Plasma Clusterin Increased Prior to Small for Gestational Age (SGA) Associated With Preeclampsia and Decreased Prior to SGA in Normotensive Pregnancies

Marion Blumenstein, Lesley M.E. McCowan, Steven Wu, Garth J.S. Cooper and Robyn A. North

Reproductive Sciences published online 28 February 2012

DOI: 10.1177/1933719111430999

Reproductive Sciences
00(0) 1-8

DOI: 10.1177/1933719111430999
http://rs.sagepub.com

Plasma Clusterin Increased Prior to Small for Gestational Age (SGA) Associated With Preeclampsia and Decreased Prior to SGA in Normotensive Pregnancies

Marion Blumenstein, PhD[1], Lesley M. E. McCowan, MBChB, PhD[2], Steven Wu, PhD[1], Garth J. S. Cooper, MBCHB, Dphil[1,3,4], and Robyn A. North, MBCHB, PhD[2,5] on behalf of the SCOPE consortium

Abstract

In our search for early biomarkers for the pregnancy complicationssmall for gestational age (SGA) and preeclampsia (PE) we analysed plasma from 19-21 weeks gestation in women recruited into the SCOPE study, a prospective cohort of nulliparous women, by differential in gel electrophoresis (DIGE). DIGE revealed the differential expression of clusterin levels and its isoforms in top6-depleted plasma of women who delivered an SGA infant but remained normotensive (SGA-NT; N = 8) compared to healthy women with an uncomplicated pregnancy outcome (Controls, N = 8). Immunosorbent enzyme-linked assay (ELISA) showed that compared to plasma clusterin levels from healthy controls [71.1 (SD 12.4) μg/mL, n = 39], clusterin was decreased in SGA-NT [58.3 (SD 11.7), N = 20, $P < 0.0001$], increased in women with SGA and PE [81.5 (SD 14.8), N = 20, $P < 0.01$], but similar in PE alone [71.2 (SD 9.4)g/ml, $P = 1.0$]. Screening for clusterin levels and/or its different isoformsmay be useful in mid-pregnancy to identify women who subsequently develop SGA but remain normotensive or who develop preeclampsia with SGA.

Keywords

difference in gel electrophoresis, small for gestational age, preeclampsia, plasma, clusterin, DIGE

Introduction

Being born small for gestational age (SGA) can represent failure of a fetus to reach its genetic growth potential in utero and is associated with increased perinatal morbidity and mortality.[1] Individuals who are SGA at birth are more likely to have cognitive and behavioral problems and, in later life, have an increased risk of the type 2 diabetes mellitus and cardiovascular disease. Small for gestational age can be broadly classified into SGA in normotensive women (SGA-NT) and SGA with hypertensive pregnancy complications, including preeclampsia (SGA-PE).[2] A study of nearly 20 000 nulliparous women showed that the majority of SGA infants (82%) are born to normotensive women, approximately 7% to women with preeclampsia (SGA-PE) and 11% to women with gestational hypertension.[3] Currently, there are no blood-based screening tests that enable early prediction of SGA pregnancies.

While aberrant placentation is a recognized common pathology in SGA-NT and SGA-PE, there is limited understanding of the differences in the maternal adaptation resulting in these divergent clinical outcomes.[4,5] Different risk factors are associated with normotensive and hypertensive SGA, with SGA-NT being associated with factors such as smoking and periconceptional nutrition and the women have a normal body mass index (BMI), whereas hypertensive SGA is associated with an elevated maternal BMI.[2] There is a substantial body of evidence that obese women with features of the metabolic syndrome have a maternal predisposition to develop the inflammatory and vascular response that culminates in preeclampsia.[4,5]

Proteomic strategies, either two-dimensional (2D) gel-based or gel-free mass spectrometry-based such as iTRAQ (isobaric tagging for absolute and relative quantification), are now

[1] School of Biological Sciences, Faculty of Science, University of Auckland, Auckland, New Zealand
[2] Department of Obstetrics & Gynecology, Faculty of Medical & Health Sciences, University of Auckland, Auckland, New Zealand
[3] Centre for Advanced Discovery and Experimental Therapeutics (CADET), School of Biomedicine, The University of Manchester, Central Manchester University Hospitals NHS Foundation Trust, Manchester, UK
[4] Maurice Wilkins Centre for Biodiscovery, Faculty of Science,University of Auckland, Auckland, New Zealand
[5] Division of Women's Health, King's College London, UK

