

Original Article

Maternal plasma protein profiles in response to oral 50-gram glucose load in mid-pregnancy: a pilot study

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Abstract: Accumulating evidence documents the initiation of diverse physiologic and biochemical response subsequent to an oral glucose load. However, significant gaps in knowledge exist in the understanding of consequences of glucose load during pregnancy, a state of insulin resistance. Using high dimensional protein arrays, we conducted a pilot proof-of-concept and feasibility study to investigate profiles of 120 plasma proteins in pre- and post- 50-gram oral glucose challenge samples. Participants (N=10) were selected from among women enrolled in a pregnancy cohort. Differences in plasma protein concentrations between pre- and post-glucose load challenge samples were evaluated using Student's T-test (paired) and mean fold change comparisons. Multiple testing adjusted p-values (i.e., false discovery rate q values) were computed using Benjamini-Hochberg (BH) corrections. Plasma haptoglobin, epidermal growth factor, hemoglobin, thrombospondin-1, and S100 protein concentrations were two to five fold higher in post-glucose load compared with pre-glucose load samples (all q -values <0.05). Among women aged >31 years (above median), post-load S100 protein was elevated 9.92-fold above pre-load concentrations, while it was elevated 4.10-fold among women aged <31 years (below median). Similarly, among women with post-load glucose concentrations <101mg/dl (below median), S100 was elevated 8.26-fold while it was elevated 3.28 fold among women with post-load glucose concentrations >101mg/dl (above median). Our study findings suggest that post-glucose load changes in plasma biomarkers represent a diverse set of cellular responses including receptor for advanced glycation end products (RAGE), inflammation, oxidative stress and adipogenesis, during mid-pregnancy. Future studies of larger populations and longer periods of follow-up are warranted.

Keywords: Plasma protein, oral glucose load, mid-pregnancy

Introduction

Abnormal glucose tolerance is a heterogeneous disorder consisting of insulin resistance, impaired insulin secretion or both. Gestational diabetes mellitus, is a condition associated with significant morbidity and mortality in the mother and her offspring [1-2]. Understanding normal and abnormal responses to glucose load will enhance characterization of underlying pathological changes that result in gestational diabetes and related complications.

Accumulating evidence from previous, primarily experimental, studies supports initiation of diverse biochemical events subsequent to glucose load [3]. These pathophysiologic events include hemodynamic, metabolic and inflamma-

tory responses. However, significant gaps in knowledge exist in the understanding of consequences of glucose challenge tests during pregnancy, itself, a state of insulin resistance, as well as potential risk factors (e.g. maternal age or body mass index) that potentially influence maternal response to glucose loading during pregnancy [4-5].

As part of antenatal care, pregnant women undergo a glucose challenge screening test (also known as a "glucola test") between 24-28 weeks of gestation for gestational diabetes screening. This presents a unique opportunity to observe maternal responses to acute glucose loading. Further, recent advances in proteomics have facilitated the development of arrays that allow simultaneous measurement of hundreds

of proteins. Using high dimensional protein arrays, we conducted a pilot proof-of-concept and feasibility study to investigate plasma protein profiles in pre- and post- oral glucose challenge plasma samples collected from participants (N=10) of a pregnancy cohort.

Materials and methods

Study participants

Study subjects were selected from participants of the Omega study, a prospective cohort study designed to investigate risk factors of pregnancy complications such as preeclampsia and gestational diabetes. Study population and data collection procedures, described before, were briefly as follows [6-7]. Participants were women who attended prenatal care clinics affiliated with Swedish Medical Center, Seattle, WA. Eligible women were those who began prenatal care before 20 weeks gestation, spoke and read English, were ≥ 18 years of age, and planned to carry the pregnancy to term and deliver at the study hospital. We randomly selected 10 participants for the Omega study cohort for inclusion in this present pilot and feasibility study. The Institutional Review Board of the Swedish Medical Center approved study protocols. All participants provided written informed consent.

