Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Magee-Womens Research Institute and Foundation

2. **Reporting Period (start and end date of grant award period):** 1/1/2012-12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Cheryl Richards, MBA

4. **Grant Contact Person's Telephone Number:** (412) 641-8932

5. **Grant SAP Number:** 4100057668

6. **Project Number and Title of Research Project:** Project 2: Glycocalyx Syndecan-1 and Preeclampsia Pathogenesis

7. **Start and End Date of Research Project:** 1/1/2012-12/31/2012

8. **Name of Principal Investigator for the Research Project:** Carl Hubel, Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ $162,465.64

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Hubel	Associate Professor	30%	$30,750.82
Snyder	Research Associate (Technician)	100%	$29,966.40

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Weissgerber	MWRI Post-doctoral fellow (now Assistant Professor at Mayo Clinic, Rochester, MN)	2%
Rajakumar	Instructor in Medicine (Harvard University)	2%
McGonigal	Research Associate (Technician)	2%
Klemencic	High School Student; 2012 MWRI Summer Research Intern (4 weeks)	100%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes_____ No____x_____

If yes, please indicate the source and amount of other funds:

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes___x_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Pregnancy Adaptation and Maternal Cardiovascular Health 2 P01 HD030367-18	☒NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	May 2012	$4,999,981 Direct Costs $7,881,787 Total Costs	$0
Glycocalyx in the Preeclampsia Syndrome 1 R21 HD077388	☒NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	October 2012	$275,000 Direct Costs $431,662 Total Costs	$0

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes___x_____ No_____

If yes, please describe your plans:

A. Preeclampsia Program Project competitive renewal

The Program Project P01 competitive renewal submitted May 2012, *"Pregnancy Adaptation and Maternal Cardiovascular Health"* (2 P01 HD030367-19) is composed of 4 projects, one headed by Susan J. Fisher of UC San Francisco, and 3 Pittsburgh, Magee-Womens Research Institute-based projects (Hubel, CA; Gandley, RE; Powers, RW), all supported by MWRI-based Administrative (A) and Clinical Core (B) units. The P01 is a combined clinical and animal model project of 5-year duration to more deeply understand the relationships of metabolic and vascular dysfunction during and after pregnancy. It emphasizes identifying placental and maternal maladaptations of pregnancy that unmask latent cardiovascular disease risk in the mother. This theme is concordant with the 2011 NICHD "Vision Workshop on Pregnancy and Pregnancy Outcomes", which emphasized that pregnancy is a

"stress test", a window into future CVD risk.

Data generated by Dr. Hubel's Research Project 2 of the Commonwealth Health Research Grant supported a major emphasis of this Program Project competitive renewal currently under NICHD SEP review. An hypothesis that P01 sub-projects II (Hubel), III (Gandley) and IV (Powers) will test is that glycocalyx (GCX) damage/*dysfunction contributes to manifestations of preeclampsia and thus is a potential target for therapy*. The endothelial GCX is an orchestrator of vascular homeostasis, protecting the vascular wall against disease. The placental syncytiotrophoblast, *in contact with maternal blood*, also has a GCX. It is proposed that the GCX is a critical factor in vascular and placental dysfunction leading to preeclampsia and CVD. *Syndecan-1 (SDC-1),* a major heparan sulfate proteoglycan (HSPG) of GCX, is highly expressed on the surface of villous syncytiotrophoblasts. *We find that syncytiotrophoblast SDC-1 is reduced in preeclampsia (Health Research project data presented in sub-project III of the Program Project). Maternal plasma concentrations of soluble SDC-1 are concomitantly reduced in preeclampsia, and reduced weeks before preeclampsia develops compared to gestational age-matched controls (data described in Hubel sub-project II, P01 Progress Report). These data suggest a role of SDC-1 dysregulation in pathogenesis.* Further, P01 sub-project IV (Powers) will determine in a mouse model if excess asymmetric dimethylargine (ADMA), a circulating inhibitor of nitric oxide synthase implicated in preeclampsia, disrupts the GCX.

We received the NIH Review Summary Statement (NICHD SEP, ZHD1 DRG-H (40)) on 11/30/2012, with an overall Impact Score of 23 and Percentile Score of 12. The bottom line (Review Panel Summary of Discussion): *"Although the individual projects did have some minor weaknesses identified by the committee, the panel was strongly enthusiastic for this program project application, and expressed confidence that it would have a strong and lasting impact on our understanding of the relationships between pregnancy, preeclampsia and cardiovascular risk in women.*" We resubmitted the P01 on January 25, 2013 (2 P01 HD030367-19 (A1)). All of the identified areas for improvement were addressed. If the amended application is funded the earliest start date will be December 2013. We have received a 1-year bridge-funding award (04/01/2013-03/31-2014) of $180,000 from the University of Pittsburgh School of Medicine to promote our chances of future NIH funding.

B. Other plans for spin-off NIH funding from this Health Research Formula Fund project.

Dr. Robin Gandley (Co-I on the Commonwealth Health Research Formula Fund project) submitted an NIH R21, with Dr. Hubel as Co-I, *"Glycocalyx in the Preeclampsia Syndrome"*. It used data generated in this Health Research Formula Fund project implicating dysfunctional endothelial GCX in the pathogenesis of preeclampsia. The R21 project will use noninvasive, side-stream darkfield imaging to quantify GCX dimensions in the sublingual microcirculation of pregnant women, (Aim 1). It tests the hypothesis that disruption and shedding of GCX leads to endothelial dysfunction, proteinuria and increased severity of preeclampsia. It will be determined whether the sublingual GCX measurements correlate with *ex vivo* assessments of GCX in the maternal vasculature (subcutaneous fat), placental syncytiotrophoblasts, vessels in the placental villi, and the umbilical vein (Aim 2), and if components of shed GCX in the circulation or urine of pregnant women change with

preeclampsia (Aim 2). The hypothesis will be tested that reduced GCX depth reflects increasing severity of preeclampsia (Aim 3). This R21 underwent first merit review on February 2013 (percentile score 34).

Dr. Hubel plans to submit an R21, "Trophoblast glycocalyx syndecan-1 and preeclampsia", capitalizing on data from the Commonwealth Formula Fund project (June or October 2013).

The R21 projects and a mouse model of glycocalyx dysfunction, will be integrated as an R01 submission (Hubel and Gandley Co-PIs; Robert W. Powers Co-I)) for October 2013.

12. Future of Research Project. What are the future plans for this research project?

The funding plans for the research project are described in 11(B) above.

1. Submission/publication of Manuscript 1:
The following article/manuscript will be submitted for peer-review in April 2013: *"Intravenous Heparin Reveals a Vascular Store of Releasable sFlt-1 and Placental Growth Factor in Non-pregnant Women of Reproductive Age".* Data regarding the release of sFlt-1 from whole blood *in vitro* by exogenous heparin (presented in Section 17, below) were generated from the Health Research project during the funding interval. Other data were presented in the original Health Research Project Strategic Plan. The research was supported by NIH P01HD030367 (Hubel CA), UL1RR024153 and UL1TR000005 (University of Pittsburgh Clinical and Translational Science Institute), and the Pennsylvania Health Research Formula Fund (Hubel CA).

2. Completion of experiments for Manuscript 2 (in progress):
*Gandley RE, McGonigal S, Myerski A, Powers RW, Jeyabalan A, *Hubel, CA (*corresponding author). Reduced soluble syndecan-1 concentration in maternal circulation precedes preeclampsia and reflects altered placental syndecan-1 kinetics.*
Here it is shown that 1) that maternal plasma concentrations of shed (soluble) syndecan-1 (s)SDC-1 rise significantly by 7-11 weeks of normal pregnancy, and reach ~25-fold higher levels during the 3rd trimester compared to 8 weeks post-partum (and 1 year post-partum) levels, implicating the placenta as the major source of circulating sSDC-1 during pregnancy, 2) compared to uncomplicated pregnancies, preeclampsia is characterized by reduced expression of SDC-1 on the apical surface (glycocalyx) of placental syncytiotrophoblast, and 3) on average, women who develop preeclampsia have lower levels of (shed) sSDC-1 in maternal plasma both at 20 weeks' gestation (*before clinically evident disease*) and 3rd trimester (during disease), especially in preeclampsia with fetal growth restriction. Syncytiotrophoblast SDC-1 expression level correlates with sSDC-1 in maternal plasma from the same patients indicating that circulating sSDC-1 directly reflects reduced SDC-1 on placental syncytiotrophoblast surface.

Work to be completed for manuscript 2: ELISA will be used to complete the measurements of sSDC-1, sFlt1, and sGPC1 in maternal plasma. Syncytiotrophoblast expression levels have been measured in placenta biopsies from 12 normal pregnant and 9 preeclampsia patients. A current weakness is the significantly earlier mean gestational age at delivery of the preeclampsia group, which could confound results as suggested by the normal gestational increase in plasma sSDC-1 with advancing gestation. A second control group will be added to address this, characterized by preterm delivery in otherwise uncomplicated pregnancies (in the absence of infection); we have

identified 8 preterm control and 8 additional preeclampsia cases (all non-smokers). The new cases and preterm controls have been group matched based on gestational age at delivery and parity. Also, comparisons of normotensive, small for gestational age (SGA/IUGR) versus gestational age-matched control groups will be completed. Immunohistochemical and mRNA analyses (SDC-1 and GPC1 (glypican-1)) will be performed on the placental tissue. We will complete the analysis of the relationship of maternal plasma sSDC-1 and placental SDC-1 immunostaining score with clinical parameters and fetal (cord) and maternal plasma triglycerides and cholesterol. The work is targeted for manuscript submission by November 2013. An abstract will be submitted (October) for the 2014 Annual Society for Gynecologic Investigation Meeting.

3. Completion of experiments for Manuscript 3 (in progress):
The in vitro experiments will show involvement of trophoblast glycocalyx HSPGs (particularly SDC-1) in placental transport of triglyceride-rich lipoproteins. The approach to show that siRNA knockdown of cell surface SDC-1 reduces VLDL binding and internalization will be modified by use of SDC-1 siRNA from Dharmacon (SMARTpool: Accell SDC1 siRNA) with negative controls, transfection agent, and nuclease free water. The work will be submitted in abstract form (October 2013) for the 2014 Annual Society for Gynecologic Investigation Meeting.

4. The reasons why women with a history of preeclampsia are at increased risk of future cardiovascular disease (CVD) remain poorly understood. Although preeclampsia itself may have lasting adverse effects, we believe that preeclampsia is primarily a harbinger/surrogate marker of heightened CVD risk. In any case, there are significant gaps in our understanding of deficiencies in vascular function that may exist in women after a preeclamptic pregnancy. For our next (2013) Commonwealth Health Research Project we will, as a logical extension, test whether maternal microvascular GCX damage correlates with peripheral arterial endothelial dysfunction and atherogenic lipids in women with prior preeclampsia compared to women with prior normal pregnancy, in the non-pregnant state 1 year after pregnancy (n=18 per group). In collaboration with researchers at the University of Pittsburgh Center for Ultrasound Molecular Imaging and Therapeutics, we will use non-invasive sidestream dark field (SDF) imaging to enable real-time intravital observation of the sublingual microcirculation. We will use SDF to quantify sublingual capillary density and capillary/venular glycocalyx (GCX) width/integrity and examine the relationship of these variables to measures of peripheral vascular function. This project will advance our understanding of the pathogenesis of preeclampsia and its link to future CVD, and may lead to therapeutic avenues.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes____x_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male	1			
Female				1
Unknown				
Total	**1**			**1**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic	1			1
Unknown				
Total	**1**			**1**

	Undergraduate	Masters	Pre-doc	Post-doc
White	1			1
Black				
Asian				
Other				
Unknown				
Total	**1**			**1**

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No____x_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes_____ No_____x_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes____x_____ No_____

If yes, please describe the collaborations:

Augustine Rajakumar PhD, Instructor of Medicine, Harvard University, Boston, MA, advised/assisted with syncecan-1 Western Blot and Western blot data analyses.