Corresponding Author:
Marion Blumenstein, University of Auckland, Kate Edger Information Commons, 11 Symonds Street Private Bag 92019, Auckland 1142, New Zealand
Email: m.blumenstein@auckland.ac.nz

widely used for the discovery of novel biomarkers in pregnancy research by comparing proteomes of biological fluids or tissue sections from healthy versus disease states (reviewed in ref 6). Through an unbiased proteomic approach for potential preeclampsia biomarkers, we and others found plasma clusterin, also known as apolipoprotein J, complement cytolysis inhibitor, complement-associated protein SP-40, or testosterone repressed prostate message 2 to be significantly more abundant in the circulation of women with preeclampsia.[7–10] Clusterin is a highly conserved glycoprotein that circulates as a moderately high-abundance protein at 90 mg/L and is associated with a subclass of high-density lipoprotein cholesterol rich in apolipoprotein A1 and cholesterol ester transfer activity.[11] In contrast to the elevated plasma clusterin levels prior to SGA-PE,[7] we found reduced plasma clusterin protein expression in a pilot proteomics study to identify novel biomarkers for SGA-NT.

Given the shared and also disparate characteristics of SGA-NT and SGA-PE, we analyzed the differential expression of the proteins in week 20 plasma from SGA-NT and healthy pregnancy outcome by differential in gel electrophoresis (DIGE). Proteomics findings were then validated by enzyme-linked immunosorbent assay (ELISA) in plasma samples at 20 weeks of gestation from 4 different patient groups, SGA-NT, SGA-PE, PE with an appropriately grown infant for gestational age (PE alone), and uncomplicated pregnancy.

Patients and Methods

Study Groups and Specimen Collection

Nested case-control studies were performed using plasma collected from pregnant women recruited into the Screening for Pregnancy Endpoints (SCOPE) study between November 2004 and July 2007 in Auckland, New Zealand.[12] The SCOPE is a prospective, multicenter cohort study of nulliparous women, the main aim of which is to develop screening tests to predict preeclampsia, SGA infants, and spontaneous preterm birth. Ethical approval was gained from local ethics committees (New Zealand AKX/02/00/364) and all women provided written informed consent. Participants were interviewed and specimens obtained at 14 to 16 and 19 to 21 weeks of gestation. Blood was collected into EDTA-Vacutainer tubes (Becton Dickinson; BD Australia/New Zealand, Auckland, New Zealand), placed on ice, centrifuged at 2400*g* for 10 minutes at 4°C and stored at −80°C within 4 hours. Women were tracked throughout pregnancy and details of pregnancy outcome recorded in the SCOPE database (MedSciNet[AB]).

The SGA was defined as birth weight <10th customized centile adjusted for maternal weight, height, parity, ethnicity, and infant sex and gestation at delivery (www.gestation.net), unless otherwise stated.[13] Infants with congenital or chromosomal anomalies were excluded. Preeclampsia was defined as systolic blood pressure ≥140 mm Hg and/or diastolic blood pressure ≥90 mm Hg on at least 2 occasions 4 hours apart after 20 weeks of gestation, but before the onset of labor, or postpartum, with proteinuria (24-hour urinary protein ≥300 mg or spot urine protein: creatinine ratio ≥30 mg/mmol creatinine or urine dipstick protein ≥2+) or any multisystem complication of preeclampsia.[14] Multisystem complications included any of acute renal insufficiency (defined as a new increase in serum creatinine concentration ≥100 μmol/L antepartum or >130 μmol/L postpartum); effects on the liver defined as raised aspartate transaminase or alanine transaminase, or both, >45 IU/L and/or severe right upper quadrant pain or liver rupture, neurological effects included eclampsia or imminent eclampsia (severe headache with hyper-reflexia and persistent visual disturbance) or cerebral hemorrhage; and hematological effects included thrombocytopenia (platelets $<100 \times 10^9$/L), disseminated intravascular coagulation or hemolysis. Uncomplicated pregnancy was defined as a pregnancy with no antenatal obstetric, medical, or surgical complications resulting in delivery at ≥37 weeks of gestation of an appropriately grown, healthy baby.