Data collection

During early pregnancy, participants were asked to complete a structured interviewer administered questionnaire regarding socio-demographic characteristics, lifestyle habits, and medical and reproductive histories. Participants also provided non-fasting blood and urine samples. Plasma samples remaining after routine 50-g oral glucose challenge screening tests gestational diabetes (tests administered between 24-28 weeks) were also collected and stored for future analyses. All collected biological samples were immediately processed and stored at -80°C until further processing. Pregnancy outcome information was abstracted from hospital and clinic medical records.

Plasma protein measurement

Plasma pre- and post- glucose load protein profile changes were evaluated using the Whatman Serum Biomarker Chip (WSBC) (Whatman Inc.,

Piscataway, NJ). The WSBC was specifically designed to conduct a high throughput comparative analysis of abundance of known plasma biomarkers and address a growing need of broad molecular profiling of biological samples. It is a single antibody capture array built on the FAST[®] Slide dual pad platform. Each slide has two identical arrays of antibodies printed in triplicates. Two-color fluorescent detection permits reproducible profiling of 120 plasma proteins (**Table 1**).

Initially, arrayed slides underwent internal QC procedures and were stored desiccated at room temperature until ready for use. Plasma samples were labeled with biotin-Universal Linkage System (ULS[™]) and fluorescein-ULS[™] at 37°C using the Two-Color Labeling and Fluorescent Detection Kit (KeraFAST, Winston-Salem, NC). Labeled lysate was purified using a ULS[™]-Trap column and incubated with WSBC in an attached dual-pad incubation chamber. The slide was then placed into a FAST Frame for further processing. Images were analyzed using Imaging Research ArrayVision software (Imaging Research Inc., St. Catharines, ON). Initial pre-processing of data involved subtraction of background signal and averaging spot replicates.

Statistical analysis

Study participant characteristics were summarized using mean (standard deviation) for continuous variables and number (%) for categorical variables. Analyses of pre- and post- glucose load protein concentrations were conducted on normalized and log₂-transformed data. Differences in plasma protein concentrations between pre- and post-glucose load challenge samples were evaluated using Student's T-test (paired) and mean fold change comparisons. A volcano plot was constructed to demonstrate distribution of Student's T-test p-values (Y-axis: $-\text{Log}_{10}$ [p-value]) and fold change (X-axis: Log_2 [fold change]) results. Multiple testing adjusted p-values (also known as false discovery rate q values) were computed using Benjamini-Hochberg (BH) corrections [8].

We conducted stratified analyses that examined plasma concentrations changes of selected proteins (that showed significant differences in the larger group) pre- and post glucose load among subgroups defined by maternal age, pre-pregnancy body mass index (BMI). These analy-