Tracey L. Weissgerber, PhD, Assistant Professor, Mayo Clinic, Rochester, MN (formerly post-doctoral fellow, MWRI), assisted with data analyses and writing of manuscript 1.

Mark Klemencic, a high school student from Mt. Lebanon High School, participated in the Magee Womens Research Institute High School Internship program during 4 weeks of the summer of 2012. Under supervision of Dr. Hubel he advanced the Health Research Project and presented his research, "*Mechanisms of VLDL Binding and Uptake by Trophoblasts"*, to MWRI faculty, staff and community invitees, during the High School Presentation day.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No___x_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No____x_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending

publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

I. OVERVIEW:

The glycocalyx (GCX) is a negatively charged, hydrated matrix of proteoglycans and glycoproteins that forms a coating on the external (apical) surface of epithelial cells and is linked to the plasma membrane. Proteoglycans of the GCX consist of core proteins (including transmembrane syndecans) and glycosaminoglycan side chains covalently bound to these core proteins. Heparan sulfate proteoglycans (HSPGs), named after the attached heparin sulfate glycosaminoglycan chains, are the most abundant proteoglycans in the GCX. The vascular endothelial GCX, in direct contact with blood, has become a major focus in understanding mechanisms of cardiovascular disease (CVD). The GCX mechanically transmits shear forces to the endothelium to regulate nitric oxide (NO) release. The GCX is a key regulator of vascular permeability, coagulation, and inflammation. The GCX is a major reservoir for extracellular enzymes, cofactors, and growth factors, and it influences their bioavailability by local electrostatic binding to the GCX HSPGs. Intravenous heparin administration can displace these proteins from GCX HSPGs into the circulation, revealing substantial sequestering by the GCX.

Evidence has accrued regarding the fragility of the GCX. States in which GCX damage is observed and implicated in pathogenesis include reperfusion injury, inflammation (sepsis, surgical trauma), atherosclerosis and diabetes. Damage of the glomerular endothelial GCX is linked to proteinuria in several non-pregnancy diseases, and endothelial GCX damage impairs NO-mediated relaxation responsiveness in the peripheral microcirculation. Sequelae of endothelial GCX damage/dysfunction include proteinuria, edema, inflammation, platelet hyperaggregation, hypercoagulation, and loss of vasodilator responsiveness, which notably are among the major manifestations of preeclampsia. Activation of GCX degrading enzymes (hyaluronidase and heparanase) as a result of hypertriglyceridemia and free fatty acids in diabetes may reduce GCX synthesis or cause chronic GCX injury. The apical surface of human placental syncytiotrophoblast, in contact with maternal blood, normally has a robust GCX.

Syndecan-1 (SDC-1) is a major cell surface HSPG involved in regulation of cell behavior. SDC-1 shedding from the GCX into the circulation is induced by acute inflammation/sepsis and other pathological conditions. As a major glycocalyx HSPG, SDC-1 has many critical functions that could be disrupted and contribute to disease progression. These include (1) regulating the activity and availability of angiogenic growth factors through tethering to heparin-binding domains, and (2) as the primary HSPG mediating hepatic and intestinal clearance of triglyceride rich lipoproteins (VLDL and lipoprotein remnants). The abundant expression of SDC-1 on microvilli of hepatocyte basal membranes positions SDC-1 to capture triglyceride-rich lipoproteins in an LDL receptor–independent and LDL-receptor related protein–independent fashion. Through similar properties, we propose that the SDC-1 on syncytiotrophoblast is vital to maternal-fetal communication and fetal nutrition.

II. RESEARCH COMPLETED:

II A. HYPOTHESES AND SPECIFIC AIMS:

Hypothesis 1. Maternal plasma concentrations of soluble SDC-1 (sSDC-1) and glypican-1 (sGPC1) are reduced both early in pregnancy (before clinical disease) and during preeclampsia compared to women whose pregnancies remain uncomplicated; the reduced plasma sSDC-1 and sGPC1 is explained by reduced expression of these glycoproteins on syncytiotrophoblast of the placenta.
Specific Aim 1a: Plasma concentrations of sSDC-1 and sGPC1 will be compared using archived maternal plasma samples obtained both prior to preeclampsia and predelivery, with matched controls from our Program Project longitudinal study. We will look for correlation of sSDC-1 and sGPC1 with soluble VEGF receptor-1 (sFlt1, an anti-angiogenic decoy receptor implicated in the pathogenesis of preeclampsia) in the same patients.
Specific Aim 1b: Villous placenta from preeclampsia and control pregnancies will be compared with regard to (a) syncytiotrophoblast cell surface immunoreactivity for SDC-1 and GPC1 and (b) protein and mRNA levels of SDC-1 and GPC1. Correlation of placenta and plasma variables will be examined.
Specific Aim 1c: Test whether quantities of sSDC-1 and sGPC1 secreted by villous placenta in explant (*in vitro*) culture differ under normoxic, hypoxic and hyperoxic conditions.

Hypothesis 2: Local retention/binding of the placenta-secreted, soluble sFlt1 is mediated by heparan sulfate components of cell surface SDC-1 on trophoblast in culture.
Specific Aim 2: To show that trophoblasts sequester substantial sFlt1 on the cell surface, and that the quantity of releasable sFlt1 from trophoblast cell surface is reduced after specific inhibition of cell surface expression of SDC-1 or by heparanase pre-treatment.

Hypothesis 3: SDC-1 mediates the binding and internalization of very low density lipoprotein (VLDL) in human trophoblasts, suggesting a role for SDC-1 in placental lipid trafficking.
Specific Aim 3a: To show that trophoblast in culture bind and internalize VLDL.
Specific Aim 3b: To examine whether siRNA knockdown of cell surface SDC-1 or heparanase pre-treatment reduces VLDL binding and internalization.

II B. METHODS AND RESULTS ACCORDING TO EACH SPECIFIC AIM.

Aim 1 General Methods—Study Subjects and Sample processing:

All participants provided written informed consent for use of their samples and de-identified clinical data under the umbrella of the Preeclampsia Program Project (PEPP study; National Institutes of Health P01 HD030367) and related ancillary studies by the same investigators. Pregnancy plasma samples were from the PEPP Project longitudinal and cross-sectional studies of women enrolled at their first prenatal visit and followed through the immediate postpartum period. These women delivered at Magee-Womens Hospital within the years 1997–2007, and were recruited from clinics and private practices (University of Pittsburgh IRB #0404159).

Non-pregnant women with prior pregnancy delivered at Magee-Womens Hospital between 1999 and 2007, were studied 6-24 months post-partum, and were not lactating. These women were PEPP participants during their pregnancy. The University of Pittsburgh Institutional Review Board (IRB) approved the postpartum study (IRB #0404185), and all subjects provided written informed consent prior to participating. Some of these women were also part of a previous study comparing myeloperoxidase concentration in the circulation, before and after intravenous heparin administration (*Gandley RE, et al. Increased myeloperoxidase in the placenta and circulation of women with preeclampsia. Hypertension. 2008; 52:387-93*) and a study in which plasma sFlt-1 and VEGF concentrations prior to heparin administration were compared (*Wolf M, et al. Preeclampsia and future cardiovascular disease: potential role of altered angiogenesis and insulin resistance. J Clin Endocrinol Metab. 2004; 89:6239-43*).

A jury of clinicians met monthly to determine pregnancy diagnosis of all the women. Criteria for gestational hypertension were an absolute blood pressure of > 140 mmHg systolic and/or > 90 mmHg diastolic. Proteinuria was defined as > 300 mg of protein in a 24-hour urine collection, or > 2+ on a voided or > 1+ on a catheterized random urine sample, or a random urine protein/creatinine ratio of > 0.3. Hyperuricemia was defined as > 1 standard deviation above normal for the given gestational age [at term > 5.5 mg/dL (3.3 mM)].

Preeclampsia is defined as: (1) new onset hypertension arising after 20 weeks' gestation, plus (2) manifesting proteinuria, plus (3) manifesting hyperuricemia, with reversal of hypertension and proteinuria postpartum. Gestational hypertensives met the blood pressure criteria but lacked significant proteinuria and hyperuricemia. Uncomplicated pregnancy controls were normotensive, without proteinuria or hyperuricemia throughout gestation, and delivered healthy babies at term, or preterm (to control for gestational age at delivery) in the absence of infection. All participants were without illicit drug use, and had no history of renal or vascular disease.

Maternal and umbilical cord (fetal) nonfasting plasma samples were collected into 10 mL sterile tubes containing 4 mmol/L potassium-ethylenediaminetetraacetic acid (EDTA) and processed within 2 hours of collection. The plasma was stored in aliquots at -80°C without thaw until further analysis.

Placental biopsies were obtained immediately after delivery from the maternal side of the placenta, in regions free of infarcts between the placental rim and cord insertion. Placental

villous tissue for explant culture studies were obtained and processed within 10 minutes after delivery from normal pregnant women who delivered healthy, full-term infants. The tissue was washed three times in room temperature buffered saline and transported to the laboratory. Cotyledons were excised and decidua and large blood vessels removed by blunt dissection. The tissue was cut into 5-10 mg pieces, washed, and then 50 mg pieces are placed into wells of a 24-well plate in phenol red-free M199 media + 2% v/v FBS. A portion of the placental villous tissue, with the decidual layer removed, was flash frozen for later RNA and protein analysis. For immunohistochemistry the remaining tissue was cut in to 0.5 cm^3 pieces and rinsed ice-cold phosphate buffer saline. After rinsing, these portions of villous tissue were flash frozen.

Statistical analyses: Cross sectional and longitudinal case-control data were analyzed by Student's unpaired t-test, or by Mann-Whitney U test for skewed variables. Longitudinal data across normal pregnancy were analyzed by repeated measures ANOVA and Student-Newman-Keuls post-hoc pairwise comparisons. Relationships among continuous variables were assessed by Spearman rank or Pearson correlation.

Specific Aim 1a: Plasma concentrations of sSDC-1 and sGPC1 will be compared using archived maternal plasma samples obtained both prior to preeclampsia and predelivery, with matched controls from our Program Project longitudinal study.