In order to detect changes in biomarkers for PE/SGA which may be greater by 19 to 21 weeks compared to our earlier sampling time point at 14 to 16 weeks, cases were randomly selected from the SCOPE database from women meeting prespecified conditions within the cohort using plasma samples from 19 to 21 weeks of gestation. Using our selection criteria for PE and SGA as outlined below for the proteomics discovery study (DIGE) and clusterin ELISA study, we did not aim at the redistribution of women into subphenotypes of preeclampsia, for example, with/without Hemolysis Elevated Liver Enzymes Low Platelet count (HELLP) syndrome and early- or late-onset disease.

Differential in Gel Electrophoresis Study

In a first step of our present investigation, a proteomics discovery study utilizing DIGE technology involving a small number of patients was used to compare the plasma proteome at 19 to 21 weeks of gestation from normotensive women who delivered an SGA baby (N = 8) with a customized birth weight centile ≤5th to ethnicity-matched controls who had uncomplicated pregnancies (N = 8), as previously described.[7] In brief, Top6 (albumin, transferrin, IgG, IgA, haptoglobin, and α1-antitrypsin) depleted plasma samples from cases and controls were labeled with 200 pmol per 50 μg of protein each with Cy3 and Cy5 Fluor minimal dyes " . . . .(GE Healthcare, Auckland, New Zealand) according to the manufacturer's protocol. To minimize dye bias, within each case and control group, one half of the samples were labelled with Cy3 and the other half with Cy5. All gels included aCy2 labelled internal standard comprising equal amounts of Top6 depleted plasma of all cases and controls in the experiment. First dimension focusing of immobilized pH gradient (IPG) strips (pH 4-7, 11cm) was performed on a Multiphor II flatbed (GE Healthcare) at 20°C for 12 hours to a total applied 54 500 V hours followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8% to 16% Criterion gels using a DoDeca cell (BIORAD) running all 8 gels at a time.

Digitized fluorescent gel images (Typhoon 9410; GE Healthcare) were analyzed in DeCyder v6.5 2D Differential Analysis software (GE Healthcare). Spot volumes, normalized

to the Cy2-labeled internal pooled standard, were exported from the DeCyder software, log-transformed (base 2), and values of cases and controls compared using a moderated *t* test implemented in Limma (Linear Models for Microarray Data) with *P* values, corrected for a false discovery rate (FDR), less than .05 considered significant as described.[7]

Spots of interest were excised from gels, digested with trypsin, and submitted for liquid chromatography–mass spectrometry (MS)/MS analysis on a QSTAR XL ESI-qTOF (Applied Biosystems, Foster City, California). Tandem MS/MS data were extracted from raw spectra using Mascot Distiller (Matrix Science, London, UK). Data were searched against the Swiss-Prot database (version 52.2, date April 14, 2007) using Mascot v2.2.0 with the following parameters. Taxonomy: human; semi-tryptic cleavage with up to 1 missed cleavage allowed; fixed modification: propionamidation; variable modification: oxidation (M), mass tolerances ± 0.1 Da, and peptide charges 2+ and 3+.

Clusterin ELISA Study

For validation of differential protein expression of clusterin in women who later developed SGA-NT in the DIGE study, plasma samples collected at 19 to 21 weeks of gestation from a larger group of women were analyzed by ELISA for total blood clusterin levels in 3 different case groups: (1) normotensive women with an SGA baby with a customized birth weight centile $\leq$5th (SGA-NT, N = 20); (2) women with preeclampsia who had an SGA baby (SGA-PE, N = 20); and (3) preeclampsia with an appropriately grown for gestational age baby (PE alone, N = 20). Controls were women who had an uncomplicated pregnancy (N = 39). For SGA-PE, 20 of the total 21 cases available were included and for the other groups, women were randomly selected from women in cohort with SGA-NT, PE alone, and uncomplicated pregnancies. Clusterin was measured in EDTA plasma collected at 19 to 21 weeks of gestation using a commercially available sandwich ELISA kit to detect human clusterin (BioVendor, Modrice, Czech Republic). Plasma was diluted $\times$3000 with buffer, according to the manufacturer's protocol. Diluted standards, cases and controls, and a quality control (QC) sample (low clusterin QC supplied by manufacturer) were pipetted, in duplicate, into the assay plate wells pre-coated with monoclonal anti-human clusterin antibody. Captured clusterin was detected with a biotin labeled second anti-human monoclonal clusterin antibody. Concentrations of unknown samples were calculated from the standard curve (range 5-160 ng/mL) and were all within the middle range (15-30 ng/mL). Duplicate samples had an average coefficient of variation below 5.5%. Interassay coefficient of variation for the QC sample (expected value 11.36 ng/mL) was 11.2% (n = 3).