Plasma protein profile changes and oral glucose load

Table 1. List of evaluated protein/biomarkers

Alpha fetoprotein	ErbB2	IL-6	Plasminogen
alpha1 anthchymotrypsin	E-Selectin	IL-7	Plasminogen Activator Inhibitor
Alpha2 macroglobulin	Estrogen Receptor	IL-8	Prostatic Acid Phosphatase
Angiogenin	Fas	Insulin	PSA (free)
Angiopoietin-2	Fas Ligand	Insulin growth factor binding protein 3	PSA (total)
Angiostatin	Ferritin	Insulin-like Growth Factor 1	PSA-ACT Complex
Apolipoprotein	Fibroblast Growth Factor -7	Interferon-gamma	RANTES
Apolipoprotein J	Fibroblast Growth Factor -basic	IP-10	S100
beta2 microglobulin	G-CSF	Kallikrein-12	Serum Albumin
Bone Sialoprotein	GM-CSF	Kallikrein-14	Sialyl Lewis X
CA125	haptoglobin	Kallikrein-5	TAG-72
CA15-3	Hemoglobin	Kallikrein-9	Tetranectin
CA19-9	Hepatocyte Growth Factor	Laminin	TGF-alpha
CA50	ICAM-1	low-density lipoprotein	TGF-beta
Carcinoembryonic antigen (G 2 specific)	IgA	MCP-1	Thrombopoietin
Carcinoembryonic antigen (G 4 specific)	IgG	MCP-2	Thrombospondin-1
Cathepsin B	IgM	MCP-3	thyroglobulin
Ceruloplasmin	IL-10	MCP-4	TIMP1
Chondroitin Sulfate	IL-12p40	M-CSF	TIMP2
Chorionic gonadotropin-alpha	IL12-p70	MIP-1alpha	TNF-alpha
Chorionic Gonadotropin-beta	IL-13	MMP-2	TNF-beta
Chromogranin	IL-17	MMP-3	Transferrin
Collagen Type 1	IL-1alpha	MMP-9	Tumor-Associated Trypsin Inhibitor
complement c4	IL1-beta	Myeloperoxidase	Tyrosinase
C-reactive protein	IL-2	Myoglobin	Urokinase Plasminogen Activator
Cyclin-dependent Kinase Inhibitor 2A	IL-2 receptor-alpha	Neuron Specific Enolase	VCAM-1
Cytokeratin Fragment 21-1 (CYFRA21-1)	IL-2 receptor-beta	Osteopontin	VE-Cadherin
Eotaxin	IL-3	PDGF (all isoforms)	VEGF
Epidermal Growth Factor	IL-4	PDGF (BB isoform only)	VEGF-D
Epidermal Growth Factor Receptor	IL-5	Placental Alkaline Phosphatase	Von Willebrand Factor

ses were conducted to evaluate differences across sub-groups characterized by potentially different risk status. In these analyses, participants were categorized into two groups based on medians of maternal age (31 years), pre-pregnancy BMI (21.4 kg/m²), and post-load glucose measurements (101 mg/dl). Student's paired T-test and fold change comparisons were repeated for each group. All statistical analyses were conducted using Microsoft Excel (Microsoft, Redmond, WA) and STATA, Version 11 (STATA, College Station, TX).

Results

The mean age of participants was 33.2 years (**Table 2**). The majority of participants were nulliparous, white and married. Volcano plot depicting results of Student's paired T-test and fold change comparisons of pre- and post-glucose load protein (N=120) concentrations indicate significant signal in our data as there were more proteins with extreme p-values than would have been expected by chance (**Figure 1**). For example, there were 11 proteins with -

Plasma protein profile changes and oral glucose load

Table 2. Selected participant characteristics

Characteristics	Number (%)
	(N=10)
Maternal age, years*	33.2 (6.1)
20-34	7 (70%)
35+	3 (30%)
Nulliparous	9 (90%)
Non-Hispanic White	8 (80%)
Married	7 (70%)
GA at glucola screening, weeks*	27.3 (1.6)
Family history hypertension	5 (50%)
Family history of diabetes	3 (30%)
Gestational diabetes	0 (0%)
Preeclampsia	2 (20%)
Smoked during pregnancy	1 (10%)
Pre-pregnancy BMI, kg/m ² *	24.5 (8.0)
< 25	7 (70%)
25-29	1 (10%)
30+	2 (20%)

Abbreviations: GA: gestational age, BMI: body mass index; *Mean (standard deviation), otherwise number (%)

Log₁₀ [p-value] more extreme than 2.079 (-Log₁₀ [1/120]), when we expected only 1 corresponding to a conservative false discovery rate of 1/120.

Pre- and post- glucose load differences in concentrations of selected proteins are shown in **Table 3**. Post-glucose load concentrations of these proteins were statistically different from pre-glucose load concentrations before multiple testing adjustments (unadjusted p-values <0.05). Of these, differences in haptoglobin, epidermal growth factor, hemoglobin, thrombospondin-1, and S100 were significant after BH-based q-value corrections for multiple testing adjustments. Concentrations of these proteins were approximately 2-fold higher among post-challenge samples, except for S100 protein which was elevated more than 5-fold.