Aim 1a, Cross-sectional, 3rd trimester maternal study, METHODS:
We tested the hypothesis that soluble (shed) sSDC-1 is increased in maternal plasma from women with preeclampsia compared to control pregnancies, reflecting acute GCX disruption in the disease. A nested, case-control comparison was conducted, comprised of 3rd trimester samples from 19 women with uncomplicated, normotensive pregnancies who delivered appropriate weight for gestational age (AGA) neonates (controls), and 17 women with preeclampsia. An additional study of 3rd trimester plasma samples from women with gestational hypertension (without proteinuria) (n= 8), women with small for gestational age (SGA) fetuses (n= 6), and uncomplicated pregnancy controls (n = 6) was performed. Cases were group matched to controls for race and gestational age at time of venipuncture. The controls for preeclampsia were different from the controls for gestational hypertension and SGA groups. The reason for this was the earlier mean gestational age at delivery of women with preeclampsia— many of whom were recruited in the labor and delivery suite. All patients were nulliparous. Clinical characteristics and sSDC-1 values are summarized in Table 1 and Table 2.
sSDC-1 plasma concentrations were measured in duplicate by ELISA (Eli-pair kit from Diaclone/Cell Sciences Inc.). We validated the sSDC-1 ELISA for use in pregnancy samples by performing sample dilution and spike-recovery tests on separate pools of preeclampsia and normal pregnancy plasma. We looked for correlation of sSDC-1 with clinical parameters.

Aim 1a, Cross-sectional, 3rd trimester maternal study, RESULTS:
By design, mean gestational age at the time of blood sampling was not different between the preeclampsia group and uncomplicated pregnancy controls (Table 1). Age, pre-pregnancy body mass index, average gestational blood pressures before 20 weeks, percentage of women who smoked cigarettes during pregnancy, and racial distribution did not differ between groups. Women with preeclampsia delivered significantly earlier, had (by definition) significantly higher pre-delivery systolic and diastolic blood pressures, and delivered smaller babies on both absolute

birth weight and birth weight percentile bases, compared to controls (Table 1). Third trimester (time of disease) plasma soluble SDC-1 levels were 2.5-fold lower in women with preeclampsia compared to controls ($P<0.01$; Table 1). Within the preeclampsia group, plasma sSDC-1 was marginally correlated with baby birth weight ($r=0.46$, $p=0.059$) and birth weight centile ($r=0.45$, $p=0.067$), and inversely correlated with pre-pregnancy body mass index ($r=-.45$, $p=0.067$). Plasma sSDC-1 concentration was positively correlated with gestational age at the time of blood sampling in preeclampsia ($r=0.53$, $p<0.03$) and control ($r=0.51$, $p<0.03$) groups. No other correlations of sSDC-1 with clinical variables were noted.

The mean baby birth weight percentile in the preeclampsia group was 20 ± 25 (SD). Seven of the 17 preeclampsia cases delivered babies that were small for gestational age, defined as birth weight percentile < 10. The mean birth weight centile of this sub-group (2.3 ± 1.7), was significantly different ($P<0.01$) compared to birth weight centile of the 10 preeclampsia cases with more AGA babies (34.3 ± 3.2). As shown in Figure 1, the low plasma SDC-1 values in the preeclampsia group were predominantly clustered in the preeclampsia sub-group with SGA infants [SDC-1 median (interquartile range): 221 (149-268)]. These values were significantly different from the controls ($p<0.01$) whereas values in the preeclampsia sub-group with AGA infants [SDC-1 median (interquartile range): 635 (255 – 779)] did not differ from the controls. These data indicate a strong association of low maternal plasma sSDC-1 with the restricted infant growth that often accompanies preeclampsia.

Preliminary comparisons of plasma sSDC-1 have been made between gestational hypertension (without proteinuria), normotensive SGA, and control groups. Clinical characteristics and plasma sSDC-1 values of these 3 patient groups are summarized in Table 2. Soluble SDC-1 did not differ between these groups (Kruskal-Wallis test). Although the sample size will be increased, these data suggest that low plasma sSDC-1 is not merely a consequence of hypertension and is more strongly related to preeclampsia with SGA than SGA alone.

Please note that plasma sSDC-1 was higher in the Controls for preeclampsia compared to the Controls for gestational hypertension and SGA groups, likely due to the later gestational age of the Controls for preeclampsia.

Aim 1a, Longitudinal soluble factor changes during uncomplicated pregnancy, METHODS:
Soluble SDC-1 concentrations were measured by ELISA in longitudinal (pregnancy and postpartum) plasma samples obtained from 8 women with uncomplicated pregnancy. The clinical characteristics of these control patients, who are different patients than all other control sets, are summarized in Table 3.

Aim 1a, Longitudinal soluble factor changes during uncomplicated pregnancy, RESULTS:
As shown in Figure 2, plasma sSDC-1 concentration increases significantly with advancing pregnancy, reaching a zenith at term, and decreases within 1-2 days postpartum. Plasma sSDC-1 at 7-11 weeks of uncomplicated pregnancy was significantly higher than values at 4-9 weeks postpartum (Figure 2). These data implicate the placenta as the primary source of elevated sSDC-1 in the maternal circulation during pregnancy.

Aim 1a, Mid-pregnancy evaluations of plasma sSDC-1 and triglycerides, METHODS:

Maternal plasma sSDC-1 concentrations were measured in gestational age-matched samples collected at mid-pregnancy from 9 women who later developed preeclampsia, 9 women who later developed gestational hypertension (without proteinuria), and from 19 normotensive controls with uncomplicated outcome. These patients comprised a nested-case subset of longitudinal patients recruited by the PEPP study. Plasma sSDC-1 was measured by ELISA in duplicate (Eli-pair kit from Diaclone/Cell Sciences Inc.). Plasma total triglycerides were measured by colorimetric assay, with quality controls provided by the vendor (Pointe Scientific, Inc.). The clinical characteristics of these patients, who are different individuals from the other patient sets, are summarized in Table 4.

Aim 1a, Mid-pregnancy evaluations of plasma sSDC-1 and triglycerides, RESULTS:
As shown in Table 4, and similar to the cross-sectional study subjects, longitudinal subjects who developed preeclampsia delivered earlier ($p<0.05$), had babies with lower birth weight percentiles ($p<0.05$), and by definition significantly higher blood pressures at admission to labor and delivery ($p<0.05$). Women with gestational hypertension also had elevated blood pressures and babies with lower birth weight percentiles ($p<0.05$), but did not deliver earlier, compared to controls. As shown in Figure 3 and Table 4, Plasma sSDC-1 concentrations were significantly reduced (36%, $p<0.05$) at 20 weeks of gestation (more than 10 weeks prior to clinically evident disease) in women who later developed preeclampsia, but not gestational hypertension, compared to controls. Median plasma triglycerides were higher at 20 weeks gestation in patients who later developed preeclampsia (mg/dl: preeclampsia 127, gestational hypertension 104, control 92), but not significantly so ($P=0.10$).

Third trimester samples have been obtained (during preeclampsia or gestational hypertension) from most of these longitudinal subjects; sSDC-1 and triglycerides are in the process of being measured.

Aim 1a, 1 Year Postpartum Evaluations of sSDC-1 and soluble Hyaluronic Acid, METHODS:
We explored whether circulating sSDC-1 and soluble hyaluronic acid (a GCX non-sulfated glycosaminoglycan) differ in plasma, 1 year after pregnancy, in non-pregnant women with a history of preeclampsia compared to women with a history of uncomplicated pregnancy. Study participants had given permission to be contacted for additional preeclampsia-related research studies upon consenting to participate in the Preeclampsia Program Project with IRB approval. Subjects underwent a history and physical examination and a urine pregnancy test. Blood was collected on the morning after an overnight fast. Soluble factors were measured in the banked plasma from 16 primiparous women with a history of preeclampsia and 20 primiparous women with a history of normal pregnancy (both on average 1 year postpartum). No women were lactating, and all were nonsmokers. There were insufficient numbers of pregnancy samples available from these women for comparison of values during and after pregnancy. Hyaluronic acid was measured by ELISA kit (Echelon Biosciences Inc., Salt Lake City, UT).

Aim 1a, 1 Year Postpartum Evaluations of sSDC-1 and soluble Hyaluronic Acid, RESULTS:
Women who developed preeclampsia are at increased risk of CVD in later years. Table 5 shows that that concentrations of sSDC-1 and soluble hyaluronic acid in maternal plasma 1 year postpartum do not differ between women with prior preeclampsia compared to prior uncomplicated pregnancies (Table 5). Note the much lower postpartum sSDC-1 levels compared

to pregnancy levels. These SDC-1 and hyaluronic data together suggest that GCX dysfunction in women with preeclampsia, in the form of shed/soluble components, is not detected after pregnancy and therefore these soluble factors will not serve as an early marker of impending CVD in the postpartum state.

Specific Aim 1b: Villous placenta from preeclampsia and control pregnancies will be compared with regard to (a) syncytiotrophoblast cell surface immunoreactivity for SDC-1 and GPC1 and (b) protein and mRNA levels of SDC-1 and GPC1. Correlation of placenta and plasma variables will be examined.

Specific Aim 1b, METHODS:

Syncytiotrophoblast Immunohistochemical Staining and Scoring:
Snap frozen placenta tissue was broken into 1cm cubes and placed into embedding medium to ensure optimal cutting temperature (OCT, Tissue-tek, Inc.). Frozen OCT-embedded placentas were sectioned at 7-µm and fixed in acetone. Endogenous peroxidase activity was quenched with treatment of 3% hydrogen peroxide. Staining was carried out using VECTASTAIN Elite ABC Kit (Vector Laboratories, Burlingame, CA). Immunostaining procedures were followed as described by the manufacturer and reagents prepared as outlined in the package insert. Slides were incubated for 1 hour at room temperature with SDC-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or glypican-1 (GPC1) antibody (NBP1-18666; Novus Biologicals), at 1:100 and stained with 3, 3-diaminobenzidine (DAB) and counterstained with hematoxylin from Vector Laboratories, Burlingame, CA. Placental biopsy tissue slices were also immunostained using rhodamine wheat germ agglutinin (WGA; Vector Labs), a lectin that binds to sialic acid and N-acetyl-D-glucosamine of hyaluronic acid of cell surface GCX. Negative controls were prepared by replacing the primary antibody with blocking serum.

Images were collected using bright field microscopy (Nikon90i) with Q imaging camera. A scoring system was developed to assess the presence of the GCX proteins on the apical surface of syncytiotrophoblasts. Scoring was on a 0-4 scale with 4 being the darkest stain seen along the entire apical surface and 0 being no stain observed. Images were scored by 2 blinded observers pre-trained to the scale, and the values averaged.

Isolation and Analysis of Placental RNA:
Total cellular RNA was isolated from placental tissue with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufactures' instructions. After isolation, double- and single-stranded DNA were removed by treatment with DNaseI (Invitrogen, Carlsbad, CA). Quality and quantity of RNA were determined by measuring absorption at 280 and 260 nm. The complementary DNA (cDNA) was synthesized from 2 µg of purified RNA and converted to cDNA using Applied Biosystems (Foster City, CA) high capacity cDNA kit. Control reactions without reverse transcriptase were performed to confirm the absence of DNA contamination. Quantitative real-time polymerase chain reaction (PCR) was performed on a TaqMan ABI PRISM 7700 sequence detector using 96-well optical plates (Applied Biosystems, Foster City, CA). Real-time PCR was performed using inventoried TaqMan Gene Expression Assays for SDC-1 (Hs00896423_m1), SDC-2 (Hs00299807_m1), glypican-1 (Hs00157805_m1) and 18s (Hs99999901_s1) (Applied Biosystems). The 18s gene expression assay was used as an endogenous reference for all

samples. All amplification cycles were performed in a 50µl reaction in duplicate with cDNA equivalent to 100ng of total RNA. Relative quantification of the target expression was performed using the comparative cycle threshold (Ct) method as described in ABI User Bulletin 2, which consists of normalization of the number of target gene copies (SDC-1) to an endogenous reference (18s) and relative to a calibrator (control pregnant placenta).