Statistical Analysis

For clinical data analyses, chi-square or Fisher exact tests were used to compare categorical variables and for continuous data, Student *t* test was used to compare groups in the DIGE study, and analysis of variance (ANOVA) with post hoc Dunnett test in the clusterin ELISA study (SAS v9.1; SAS Institute Inc, Wellington, New Zealand). Proteomics data were analyzed by Mann-Whitney tests and the moderated *t*-test implemented in Limma (Linear Models for Microarray Data) as previously described whereby a FDR-corrected *P* value <.05 was considered significant.[7] Comparison of clusterin levels (ELISA results) between the 4 different patient groups was performed by ANOVA, with post hoc Dunnett test. Significance was defined as $P < .05$.

Results

Among the 2065 nulliparous women with a singleton pregnancy who were recruited into the SCOPE study in Auckland, pregnancy outcome was known in 2032 (98.4%). In this cohort, there were 162 (8%) SGA-NT pregnancies, 21 (1%) SGA-PE pregnancies, and 64 (3.2%) women with PE alone.

Differential in Gel Electrophoresis Analysis of Week 20 Plasma

Maternal characteristics and fetal outcomes are shown in Table 1. There were no significant differences in maternal characteristics between the healthy control group (N = 8) and women who later developed SGA-NT (N = 8). However, 2 women (25%) in the control group are current smokers with none in the patient group. Due to the nature of gel-based proteomics discovery studies involving small numbers of patients, possible confounding maternal factors for example smoking were not further analyzed statistically.

A clusterin isoform with an approximate isoelectric point (pI) of 4.9 (Spot 501; SwissProt accession P10909; Figure 1A and B) was decreased 2-fold in the SGA-NT group compared to controls, $P < 0.05$. As this was a low abundance spot, it was detectable in only 4 of 8 gels (Figure 1B). This isoform mapped to a unique peptide in the clusterin β chain (AA residue 183-194). To compare, we have shown 2 isoforms of clusterin (spots 17 and 57 at pI $\sim$5.2 to 5.3; NCBInr 2006/07 accession gi|42716297) that we previously reported as increased 2.4- to 3-fold prior to developing SGA-PE (Figure 1B).[7] The isoforms present in spots 17 and 57 had an approximate pI of 5.2 and mapped to unique peptides (AA residues 386-425) in the α-chain of clusterin.

Plasma Clusterin Prior to SGA-NT, SGA-PE, and PE Alone

The clinical characteristics of women and their infants in the clusterin ELISA study are summarized in Table 2. Thirty-nine controls were included, as the ELISA was not performed in one control. Nineteen women with SGA-PE and PE alone had proteinuria, with the remaining woman in each group diagnosed by the presence of multisystem complications. One or more multisystem complication developed in 10 (50%) women with PE alone and 8 (40%) of those with SGA-PE. The uterine

Table 1. Maternal Characteristics and Pregnancy Outcome in Proteomics Study[a]