In stratified analyses (**Table 4**), we observed that post/pre glucose load changes in S100 protein concentrations were different among subgroups defined by above-below median values for maternal age (< 31 vs. ≥31 years) or post-load plasma glucose concentrations (< 101 vs. ≥101 mg/dl). Among women aged >31 years, S100 was elevated 9.92-fold while it was elevated 4.10-fold among women aged <31 years. Similarly, among women with post-load glucose concentrations <101mg/dl, S100 was elevated 8.26-fold while it was elevated 3.28 fold among women with post-load glucose concentrations >101mg/dl.

Discussion

In this pilot study conducted among pregnant women in mid-gestation, we found that plasma haptoglobin, epidermal growth factor, hemoglobin, thrombospondin-1, and S100 protein concentrations were two to five fold higher post-glucose load compared with pre-glucose load concentrations. Maternal age and post-load glucose levels appeared to modify changes in S100 protein concentrations.

Investigators have previously described significant metabolic changes that occur after an oral glucose challenge [9-10]. These changes comprise a complex biochemical and physiologic processes involving multiple organs, tissues and systems [9-10]. However, few previous studies have investigated proteomic changes in relation to blood glucose levels in pregnant women [11]. Graca et al demonstrated higher glucose concentrations and lower concentrations of acetate, formate, creatinine and glycerophosphocholine in second trimester amniotic fluid among women who later developed gestational diabetes [12]. Other investigators have noted that placental peptides in maternal serum and amniotic fluid blunt effects of insulin on glucose and possibly play significant roles in development of gestational diabetes and its complications [13]. Much remains unexplained in this area of research given the profound changes expected with glucose load and the unique metabolic and physiologic background of pregnancy.

In our study, we found changes in concentrations of a number of proteins that represent the diverse cellular and likely molecular response to a 50-g oral glucose load. S100, a pro-

Plasma protein profile changes and oral glucose load

Table 3. Proteins with significant changes in pre and post glucose load comparisons

Proteins	Fold Change*	Students' T-test p-value	BH Corrected p-value (q value)
Haptoglobin	2.09	2.07×10^{-6}	2.49×10^{-4}
Epidermal growth factor	1.99	3.83×10^{-6}	2.30×10^{-4}
Hemoglobin	2.18	4.85×10^{-6}	1.94×10^{-4}
Thrombospondin-1	2.01	6.85×10^{-5}	2.06×10^{-3}
S100	5.84	1.70×10^{-3}	4.08×10^{-2}
Low-density lipoprotein	-1.15	2.76×10^{-3}	5.51×10^{-2}
PDGF (BB isoform only)	-1.15	3.78×10^{-3}	6.49×10^{-2}
TAG-72	-1.93	3.98×10^{-3}	5.98×10^{-2}
Angiotensin-1	-1.17	4.64×10^{-3}	6.19×10^{-2}
Estrogen Receptor	-1.26	6.00×10^{-3}	7.18×10^{-2}
Tetranectin	-1.23	7.37×10^{-3}	8.04×10^{-2}
IL-17	-1.13	8.96×10^{-3}	8.96×10^{-2}