Western Blot Comparisons of SDC-1 in Human Placenta:
Twenty to thirty milligrams of frozen pulverized placenta tissue were homogenized by sonication (UltrsonicUltrasonic Processor, Tekmar, Cincinnati, OH) in 1X Laemmli buffer (50 mM Tris HCl, pH 6.8, 2% SDS, 10% Glycerol) containing 5 mM DTT, 0.5 mM phenyl methyl sulfonyl fluoride (PMSF), 1mM sodium vanadate and one µl per ml of protease inhibitors cocktail (1000X, Protease Inhibitor Cocktail Set III, Calbiochem, San Diego, CA). The crude homogenate was centrifuged at 13,400x g at 4ºC for 15 min. Protein estimation was carried out on the supernatant using the Pierce BCA protein assay (Rockford, IL). 40µg of protein per lane was used for the Western blot analysis. Total protein samples were loaded in a volume containing the desired amount of protein, 5% ß-mercaptoethanol and tris-glycine SDS sample buffer (Invitrogen, Carlsbad, CA). All preparations were boiled for 5 min and then briefly centrifuged. The proteins were separated on 7.5% SDS-polyacrylamide gel at 120 V for 2 h. The proteins were transferred to polyvinylidene fluoride membranes (Immobilon; Millipore, Bedford, MA) using a wet transfer system (Owl Separation System). Detection of protein was carried out after blocking the membranes in a solution of 5% nonfat dry milk in 10 mM Tris (pH 7.4), 150 mM NaCl (TBS)-0.05% Tween-20 (TBS-T). Blots were then incubated with a rabbit polyclonal antibody against human SDC-1 (Santa Cruz Biotechnology, Inc., CA) at a dilution of 1:500 in blocking solution for 1hr at room temperature. The blots were rinsed five times for 5 minutes each with TBS-T and incubated with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnolgy Inc., CA) at 1:5000 in blocking solution for 1 hour at room temperature. After appropriate washing, protein bands were detected by enhanced chemiluminescence (Santa Cruz Biotechnolgy Inc., CA) and exposed to x-ray film (Eastman Kodak Co, Rochester, NY).

Specific Aim 1b, RESULTS:

SDC-1 protein immunofluorescence on the apical surface of syncytiotrophoblast of placental villous biopsies was significantly ($p<0.05$) lower in preeclampsia compared to uncomplicated pregnancy control placentas (Table 6). Representative digital images are given in Figure 4. This indicates a deficit in SDC-1 protein in preeclampsia.

The placental SDC-1 protein score in control patients was significantly correlated with soluble SDC-1 concentration in maternal plasma from the same patients ($r= 0.66$, $p<0.02$) supporting the hypothesis that the soluble SDC-1 in the maternal circulation directly reflects SDC-1 expression on the surface of the placenta. The placental SDC-1 protein score in control patients was significantly correlated with both baby birth weight percentile ($r=0.66$, $p<0.02$) and absolute baby weight ($r=0.77$, $P<0.01$). The placental SDC-1 protein score in preeclampsia patients correlated significantly with both birth weight percentile ($r=0.73$, $p< 0.03$) birth weight ($r=0.70$, $p<0.04$). The placental SDC-1 protein score also correlated with birth weight percentile in the SGA patients ($r=0.81$, $p=0.05$). These data support the hypothesis that placental SDC-1 is involved in (or reflects) placental nutrient transfer to the fetus (Aim 3).

In the preeclampsia group, lower (poorer) placental SDC-1 scores correlated with higher mean pre-delivery systolic blood pressures ($r=-0.73$, $p<0.03$). In controls, lower placental SDC-1 scores correlated with higher mean pre-delivery systolic blood pressures ($r=-0.89$, $p<0.001$).

Western blot assay for SDC-1 protein in placenta protein extracts (Figure 5) was consistent with the syncytiotrophoblast immunofluorescence scoring data (reduced SDC-1 in preeclampsia placenta compared to uncomplicated, normotensive control placenta).

Villous stromal and fetal capillary endothelium stained diffusely for wheat germ agglutinin whereas syncytiotrophoblast were strongly positive. Strong glypican-1 immunoreactivity was observed in fetal villous vessels but was more sporadic on syncytiotrophoblast. However, no significant group differences in immunostaining intensity were observed.

RNA was isolated from the 3rd trimester placental biopsies. SDC-1, syndcan-2 (SDC-2), and glypican-1 (GPC1) mRNA content was determined by RT-PCR. There was no significant difference observed between any of the groups (Table 6). These data suggest that gene expression at the mRNA level does not differ between groups; therefore mRNA expression is not the explanation for the significantly lower SDC-1 protein in preeclampsia placentas.

Specific Aim 1c: Test whether quantities of sSDC-1 and sGPC1 secreted by villous placenta in explant (*in vitro*) culture differ under normoxic, hypoxic and hyperoxic conditions.

Specific Aim 1c, METHODS:
We tested whether quantities of SDC-1 secreted by villous placenta in explant (*in vitro*) culture differ under normoxic, hypoxic and hyperoxic conditions. Villous explant tissue from term normal pregnancies (n=3) were incubated (50 mg tissue/well in 24-well plates) for 24 hours in 3 different oxygen environments: 1-2% oxygen (hypoxic), 8% oxygen (placental normoxic) and 21% oxygen (hyperoxic) hypoxic in phenol red-free M199 media + 2% v/v FBS. Soluble SCD-1 was measured by ELISA in the resulting conditioned media.

Specific Aim 1c, RESULTS:
A graded response was observed with declining oxygen content (SDC-1, mean ng/mL ± SD: 21% oxygen, 207 ± 53; 8% oxygen, 291 ± 46; 1-2% oxygen 462 ± 265) suggesting that hypoxia stimulates SDC-1 shedding/ release from placenta. Three more experiments will be completed before results are evaluated statistically. These data do not support the hypothesis that placental hypoxia, implicated in preeclampsia, explains the lower placental SDC-1 expression and lower maternal plasma sSDC-1 in preeclampsia.

Hypothesis 2: Local retention/binding of the placentally-secreted, soluble VEGF receptor-1 (sFlt1) is mediated by heparan sulfate components of cell surface SDC-1 on trophoblast in culture.

Specific Aim 2: To show that trophoblasts sequester substantial sFlt1 on the cell surface, and that the quantity of releasable sFlt1 from trophoblast cell surface is reduced after specific inhibition of cell surface expression of SDC-1 or by heparanase pre-treatment.

Specific Aim 2, Initial heparin study:
The Formula Fund project was stimulated by pilot data that endothelial and trophoblast HSPGs bind/sequester sFlt1— the anti-angiogenic, soluble decoy receptor (also known as VEGF receptor-1) implicated in preeclampsia. Data on effects of intravenous heparin administration on circulating angiogenic factor profile in non-pregnant women of childbearing age were presented in the Strategic Research Plan for this project. Blood concentrations of sFlt1 increased ~250-fold shortly after intravenous heparin administration, equivalent to 3rd trimester normal pregnancy levels, and of sufficient quantity to reduce concentrations of free VEGF to near zero. Measurements of heparin-induced release of sFlt1 from whole blood *in vitro* were performed during the 2nd half of the funding period. Germane to Aim 2. The Methods and Results are described below. It was concluded that substantial reserves of heparin-releasable sFlt1 and placental growth factor exist, likely electrostatically bound to HSPGs of the vascular endothelial (but not blood cell) GCX. Endothelial surface HSPGs may thus regulate angiogenic factor bioavailability *in vivo*. A research article describing the project will be submitted April 2013 (1st author: Weissgerber TW; senior and corresponding author Hubel CA).

Specific Aim 2, Initial heparin study, blood sample incubations, METHODS:
We added heparin to whole blood samples from pregnant and non-pregnant women to test the hypothesis that blood cells or other plasma components do not contribute appreciably to releasable stores of sFlt-1. We obtained blood samples from four women prior to delivery (mean ± standard deviation: 38.4 ± 2.1 weeks gestation), and seven women between 6 and 21 months post-partum (13 ± 5 months). Blood was collected in vacutainers containing EDTA. Pre-delivery samples were treated under six conditions: (1) Plasma was isolated within 2 hours of venipuncture; (2a-c) Whole blood was incubated for 24 hours at 37°C before plasma was isolated and frozen with no additives (2a), or after adding 20 U/ml of unfractionated (2b) or low molecular weight (2c) heparin to the plasma; (3a,b) Whole blood was incubated with 20 U/ml of unfractionated (3a) or low molecular weight (3b) heparin for 24 hours at 37°C before the plasma was isolated. Plasma for all conditions was frozen at -80°C for later analysis. Post-partum samples were treated under four conditions (1, 2a, 2b, 3a), excluding the two conditions involving treatment with low molecular weight heparin (2c, 3b). Plasma sFlt1 was measured by ELISA kit (R&D Systems). Effects of treatments were determined by Wilcoxon signed rank test.

Specific Aim 2, Initial heparin study, blood sample incubations, RESULTS:
Data are shown in Figure 6. In pre-delivery samples, plasma sFlt1 was slightly but significantly lower in samples processed within 2 hours than in samples in which whole blood was incubated for 24 hours, or in which whole blood was incubated for 24 hours and LMWH was then added to plasma. Incubating whole blood or plasma with LMH or UFH did not further increase sFlt1; in fact, sFlt1 was significantly lower in all conditions in which UFH or LMWH was added to plasma than in samples incubated for 24 hours without heparin ($p<0.05$). In post-partum samples, plasma sFlt1 was slightly higher in all conditions in which whole blood was incubated for 24 hours compared to samples processed immediately ($P<0.05$). However, adding UFH to whole blood or plasma had no additional effect on plasma sFlt1. These data show negligible heparin-induced release of sFlt1 from (sFlt1 binding to) blood cells or other plasma components.

Specific Aim 2, Cell culture experiments, METHODS:
Human uterine microvascular endothelial cells (HUtMVEC) and the 1st trimester trophoblast cells (HTR8 sV-neo) were washed with low-serum media and then treated with media plus vehicle or 10 U/mL of unfractionated heparin or 10 U/mL of low molecular weight heparin for 30 minutes, and the conditioned media was then collected and assayed for sFlt1 by ELISA. Alternatively, cells were treated with vehicle or equivalent molar concentrations of the two types of heparin ($1x10^{-5}$M = 10 U/mL low molecular weight and 46 U/mL unfractionated heparin).

Specific Aim 2, Cell culture experiments, RESULTS:
Data are shown in Figure 7. Heparin caused a ~4x increase in sFlt1 in the media over baseline, suggesting substantial sequestering of heparin-releasable sFlt1 on the GCX of both cell types. HSPGs thus mediate a pro-angiogenic state by sequestering sFlt1 on the trophoblast and endothelial cell apical surface (GCX); heparin treatment releases sFlt1 into circulation because of competitive binding at the sFlt1 heparin binding site.