	Controls, n = 8	SGA-NT, n = 8	*P* Value[b]
Maternal characteristics			
Age, years	30.3 (5.5)	31.5 (5.9)	.67
Caucasian	7 (88%)	7 (88%)	1.0
Body mass index, kg/m^2	25 (4)	24 (5)	.82
Systolic blood pressure at 15 weeks, mm Hg	106 (11)	106 (10)	.95
Diastolic blood pressure at 15 weeks , mm Hg	60 (11)	65 (11)	.35
Current smoker	2 (25%)	0 (0%)	.47
Uterine RI at 20 weeks	0.57 (0.09)	0.58 (0.10)	.86
Gestation at sampling, weeks	20.0 (0.7)	19.9 (0.4)	.77
End of pregnancy			
Systolic blood pressure, mm Hg	117 (7)	120 (9)	.43
Diastolic blood pressure, mm Hg	69 (5)	72 (5)	.23
Fetal outcome			
Gestation at delivery, weeks	40.4 (1.7)	39.4 (1.4)	.23
Birthweight, g	3514 (475)	2718 (315)	.001
Customized birthweight centile	47.6 (25.6)	3.3 (1.8)	.002
Admission to neonatal unit	0 (0%)	1 (13%)	1.0

Abbreviations: SD, standard deviation; RI, resistance index.
[a] Values are mean (SD), or number (%).
[b] *P* value by chi-square or Fisher exact test (as appropriate) for categorical data, by Student *t* test for continuous data.

artery resistance index was increased in the SGA-NT and SGA-PE groups compared to the uncomplicated pregnancy group. There were no perinatal deaths.

At week 20 of gestation, plasma clusterin levels were significantly different across all 4 patient groups ($P < .0001$; Figure 2). Compared to plasma clusterin levels in controls (71.1 [standard deviation, SD 12.4] μg/mL), clusterin was decreased in women who later developed SGA-NT (58.3 [SD 11.7] μg/mL, $P < .0001$), increased in SGA-PE (81.5 [SD 14.8] μg/mL, $P < .01$) but was similar in women with PE alone (71.2 [SD 9.4] μg/mL, $P = 1.0$).

Discussion

Our study demonstrates for the first time that plasma clusterin is decreased at 20 weeks of gestation in women who deliver an SGA infant but remain normotensive (SGA-NT group). This is in contrast to the higher levels of plasma clusterin prior to SGA when the mother later develops preeclampsia (SGA-PE) and confirms our previous findings using DIGE.[7] Clusterin levels measured by ELISA were unchanged prior to the onset of preeclampsia with an appropriately grown infant. To our knowledge, this is the first study to utilize a proteomic approach to search for early pregnancy plasma biomarkers indicative of SGA, defined by customized birth weight centiles and subcategorized according to whether the mothers remained normotensive or developed preeclampsia.

Consistent with our findings in maternal plasma, clusterin has been found to be overexpressed by the syncytiotrophoblast and villous endothelial cells in preeclampsia.[15] An alternative source of clusterin in the circulation may be activated platelets.[16] Moreover, a clusterin gene polymorphism has been linked to preeclampsia.[17] Clusterin modifies several biological functions that have the potential to modulate the maternal response to placental ischemia including scavenging of denatured proteins and cellular microparticles in the circulation, regulation of apoptosis and angiogenesis, enhancement of cholesterol efflux from foam cells, inhibition of proinflammatory gene expression via interference with nuclear factor-kappa β cells, acting as a heat shock protein to protect cells from stress and regulation of complement cascade through blockade of the terminal membrane attack complex thereby preventing cellular lysis.[18–22] Our observation of lower plasma clusterin at 20 weeks of gestation in SGA-NT pregnancies may indicate a mechanism protecting against the development of preeclampsia.[19,20,22] The precise biological implications of our findings are, however, not obvious as the actions of clusterin appear to be dependent on isoform and cellular context.[22]

Clusterin is encoded by a single-copy gene and is present in 2 isoforms (isoform 1 and isoform 2) resulting from alternative splicing. The primary translation product is detectable at ~60 kDa as a glycosylated pre-secretory clusterin (CLU) protein.[22] Following bridging by 4 to 5 disulphide bonds, cleavage, and dense glycosylation, the mature secretory clusterin (sCLU) is produced. This 75 to 80 kDa heterodimer consists of two 35 to 40 kDa disulphide-linked α and β chains, with an estimated 20% to 30% of its molecular mass comprised of *N*-linked carbohydrate attached at 6 to 7 glycosylation sites.[23] The second clusterin gene transcript encodes a nuclear protein (nCLU), derived from isoform 1.[22,24] There is emerging evidence that the ratio of sCLU:nCLU is key in determining the biological action.[22] Clusterin inhibits and promotes angiogenesis and exerts pro- and anti-apoptotic actions.[22,25,26] These opposing actions can in part be explained by functional differences of the clusterin splice variants (sCLU generally has a pro-survival role, whereas nCLU is pro-apoptotic), but their actions are also