*Fold change comparing post/pre load samples

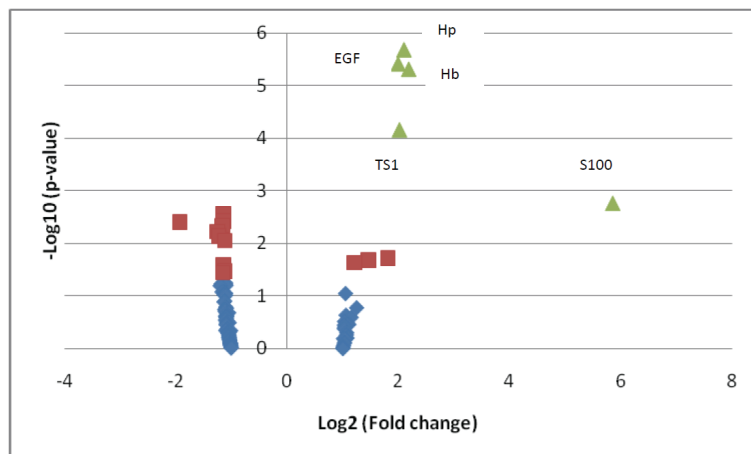


Figure 1. Volcano Plot. Distribution of Students' T-test p-value (Y-axis: $-\text{Log}_{10}$ [p-value]) and fold change (X-axis: Log_2 [absolute fold change]) results comparing pre- and post-glucose load protein concentrations. The volcano plot indicates signal in the data as there were more proteins with extreme p-values than would have been expected by chance. For example, there were 11 proteins with $-\text{Log}_{10}$ [p-value] more extreme than 2.079 ($-\text{Log}_{10}$ [1/120]), when we expected only 1 corresponding to a conservative false discovery rate of 1/120. Green proteins are proteins Students T-test p-values < 0.05 after BH-false discovery rate correction. Purple proteins are proteins with uncorrected t-test p-values < 0.05. Abbreviations: hp=haptoglobin, EGF=epidermal growth factor, hb=hemoglobin, TS1=thrombospondin-1.

inflammatory member of the calgranulin family (a calcium binding protein), has been identified to be a ligand of the receptor for advance glycation end products (RAGE) [14-16]. RAGE is a pattern recognition receptor that binds to multi-

ple ligands influencing activation of biochemical cascades that initiate and stimulate chronic stress pathways and repair [15-16]. RAGE activation and regulation have been associated with various chronic disorders including diabetes, vascular diseases, cancer and neurodegenerative diseases, possibly through mechanisms involving inflammatory response [15-16]. The RAGE pathway interacts with cytokine-, lipopolysaccharide-, oxidized LDL- and glucose-triggered cellular reactions to sustain a prolonged cellular inflammatory response driven by activation of the proinflammatory transcription factor, $\text{NF}\kappa\text{-}\beta$ [16]. In addition, S100 protein, similar to other RAGE ligands, may bind to non-RAGE receptors or act via receptor-independent mechanisms [16-17]. Our finding suggests a role for S100 protein in the relationship between glucose homeostasis and RAGE related inflammatory pathophysiological mechanisms in pregnancy.

Both hemoglobin and haptoglobin/haptoglobin were elevated in post-glucose load plasma in our study. Hemoglobin is an iron-containing oxygen-transporting metalloprotein,

Plasma protein profile changes and oral glucose load

Table 4. Proteins with significant changes in pre and post glucose load comparisons

Proteins	Maternal age (years)		Pre-pregnancy BMI (kg/m ²)		Blood glucose (mg/dl)	
	≤31	>31	<21.4	>21.4	<101	>101
Haptoglobin	1.92 (0.000)	2.37 (0.005)	2.02 (0.001)	2.17 (0.003)	2.05 (0.005)	1.90 (0.014)
Epidermal growth factor	1.90 (0.000)	2.12 (0.015)	1.92 (0.000)	2.06 (0.006)	1.94 (0.004)	1.82 (0.009)
Hemoglobin	2.04 (0.001)	2.40 (0.007)	1.96 (0.003)	2.42 (0.002)	2.02 (0.020)	2.08 (0.007)
Thrombospondin-1	2.00 (0.001)	2.03** (0.057)	2.04 (0.002)	1.98 (0.021)	1.95 (0.001)	1.84 (0.038)
S100	4.10** (0.067)	9.92 (0.008)	6.41 (0.021)	5.32** (0.071)	8.26 (0.048)	3.68** (0.179)