Hypothesis 3: SDC-1 mediates the binding and internalization of VLDL in human trophoblasts, suggesting a role for SDC-1 in placental lipid trafficking.

Specific Aim 3a: To show that trophoblast in culture bind and internalize VLDL.
Specific Aim 3b: To examine whether siRNA knockdown of cell surface SDC-1 or heparanase pre-treatment reduces VLDL binding and internalization.

Specific Aim 3a-b, METHODS:
We developed assays to test whether heparin sulfate proteoglycans, and specifically SDC-1, mediate the binding and internalization of VLDL in human trophoblasts or endothelial cells, suggesting a role for SDC-1 in placental lipid trafficking. Briefly, sub-confluent HTR8 trophoblast cells or human uterine microvascular endothelial cells were treated with 0-50 µg/mL of DiI labeled VLDL (Kalen Biomedical) in basal media with 2.5 mg/mL lipoprotein-free serum (Biomedical technologies, Inc.). Cells were incubated at 37ºC (3 hours) for uptake experiments and 4ºC for surface binding experiments. Wells were rinsed 3 times with PBS and then cell lysis reagent (1 g/L SDS dissolved in 0.1 M NaOH) was added with incubation for 1 hour. Fluorescence and cellular protein was simultaneously measured and data expressed as ng cell associated diI-VLDL per mg of cell protein, adapted from a published assay for DiI-LDL (*Teupser D, et al. Determination of LDL- and scavenger-receptor activity in adherent and non-adherent cultured cells with a new single-step fluorometric assay. Biochim Biophys Acta 1996;1303:193-8*).

Specific Aim 3a, RESULTS:
Figure 8 shows the cell-free DiI-VLDL standard curve and Figure 9 shows the concentration-dependent uptake and surface binding of DiI-VLDL or DiI-acetylated LDL by the HTR8 and endothelial cells, with the plateau of each consistent with receptor-mediated internalization.

Specific Aim 3b, RESULTS:
Figures 10 and 11 show that uptake of DiI-VLDL (5 microgram protein/mL media) by HTR8 cells is inhibited by 1 U/mL exogenous unfractionated heparin or the enzyme heparanase. This implicates cell surface heparin sulfate proteoglycans in VLDL internalization.
Finally, we show that the trophoblast HTR8 cells express SDC-1 (Figure 12).

Specific Aim 3: Analysis of the relationship between maternal and umbilical cord (fetal) blood Lipid levels, METHODS:
As an indirect test of the hypothesis that uptake of triglyceride rich lipoproteins is impeded in preeclampsia, the relationship between maternal plasma and umbilical cord (fetal) plasma concentrations of total triglycerides and cholesterol was examined. Plasma total triglycerides and cholesterol were measured using reagents by colorimetric manual method (Pointe Scientific). Eight of the 11 preeclampsia cases and 12 of the 23 controls were the same as cross sectional patients studied in Aim 1a. Clinical characteristics and lipid data are given in Table 7.

Specific Aim 3: Analysis of the relationship between maternal plasma and umbilical cord (fetal) blood Lipid levels, RESULTS:
As shown in Table 7, maternal lipid levels were higher than cord lipid levels. Triglyceride concentrations in maternal plasma were significantly higher in preeclamptics compared to controls whereas cholesterol concentrations did not differ. In contrast, cholesterol concentrations in fetal (cord) plasma were significantly higher in preeclamptics whereas cord triglyceride concentrations did not differ between groups. This is plausibly consistent with the hypothesis that trans-placental transfer of triglyceride-rich lipoproteins (VLDL and IDL) from maternal to fetal circulations is diminished in preeclampsia. It might also be consistent with reports that GCX disruption is associated with impaired LDL retention by the GCX and transcellular leakage of cholesterol (LDL) into the intima (*van den Berg, et al. Pflugers Arch 2009; 457:1199-1206*).

II C. SUMMARY OF PROGRESS:

Aim 1. Compared to uncomplicated pregnancies, preeclampsia is characterized by reduced expression of the major HSPG SDC-1 on the apical surface (GCX) of placental syncytiotrophoblast. Maternal plasma concentrations of shed (soluble) (s)SDC-1 rise significantly by 7-11 weeks of normal pregnancy, and reach ~25-fold higher levels during the 3rd trimester, compared to 8 weeks post-partum (and 1 year post-partum) levels, implicating the placenta as the major source of circulating sSDC-1 during pregnancy. On average, women who later develop preeclampsia have lower levels of (shed) sSDC-1 in maternal plasma at 20 weeks' gestation (*before clinically evident disease*) [ng/mL, median (interquartile range): *Normal pregnant* (n=19) 272 (245-595), *Preeclampsia* (n=9) 174 (136-232), *Non-proteinuric gestational hypertension* (n=9) 242 (148-369); Kruskal-Wallis ANOVA on ranks with Dunn's post hoc, *$p< 0.05$ Preeclampsia vs. Normal pregnant]. Preeclampsia vs. control differences are also evident pre-delivery, especially preeclampsia with fetal growth restriction. The placental syncytiotrophoblast SDC-1 expression level in normal pregnancies correlates directly with sSDC-1 in maternal plasma from the same patients, suggesting that sSDC-1 concentration in the maternal circulation during pregnancy directly reflects reduced SDC-1 on the surface of the placenta.

Aim 2. An intravenous bolus of unfractionated heparin increases soluble VEGF receptor-1 (sFlt1) in non-pregnant women to concentrations higher than those observed in the third trimester of pregnancy. However, the size of the vascular store of releasable sFlt1, while sufficient to drastically reduce plasma concentrations of free VEGF, does not differ between non-pregnant women with a history of preeclampsia vs. uncomplicated pregnancy. Similarly,

placental growth factor increases several-fold in non-pregnant women after heparin, but the post-heparin levels are not different between non-pregnant women with a history of preeclampsia vs. uncomplicated pregnancy. HSPGs of the vascular endothelial GCX thus comprise substantial non-placental reservoir of sFlt1 that could contribute to elevated sFlt1 and angiogenic imbalance in preeclampsia or other endothelial dysfunction states.

We found that HSPGs sequester sFlt1 on both trophoblast and endothelial cell surface (GCX), and that heparin treatment releases sFlt1 into circulation because of competitive binding at the sFlt1 heparin-binding site. In these experiments, HUtMVEC cells and the 1st trimester trophoblast cell line (HTR8) were washed with low-serum media and then treated with unfractionated heparin or low molecular weight heparin, and the conditioned media was then collected and assayed for sFlt1. Heparin caused a ~4x increase in sFlt1 in the media over baseline supporting our hypothesis of substantial sequestering of heparin-releasable sFlt1 on the surface of both cell types.

Aim 3: Data suggest a role for trophoblast GCX HSPGs (such as SDC-1) in transport of triglyceride-rich lipoproteins (that may fail in preeclampsia, especially with IUGR). VLDL binding (4°C) and uptake (37°C) by trophoblast cells was concentration-dependent and saturable, and inhibited by pre-treatment of cells with heparin or heparanase. This may parallel mechanisms of liver VLDL transport (*Stanford KI, et al. J Clin Invest.* 2009; 119:3236-45).

The role/interactions of GCX dysfunction—of trophoblast and endothelium—in the pathophysiology of preeclampsia and later-life CVD is a new frontier.

II D. TABLES

Table 1. Clinical and Plasma Soluble SDC-1 Data; Cross-sectional Control and Preeclampsia Pregnancy Groups

	Normotensive Controls (n=19)	Preeclampsia (n=17)	P value
Age (years)	26 ± 5	27 ± 7	NS
BMI pre-pregnancy (kg/m^2)	26.4 ± 6.5	27.3 ± 4.8	NS
Gestational weeks at venipuncture	32.5 ± 3.4	32.5 ± 3.7	NS
Gestational weeks at delivery	40.1 ±1.5	32.9 ± 3.5	<0.01
Early gestational BP (<20wks.)			
Systolic (mm Hg)	112 ± 9	114 ± 7	NS
Diastolic (mm Hg)	69 ± 6	71 ± 5	NS
Pre-delivery BP:			
Systolic (mm Hg)	120 ± 9	157 ± 12	<0.01
Diastolic (mm Hg)	71 ± 12	96 ±9	<0.01
Birth weight percentile	55 ± 27	20 ± 25	<0.01
Uric acid (mg/dL)	----	6.1 ± 1.0	---
Cigarette smokers (%)	26%	18%	NS
Race (% black)	21%	32%	NS
Sampling prior to labor (%)	100%	100%	NS
Soluble syndecan-1 ng/mL (median and IQR)	758.4 (597.6-947.4)	*280.8 (187.8-705.8)	P<0.001

Continuous variables are given as mean (SD) or median (interquartile range; IQR);
NS, not significant;
BMI, body mass index;
Birth weight percentiles were calculated using local (Western PA) race- and gender-specific growth data adjusted for gestational age at delivery.

Table 2. Clinical and Plasma Soluble Syndecan-1 Data; Cross-sectional Control, Gestational Hypertensive, and Small for Gestational Age (SGA) Pregnancy Groups

	Normotensive Controls (n=6)	Gestational Hypertension (n=8)	SGA (n=6)	P value
Age (years)	29 ± 8	29 ± 8	23 ± 5	NS
BMI pre-pregnancy (kg/m^2)	24.4 ± 3.3	26.4 ± 8.8	21.3 ± 1.6	NS
Gestational weeks at venipuncture	39.0 ± 1.2	38.6 ± 2.1	38.5 ± 1.5	NS
Gestational weeks at delivery	39.1 ±1.2	39.8 ± 1.2	38.5 ± 1.5	NS
Early gestational BP (<20wks.)				
Systolic (mm Hg)	117 ± 9	117 ± 8	107 ± 9	NS
Diastolic (mm Hg)	70 ± 4	69 ± 6	67 ± 6	NS
Pre-delivery BP:				
Systolic (mm Hg)	123 ± 14	#146 ± 13	107 ± 8	<0.05
Diastolic (mm Hg)	71 ± 8	*89 ± 10	61 ± 11	<0.05
Birth weight percentile	44 ± 32	51 ± 34	*2.9 ± 2.2	<0.05
Cigarette smokers (%)	0%	0%	17%	NS
Race (% black)	17%	25%	33%	NS
Soluble syndecan-1 ng/mL (median and interquartile range)	1358 (1098-1546)	923 (766-1101)	1177 (708-1908)	NS

Continuous variables are given as mean (SD) or median (interquartile range; IQR);
NS, not significant;
BMI, body mass index;
Birth weight percentiles were calculated using local (Western PA) race- and gender-specific growth data adjusted for gestational age at delivery;
P values: *Indicated group versus the other two groups; # Gestational Hypertension versus SGA.