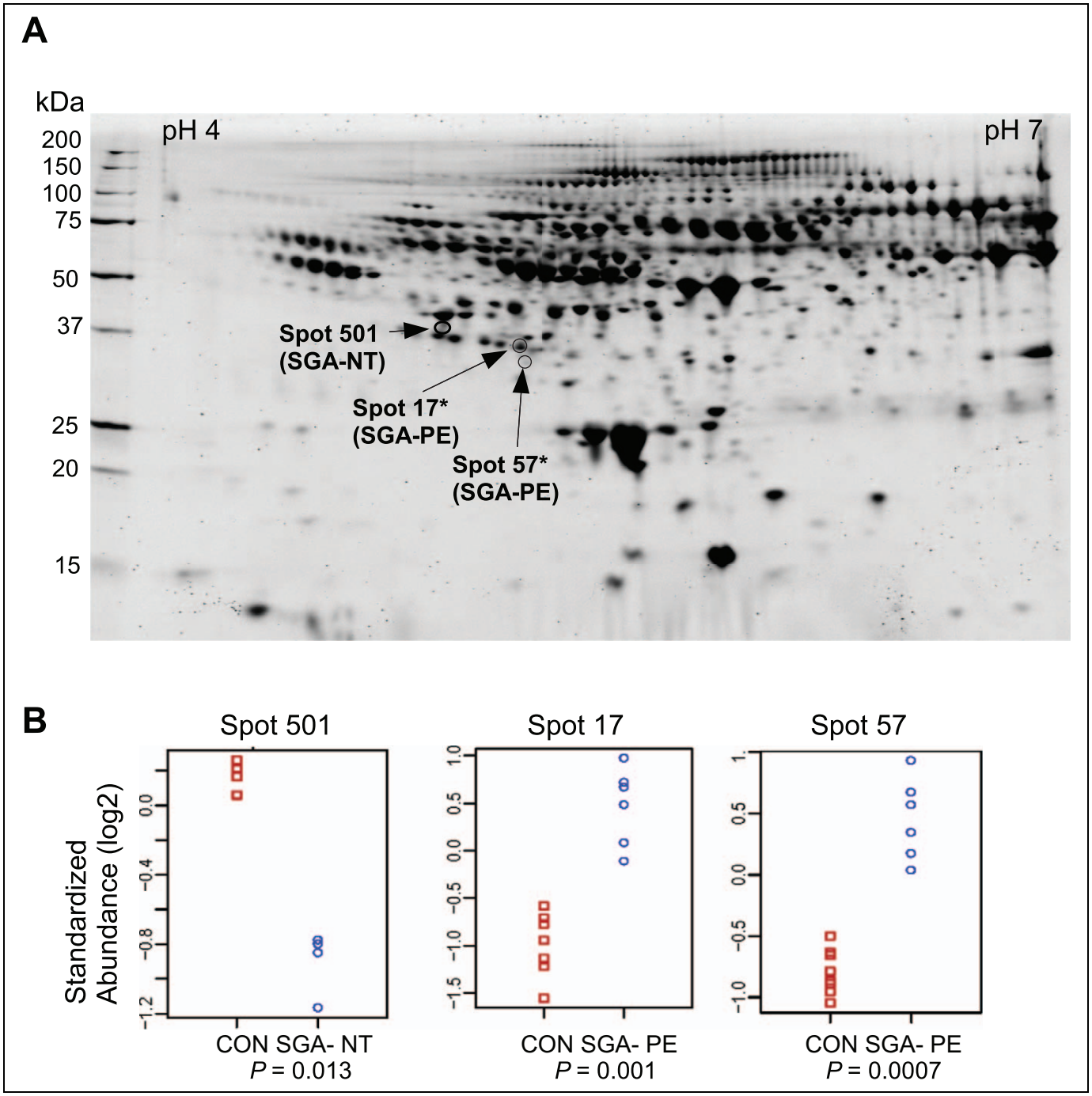

Figure 1. Clusterin isoforms identified following DIGE analysis of early pregnancy plasma depleted of the 6 most abundant proteins. A, Representative image of a digitized fluorescent two-dimensional gel loaded with 150 μg of plasma at week 20 of gestation prior to disease. Clusterin was identified by LC-MS/MS in 3 different protein spots associated with preeclampsia complicated by SGA (SGA-PE) and normotensive SGA (SGA-NT). B, Plots of Cy2-standardized spot volumes of the 3 clusterin isoforms identified in (A). False discovery rate corrected *P* values using a moderated *t* test implemented in Limma are shown.*Numbering for protein spots as previously published.[6] DIGE indicates differential in gel electrophoresis; LC-MS, liquid chromatography-mass spectrometry; SGA, small for gestational age.

influenced by the clinical disease state, tissue or cell type.[22,24] Furthermore, it is likely the glycosylation state of sCLU plays an important role in its biological activity.[27]

Posttranslational modification of plasma sCLU has long been known to contribute to the observed mass variations of clusterin in proteomic studies. The 2D-gel maps of human plasma annotate at least 16 clusterin isoforms that reflect posttranslational modifications, in particular glycosylation, and/or the in vitro reduction of sCLU into α or β chains (http://au.expasy.org/swiss-2dpage/viewer&map=PLASMA_HUMAN&ac=P10909). Our DIGE

Table 2. Maternal Characteristics and Pregnancy Outcome in the Study of Plasma Clusterin Measured by ELISA[a]

	Controls n = 39	SGA-NT n = 20	SGA-PE n = 20	PE-Alone[b] n = 20	*P* Value[c]
Maternal Characteristics[d]					
Age, years	30.6 (3.6)	29.9 (5.3)	31.2 (5.8)	29.4 (3.8)	.58
Ethnicity—Caucasian	32 (82%)	17 (85%)	14 (70%)	16 (80%)	.65