*Fold change comparing post/pre glucose load samples (Students' T-test p-values). Cut offs are based on medians of distributions. **Not statistically significant

while haptoglobin, a plasma protein, binds to hemoglobin to form a stable complex that facilitates heme iron recycling. Free hemoglobin, which catalyzes the generation of reactive oxygen species, and haptoglobin that binds to it, play key roles in oxidative stress and related complications [18-19]. Reactive oxygen species promote endothelial activation, inflammation, and eventual endothelial dysfunction, events that have been closely linked to pregnancy related disorders including gestational diabetes [18, 20-21]. Member of our team recently reported that early pregnancy maternal urinary 8-hydroxydeoxyguanosine (8-OHdG) concentrations, a biomarker of systemic oxidative DNA damage and repair, were associated with increased GDM risk later in pregnancy [22]. Additionally, we and others have recently reported that maternal diets high in heme iron are positively associated with GDM risk [23-24]. These emerging findings, coupled with our present findings of elevated concentrations of both hemoglobin and haptoglobin, suggest that oxidative stress (both chronic and acute oxidative stress) is likely to be an important pathophysiological process involved in the etiology of gestational diabetes.

Thrombospondin-1, a protein that plays a role in platelet aggregation, angiogenesis and tumorigenesis, is an adipokine that has been correlated with adipose tissue inflammation [25]. Previous reports from lab-based experiments indicate that both glucose and insulin modify thrombospondin-1 expression in primary adipocytes [26]. These reports, along

with our findings, support a potential role for thrombospondin-1 in adipocyte-related glucose homeostasis and potential complications during pregnancy.

Epidermal growth factor is an insulin-like growth factor that plays a role in cell growth, proliferation, and differentiation [27]. It is involved in glucose absorption, glucose storage and glucose transport [28]. Investigators have related epidermal growth factor to a number of diabetic complications including diabetic kidney disease [28]. In addition, in pregnancy, epidermal growth factor has been implicated in diabetes related macrosomia [29]. Thus, based on our findings, epidermal growth factor may represent yet another mechanism by which glucose load influences maternal physiology, pregnancy course and/or outcome.

Investigators have put forth thesis supporting presence of individual differences in the type and extent of physiologic or pathophysiologic cascades that are initiated post-glucose load, even when the ultimate objective was achieving glucose homeostasis [9]. These differences could potentially be related to other metabolic characteristics including participants' age and adiposity. For instance, the response that follows interaction of a ligand with RAGE is known to depend on the ligand, the environment or developmental stage of study participants [15-16]. In our study, we found potential evidence for maternal age-related differences in changes S-100 protein concentration in response to the 50-gram oral glucose load. Considerations of

these characteristics may strengthen predictive and diagnostic models for gestational diabetes or other glucose related complications of pregnancy [12].

Some limitations of our pilot study deserve mention. Participants of our study included 2 subjects who developed preeclampsia. We conducted sensitivity analyses excluding the 2 affected participants and found comparable results. We had limited statistical power to detect more subtle biochemical changes in response to the glucose challenge. Consequently, we may have missed subtle, though important changes in protein profiles and our findings may be susceptible to a common problem in proteomics studies which is failure of pattern reproducibility in the face of experimental and individual variations [11]. Larger, more highly statistically powered studies, with allowances for replication in independent populations, will address these limitations. Finally, our findings relate only to acute responses to glucose load and we were not able to examine chronic responses.

In summary, we found that plasma biomarkers that represent a diverse set of cellular responses including RAGE, inflammation, oxidative stress and adipogenesis were dysregulated post-glucose load during mid-pregnancy. Such investigations have the potential to aid in classification of metabolic states, revealing new pathways, and potentially improving sensitivity and specificity for detection of abnormalities. Future studies that involve larger study populations and longer periods of follow-up are warranted.

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Plasma protein profile changes and oral glucose load

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