Table 3. Clinical Characteristics of Controls in Longitudinal Serial Sampling Study

	Uncomplicated Pregnancy Controls (n=8)
Age (years)	25 ± 6
BMI pre-pregnancy (kg/m^2)	29.2 ± 6.3
Gestational weeks at delivery	39.7 ± 1.5
Early gestational BP (<20wks.)	
Systolic (mm Hg)	114 ± 11
Diastolic (mm Hg)	69 ± 8
Pre-delivery BP:	
Systolic (mm Hg)	130 ± 17
Diastolic (mm Hg)	74 ± 11
Birth weight percentile	59 ± 35
Cigarette smokers (%)	38%
Race (% black)	25%
Labor @ time of sampling (%)	17%

Continuous variables are given as mean (SD).
BMI, body mass index

Table 4. Clinical and Mid-pregnancy Soluble Syndecan-1 Data; Control, Gestational Hypertensive, and Preeclampsia Pregnancy Groups

	Normotensive Controls (n=19)	Gestational Hypertension (n=9)	Preeclampsia (n=9)	P-value
Age (years)	25 ± 4	24 ± 6	26 ± 8	NS
BMI pre-pregnancy (kg/m^2)	29.5 ± 3.6	28.6 ± 5.7	31.1 ± 7.1	NS
Gestational weeks at venipuncture	*20.4 ± 1.9*	*20.3 ± 2.3*	*20.0 ± 1.4*	NS
Gestational weeks at delivery	39.3 ±2.7	39.9 ± 1.2	#36.0 ± 1.3	<0.05
Early gestational BP (<20wks.)				
Systolic (mm Hg)	113 ± 7	122 ± 10	116 ± 6	NS
Diastolic (mm Hg)	68 ± 6	73 ± 7	72 ± 4	NS
Pre-delivery BP:				
Systolic (mm Hg)	125 ± 8	*147 ± 8	*154 ± 17	<0.05
Diastolic (mm Hg)	72 ± 7	*88 ± 6	*99 ± 7	<0.05
Birth weight percentile	71 ± 18	*38 ± 15	*30 ± 29	<0.05
Cigarette smokers (%)	0%	0%	0%	NS
Race (% black)	47%	44%	56%	NS
Soluble syndecan-1 ng/mL (median and interquartile range)	272 (245-595)	242 (148-369)	*174 (136-232)	<0.05

Continuous variables are given as mean (SD) or median (interquartile range; IQR);
NS, not significant;
BMI, body mass index;
Birth weight percentiles were calculated using local (Western PA) race- and gender-specific growth data adjusted for gestational age at delivery;
P values: #Indicated group versus the other two groups; *Indicated group versus Control.

Table 5. Clinical Characteristics and Plasma Soluble GCX Factor Concentrations, Non-pregnant Women 1 Year After Preeclamptic or Normal Pregnancy

Mean (±SD) or median (interquartile range)	Prior preeclampsia (n=16)	Prior normal pregnancy (n=20)	P-value
Age (years)	29 ± 4	26 ± 6	NS
Body mass index (kg/m^2)	29.9 ± 6.9	28.1 ± 6.4	NS
Days post delivery	389 ± 114	388 ± 151	NS
Systolic BP (mm Hg)	*119 ± 10	109 ± 8	$p<0.05$
Diastolic BP (mm Hg)	*81 ± 10	73 ± 9	$p<0.05$
Oral Contraceptives Use (%)	50%	60%	NS
Black Race (%)	32%	20%	NS
Plasma Soluble syndecan-1 (ng/mL)	23 (18-35)	26 (18-33)	NS
Plasma Soluble hyaluronic Acid (ng/mL)	46 (36-56)	51 (40-59)	NS

Continuous variables are given as mean (SD) or median (interquartile range; IQR);
NS, not significant;
BMI, body mass index

Table 6. Clinical and Placental Characteristics by Group

Variable	Normal Pregnant Controls (n=12)	Preeclampsia (n=10)	SGA (birthweight <5th centile) (n=6)
Maternal age (years)	27±5	29±5	23±5
Pre-pregnancy BMI (kg/m^2)	25.6±5.2	27.0±3.9	21.3±1.6 (p=0.1)
Gestational age at delivery (weeks)	39.1±2.0	*33.2±3.9 (p<0.01)	38.5±1.5
Race (% African American)	25%	20%	33%
Birth weight centile (%)	41±24	25±27 (p=0.051)	*3 ± 2 (p<0.01)
Syncytiotrophoblast Syndecan-1 immunoreactivity #	3.3 (2.1-4.0)	*2.0 (1.9-2.6) (p<0.05)	2.5 (2.0- 3.0)
Syncytiotrophoblast Glypican-1 immunoreactivity #	3.5 (1.4-3.5)	3.0 (2.0- 3.5)	3.0 (2.0 – 3.0)
Syncytiotrophoblast Wheat germ agglutinin immunoreactivity #	3.0 (2.5-4.0)	2.0 (1.8- 3.0)	2.0 (1.0 -3.0)
Syndecan-1 2-ΔΔCt mRNA ##	0.37 ± 0.26	0.33 ± 0.24	0.22 ± 0.10
Syndecan-2 2-ΔΔCt mRNA ##	0.67 ± 0.44	0.48 ± 0.43	0.71 ± 0.85
Glypican-1 2-ΔΔCt mRNA ##	0.62 ± 0.42	0.29 ± 0.21 (p=0.13)	0.49 ± 0.50
Plasma Soluble syndecan-1 (CD138) (ng/mL)	1401 (665- 2361)	*540 (399- 785) (p<0.02)	1177 (699-2003)

Data are mean ± SD or Median (interquartile range). *: p<0.05 compared to controls; N.S. : not significant; marginal P-values given are vs. controls. Mann-Whitney U or Student's t-test for preeclampsia or SGA versus control comparisons.
#: semi-quantitative ordinal (0 to 4+) scale by blinded observer, with absent staining considered scored as zero; ##: placental villous biopsy tissue mRNA content determined by RT-PCR, units relative to a normal pregnancy placenta=1.0; BMI: body mass index.

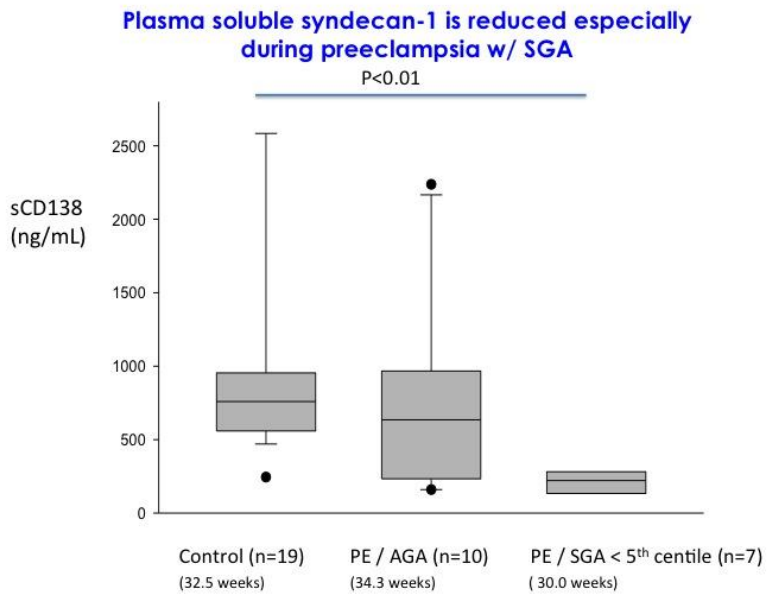
Table 7. Clinical and Lipid Data of Cross-Sectional Pregnancy Groups for Aim 3, Analysis of the Relationship Between Cord and Maternal Lipids

	Normotensive Controls (n=23)	Preeclamptics (n=11)	P value
Age (years)	26 ± 6	25 ± 5	NS
BMI pre-pregnancy (kg/m^2)	24.9 ± 4.8	28.3 ± 5.5	NS
Gestational weeks at venipuncture	35.3 ± 4.6	35.0 ± 3.4	NS
Gestational weeks at delivery	39.6 ± 1.9	35.2 ± 3.4	<0.05
Early gestational BP (<20wks.)			
Systolic (mm Hg)	112 ± 8	119 ± 6	NS
Diastolic (mm Hg)	70 ± 6	74± 3	NS
Pre-delivery BP:			
Systolic (mm Hg)	120 ± 8	154 ± 10	<0.01
Diastolic (mm Hg)	68 ± 11	97 ± 8	<0.01
Birth weight percentile	48 ± 28	26 ± 22	<0.01
Cigarette smokers (%)	17%	9%	NS
Race (% black)	22%	36%	NS
Maternal plasma triglyceride (mg/dL)	173 (132-193)	*250(149-371)	<0.04
Cord plasma triglyceride (mg/dL)	22.1 (18-44)	31.9 (26-80)	NS
Maternal plasma cholesterol (mg/dL)	238 ± 37	231 ± 59	NS
Cord plasma cholesterol (mg/dL)	62 ± 15	*73 ± 12	<0.04

Continuous variables are given as mean (SD) or median (interquartile range).
NS, not significant; BMI, body mass index

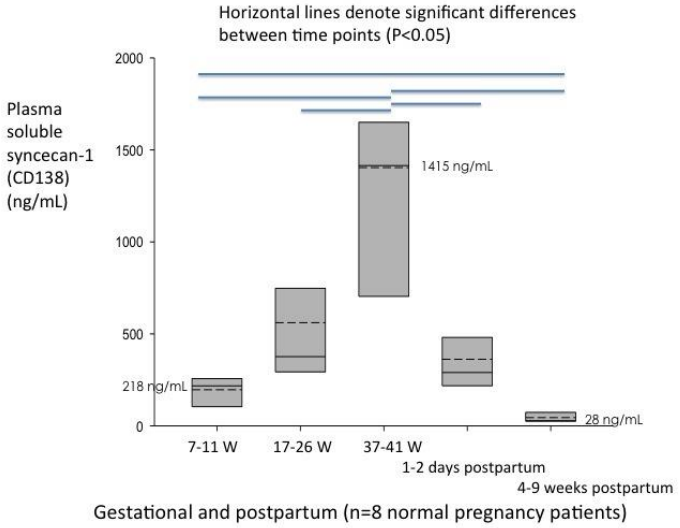
II E. FIGURES

Figure 1.



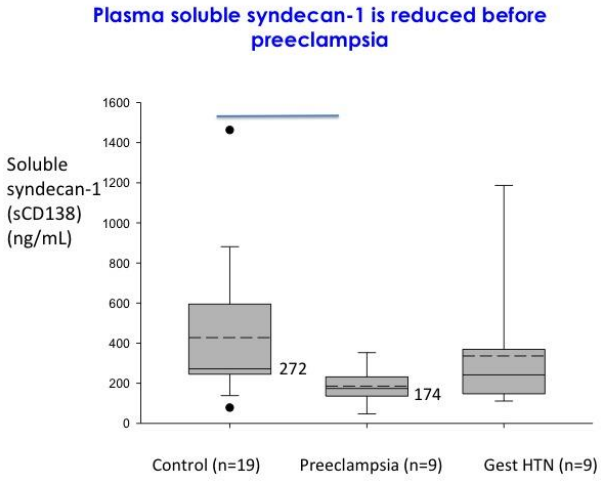
Legend, Figure 1. Box-plot of 3rd trimester plasma soluble syndecan-1 (sSDC-1) concentrations (y-axis) in gestational age-matched women with uncomplicated pregnancies (controls) compared to preeclampsia without small for gestational age (SGA) and preeclampsia with SGA (SGA defined as infant birth weight percentile less than 10; all were less than 5th centile). The top, bottom, and solid line through the interior of the box correspond to the 75th percentile, 25th percentile, and 50th percentile (median), respectively. The whiskers (t bars) on the bottom and top denote the 10th percentile and 90th percentile, respectively. Closed boxes denote the arithmetic mean. Significant difference is denoted by the horizontal line above the boxes. Note that the sSDC-1 value in the preeclampsia+SGA subgroup (mean gestation age 30 weeks) approximates the value seen during 7-11 weeks of normal pregnancy (Fig. 2).