Body mass index, kg/m^2	23 (3)	23 (5)	28 (6)[e]	28 (7)[e]	<.0001
Waist circumference, cm	82 (8)	81 (13)	93 (14)[e]	92 (16)[e]	<.001
Systolic blood pressure, mm Hg	106 (9)	105 (7)	114 (12)[e]	115 (13)[e]	<.001
Diastolic blood pressure, mm Hg	63 (7)	64 (8)	71 (11)[e]	71 (10)[e]	<.001
Current smoker	0 (0%)	1 (5%)	1 (5%)	1 (5%)	.36
Uterine RI at 20 weeks	0.53 (0.08)	0.60 (0.09)[e]	0.62 (0.13)[e]	0.59 (0.10)	<.01
Umbilical RI at 20 weeks	0.70 (0.05)	0.73 (0.04)	0.71 (0.04)	0.69 (0.06)	.07
Gestation at sampling, weeks	20.0 (0.7)	20.0 (0.6)	20.0 (0.7)	20.1 (0.6)	.93
End of Pregnancy					
Systolic blood pressure, mm Hg	119 (9)	117 (9)	159 (19)[e]	163 (11)[e]	<.0001
Diastolic blood pressure, mm Hg	75 (8)	73 (8)	104 (7)[e]	104 (8)[e]	<.0001
Fetal Outcome					
Gestation at delivery, weeks	40.0 (1.1)	38.8 (1.4)	36.0 (3.3)[e]	37.1 (2.7)[e]	<.0001
Preterm birth (<37 weeks)	0 (0%)	2 (10%)	12 (60%)	7 (35%)	<.0001
Birthweight, g	3531 (333)	2470 (286)[e]	2125 (592)[e]	2993 (753)[e]	<.0001
Customized birthweight centile	53.8 (20.0)	2.1 (1.3)[e]	3.3 (2.8)[e]	45.9 (28.2)	<.0001
Admission to neonatal Unit	0 (0%)	4 (20%)	9 (45%)	6 (30%)	<.001

Abbreviations: SGA-PE, small for gestational age complicated with preeclampsia; SGA-NT, normotensive SGA; SD, standard deviation; ANOVA, analysis of variance; RI, resistance index.
[a] Values are mean (SD) or number (%).
[b] PE alone, preeclampsia with a baby appropriately grown for gestational age.
[c] Chi-square or Fisher exact tests was used to compare categorical variables. Continuous data were analyzed using ANOVA with Dunnett *t* test for comparisons of case groups versus the control group.
[d] Measured at 14 to 16 weeks unless otherwise specified.
[e] $P < .05$ comparison between case and control.

