between 20th and 36th week for CTRL (Panel A), GDM (Panel B) and PGD (Panel C) groups are shown. Whereas more than 5-fold reduction in the number of unique proteins identified between 20th and 36th week is observed in CTRL (109 versus 20); it is approximately half in GDM (134 versus 70), but remains almost unchanged in PGD (118 versus 101).