Figure 2.



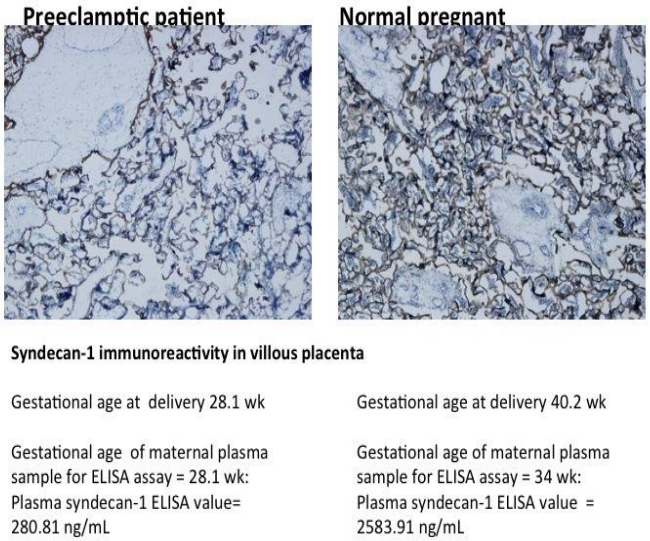
Legend, Figure 2. Plasma SDC-1 concentrations with advancing gestation and postpartum (n=8). Significant differences between time points are denoted by the horizontal lines above the boxes. The top, bottom and horizontal lines through the interior of each box denote the 75th, 25th and 50th percentile, respectively.

Figure 3.



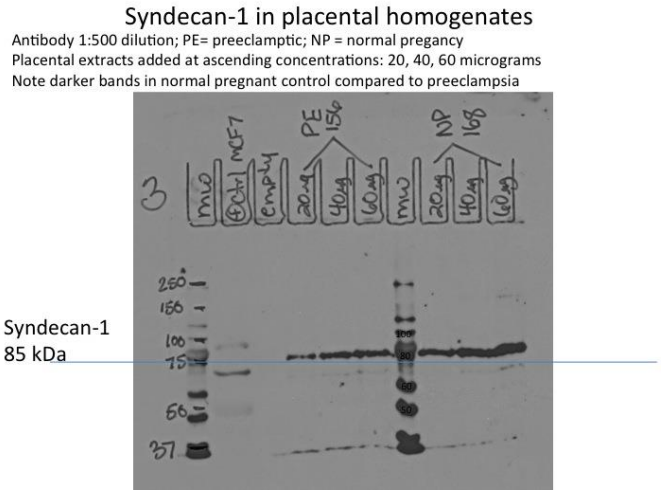
Legend, Figure 3. Box-plot of sSDC-1 concentrations in gestational age-matched mid-pregnancy (20 week) plasma samples (y-axis) from women with uncomplicated pregnancy, preeclampsia, or gestational hypertension (Gest HTN). The top, bottom, and solid line through the interior of the box correspond to the 75th percentile, 25th percentile, and 50th percentile (median), respectively. The whiskers (t bars) on the bottom and top denote the 10th percentile and 90th percentile, respectively. Closed boxes denote the arithmetic mean. Significant difference between the preeclampsia and controls groups ($p<0.05$) is indicated by the vertical line.

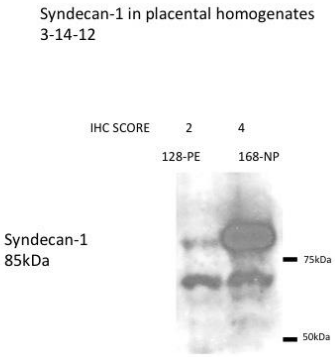
Figure 4.



Legend, Figure 4. SDC-1 immunoreactivity in representative preeclamptic (left) compared to normal pregnancy (right) villous tissue. Note more intense staining of syncytiotrophoblast of normal pregnancy placenta. Soluble SDC-1 was measured in maternal plasma from the same patients and was likewise higher in normal pregnancy.

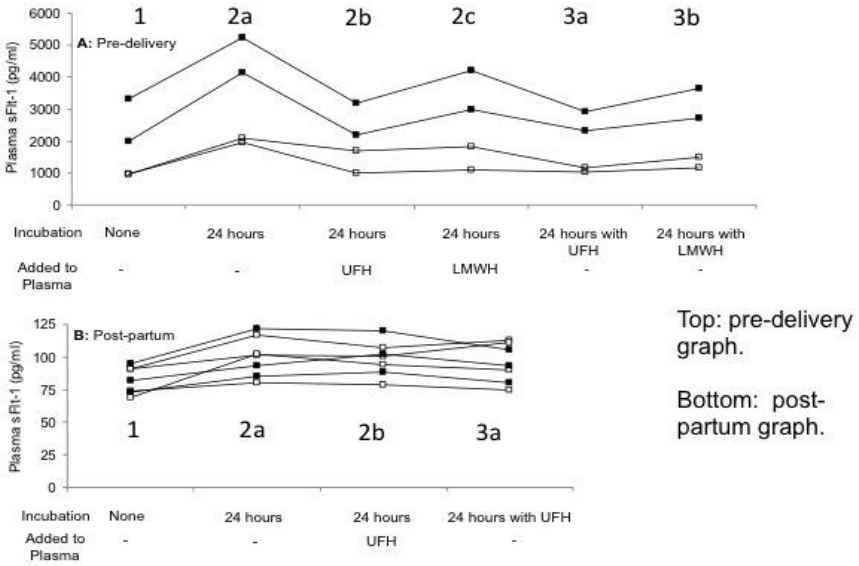
Figure 5.





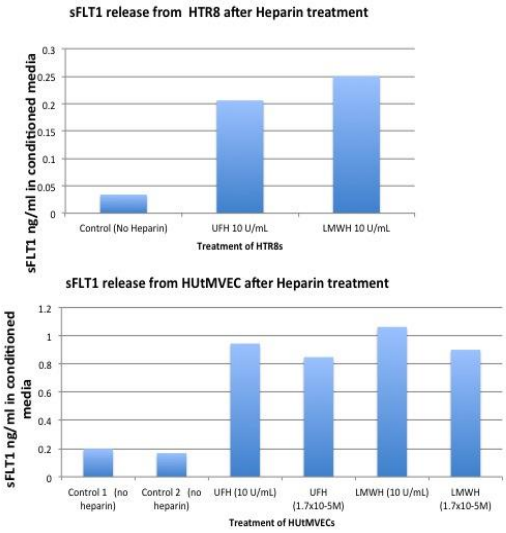
Legend, Figure 5. Top panel: Sample Western blot showing darker bands corresponding to SDC-1 at 85 kDa in preeclampsia placental tissue homogenate, and with dose-response from 20, 40 and 60 micrograms. PE: preeclampsia; NP: normal (uncomplicated) pregnant. MCF7: positive control breast cancer cell line extract. Extracts were from same two patients as used in Figure 4. Bottom panel: Sample Western blot showing darker bands corresponding to SDC-1 at 85 kDa in preeclampsia (128-PE) compared to normal (168-NP) placental tissue homogenate.

Figure 6.



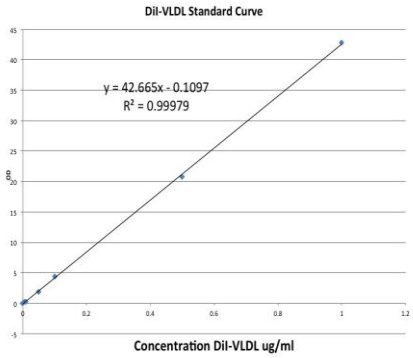
Legend, Figure 6. Pre-delivery samples were treated under six conditions: (1): Plasma was isolated within 2 hours of venipuncture; (2a-c): Whole blood was incubated for 24 hours at 37°C before plasma was isolated and frozen with no additives (2a), or after adding 20 U/ml of unfractionated (2b) or low molecular weight (2c) heparin to the plasma; (3a,b): Whole blood was incubated with 20 U/ml of unfractionated (3a) or low molecular weight (3b) heparin for 24 hours at 37°C before the plasma was isolated. Plasma for all conditions was frozen at -80°C for later analysis. Post-partum samples were treated under four conditions (1, 2a, 2b, 3a), excluding the two conditions involving treatment with low molecular weight heparin (2c, 3b). In pre-delivery samples, plasma sFlt1 was slightly but significantly lower in samples processed within 2 hours than in samples in which whole blood was incubated for 24 hours, or in which whole blood was incubated for 24 hours and LMWH was then added to plasma. Incubating whole blood or plasma with LMH or UFH did not further increase sFlt1; sFlt1 was significantly lower in all conditions in which UFH or LMWH was added to plasma than in samples incubated for 24 hours without heparin ($p<0.05$). In post-partum samples, plasma sFlt1 was slightly higher in all conditions in which whole blood was incubated for 24 hours compared to samples processed immediately ($P<0.05$). However, adding UFH to whole blood or plasma had no additional effect on sFlt1.

Figure 7.



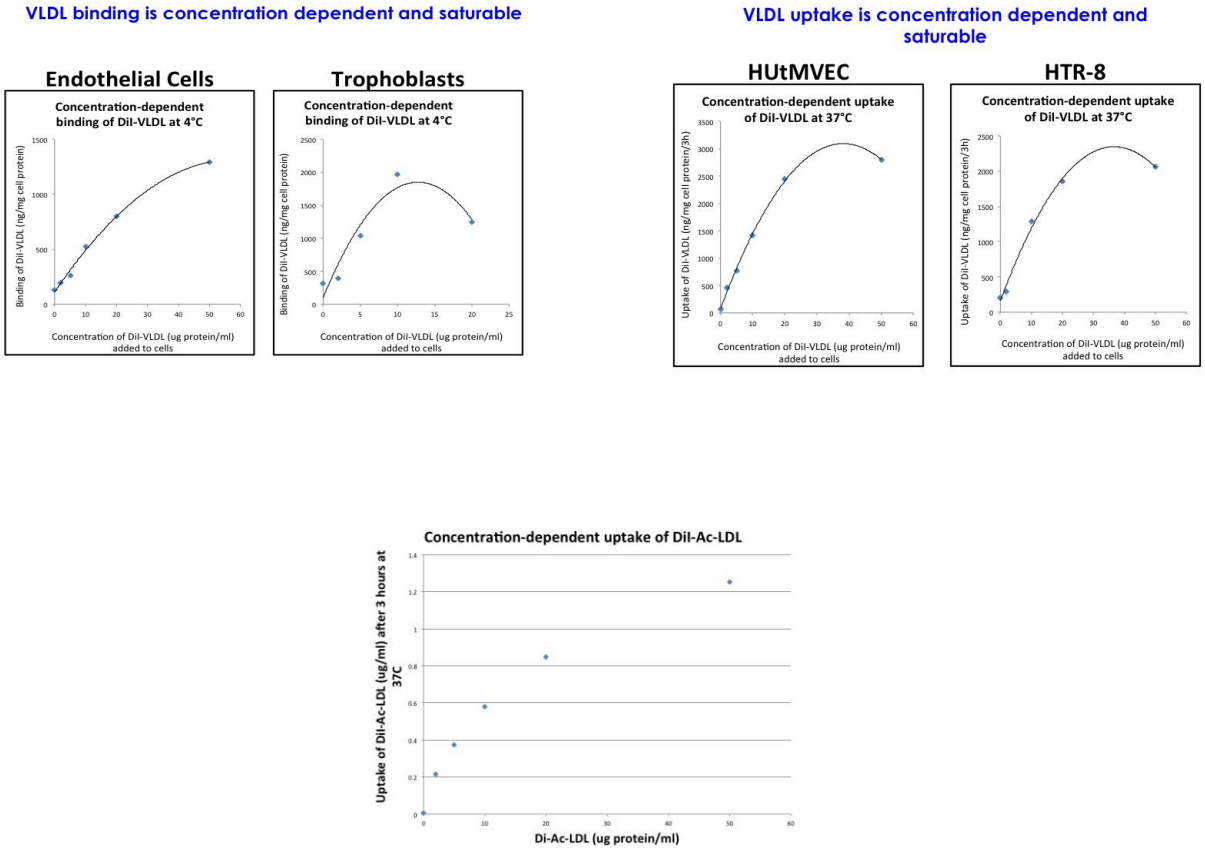
Legend, Figure 7. Human uterine microvascular endothelial cells (HUtMVEC) or 1st trimester trophoblast (HTR8) were washed with low-serum media and then treated with vehicle or 10 U/mL of either unfractionated or low-molecular weight heparin for 30 minutes, and the conditioned media was then collected and assayed for sFlt1 by ELISA. HUtMVEC were also treated with vehicle or equivalent molar concentrations of the two types of heparin ($1x10^{-5}$M = 10 U/mL low molecular weight and 46 U/mL unfractionated heparin). All heparin doses caused a ~4x increase in sFlt1 in the media over baseline.

Figure 8.



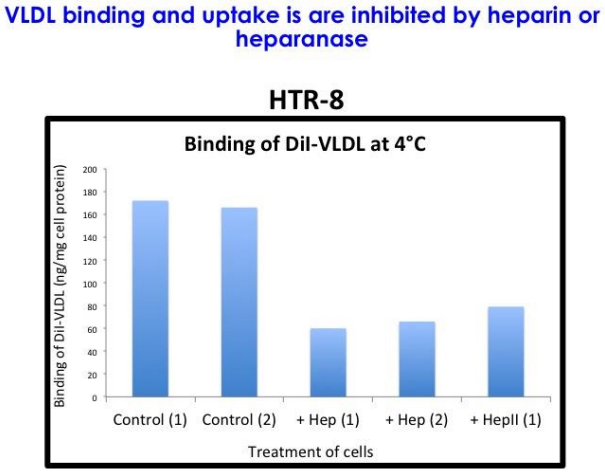
Legend, Figure 8. Fluorescence of DiI-labeled VLDL dissolved in cell lysis reagent. The fluorescence intensity was linear over a range of 0.005 to 10 micrograms VLDL protein/mL. Linearity of 0.005 to 1 microgram is depicted.

Figure 9.



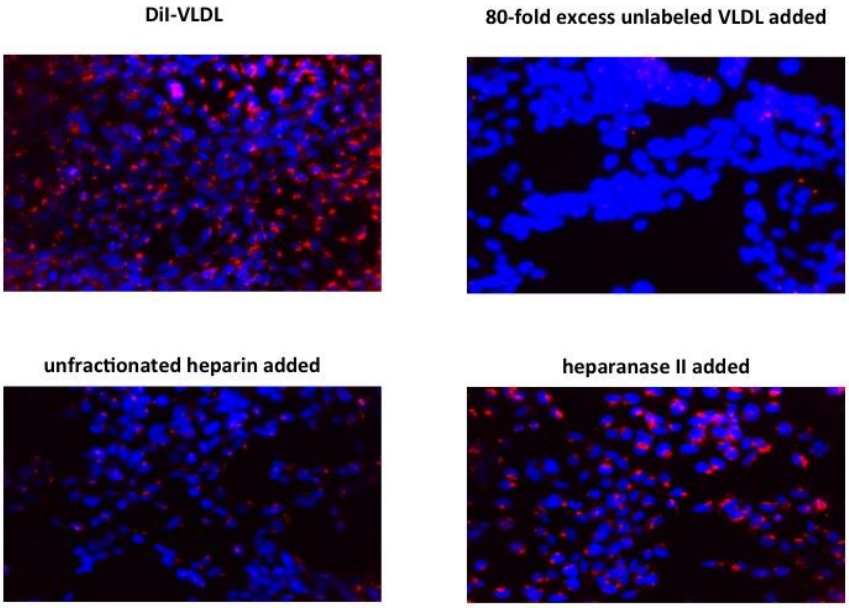
Legend, Figure 9. Top: Concentration-dependent cell surface binding (top left panels) or cellular uptake (top right panels) of DiI-VLDL to human uterine microvascular endothelial cells or human HTR8 SV-neo trophoblast line cells. Bottom: Concentration-dependent cell uptake of DiI-acetylated LDL to human endothelial cells. Cells were incubated with 0-50 micrograms of lipoprotein /mL for 3 hours at 37 degrees. Cells were washed and then lysed in lysis reagent and DiI fluorescence of lysis reagent measured in fluorescence plate reader. Values not corrected for non-specific (non-receptor mediated) uptake, which is <10%.

Figure 10.



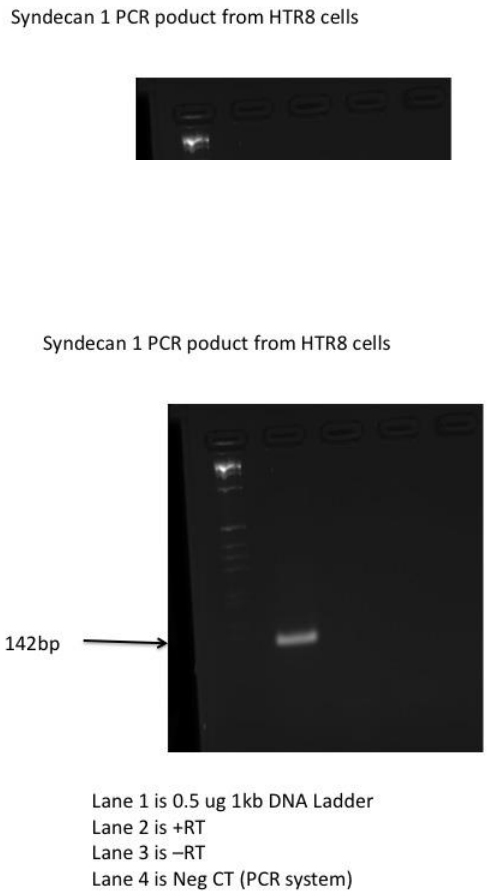
Legend, Figure 10. DiI-VLDL uptake into cytoplasm of HTR8 trophoblast cells in culture measured by colorimetric microplate assay. Hep (1) and Hep (2): duplicate pre-treatments of cells with exogenous unfractionated heparin (1 U/ml) for 2 hours followed by 3 hours incubation with 5 microgram/mL diI-VLDL. HEPII (1): pre-treatments of cells with exogenous recombinant heparanase I and III (15 mU/mL, 2 hours) prior to diI-VLDL incubation. Data are consistent with uptake partially dependent upon cell surface heparan sulfate proteoglycans.

Figure 11.



Legend, Figure 11. Fluorescence microscopic visualization of DiI-VLDL uptake (red) into cytoplasm of HTR8 trophoblast cells in culture. Blue DAPI staining reveals cell nuclei. Top Right panel illustrates the inhibition of VLDL uptake by preincubation of cells with an 80-fold excess of unlabeled VLDL. Bottom left and right panels illustrate the inhibition of VLDL uptake by preincubation of cells with exogenous heparin or heparanase, respectively. Data are consistent with uptake partially dependent upon cell surface heparan sulfate proteoglycans.

Figure 12.



Legend, Figure 12. HTR8 trophoblast cells contain the message (mRNA, Lane 2) for SDC-1.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
____x_No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___x__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___x__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two

publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐ Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____x_____ No_____

If yes, please describe your plans:

1. Manuscript 1:
An article/manuscript has been drafted for submission in April 2013: *"Vascular Stores of Releasable sFlt-1 and Placental Growth Factor: Substantial but Not Different Between Non-pregnant Women After Preeclampsia or Uncomplicated Pregnancy".* It suggests that endothelial surface HSPGs are an important determinant of angiogenic factor regulation in vivo. This research was supported by the NIH Grants P01HD030367 (C. A. Hubel), UL1RR024153 and UL1TR000005 (University of Pittsburgh Clinical and Translational Science Institute), and the Commonwealth of Pennsylvania Health Research Formula Fund (C. A. Hubel).

2. Manuscript 2, in preparation:
Gandley RE, McGonigal S, Myerski A, Powers RW, Jeyabalan A, *Hubel, CA (*corresponding author). *"Reduced soluble syndecan-1 concentration in the maternal circulation precedes preeclampsia and reflects altered placental syndecan-1 kinetics".*

3. Manuscript 3, in preparation:
Hubel CA, Klemencic M, Myerski A, McGonigal S, Rajakumar A, Gandley RE, Powers RW. *"Heparan sulfate proteoglycans mediate binding and uptake of triglyceride-rich lipoproteins by trophoblasts: potential implications for fetal growth restriction".*

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The median soluble syndecan-1 concentration in maternal plasma at mid-gestation is significantly lower in women who later develop preeclampsia, but not gestational hypertension without proteinuria, compared to women with uncomplicated pregnancy outcome. This discovery might eventually have utility in prediction or differential diagnosis of preeclampsia.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___x_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No___

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No____x_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

Carl A. Hubel is a tenured Associate Professor of Obstetrics, Gynecology & Reproductive Sciences, with a secondary appointment in the Department of Environmental and Occupational Health, University of Pittsburgh. Dr. Hubel earned his Ph.D. degree from the University of Vermont, Department of Physiology and Biophysics, in 1988. Dr. Hubel's research has centered upon understanding the pathogenesis of the pregnancy disorder preeclampsia, and why women who have experienced preeclampsia are at heightened risk for developing heart disease in later life. This research has progressively provided evidence that dyslipidemia, oxidative stress, chronic inflammation and angiogenic imbalances interact to promote vascular dysfunction both during and after preeclampsia. Dr. Hubel is currently Program Director of the NICHD-sponsored Program Project, "Mechanisms of Preeclampsia: Impact of Obesity", and thus leads the country's largest program studying mechanisms of preeclampsia. One current focus of his research is to understand the role of vascular glycocalyx dysfunction in preeclampsia and in the increased risk of later-life cardiovascular disease among women who have had preeclampsia.