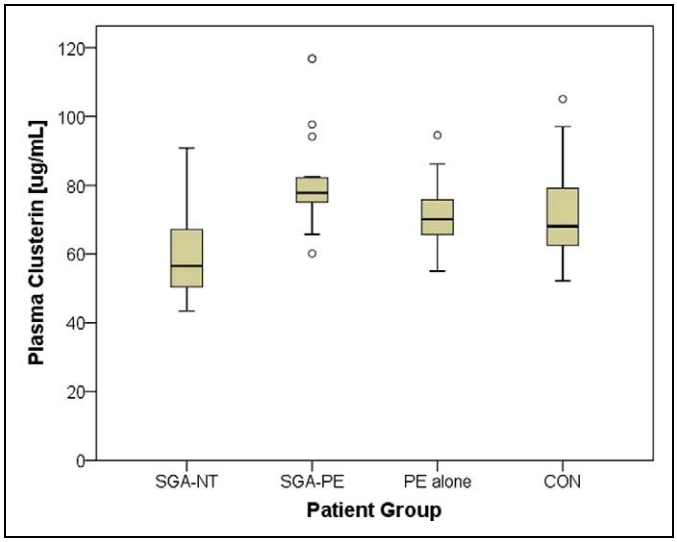

Figure 2. Box plots of plasma clusterin measured by ELISA at week 20 of gestation prior to normotensive SGA (SGA-NT), SGA associated with preeclampsia (SGA-PE), preeclampsia with an appropriately grown baby for gestational age (PE alone) and controls (CON). Compared to mean plasma clusterin in controls (71.1 [SD 12.4] μg/mL), clusterin levels were lower in SGA-NT (58.3 [SD 11.7] μg/mL, $P < .0001$), increased in SGA-PE (81.5 [SD 14.8] μg/mL, $P < .01$) and unchanged in PE alone (71.2 [SD 9.4] μg/mL, $P = 1.0$). ELISA indicates enzyme-linked immunosorbent assay; SGA, small for gestational age; SD, standard deviation.

data indicate downregulation of the clusterin β chain in SGA-NT, whereas the α chain was increased prior to SGA-PE. In SGA-NT, a shift in isolectric point indicates the presence of a more acidic isoform of clusterin, possibly due to the addition of sialic acids as part of its carbohydrate structures.[23] Many placenta-derived proteins are densely sialylated.[28] We postulate that this altered pattern in glycosylation may contribute to any role clusterin plays in the development of the maternal response in preeclampsia or protection from maternal complications in normotensive SGA pregnancies. Another 2D-gel proteomic study of plasma from neonates demonstrated a reduced glycosylation/sialylation pattern of fetuin-A in intrauterine growth restricted babies. [29] Karamessinis and co-workers speculate that reduced fetuin-A sialylation may impair bone formation and cellular adhesion in intrauterine growth restricted foetuses. Furthermore, differentially glycosylated clusterin isoforms are thought to be of potential value as tumor markers.[30]

Recently, Auer and coworkers used iTRAQ to determine differences in the plasma proteomes from women near term with isolated intrauterine growth restriction, isolated preeclampsia, and preeclampsia with intrauterine growth restriction.[10] They reported that clusterin was increased in preeclampsia but did not identify decreased levels in isolated intrauterine growth restriction. Our results may differ due to sampling at different gestational ages (pre disease here vs term in the Auer study) and/or experimental design differences. As clusterin was elevated in

preeclampsia with SGA and 60% of this group had preterm preeclampsia, it may be that clusterin is a marker for early-onset preeclampsia. This will require further studies designed to investigate plasma clusterin levels prior to early-onset preeclampsia.

In summary, plasma clusterin at 20 weeks of gestation was decreased in women prior to developing SGA-NT but increased prior to SGA-PE. Furthermore, the disparate glycosylation pattern in the 2 conditions warrants further investigation into the complex biology of clusterin. Of particular interest is whether differences in isoform expression and glycosylation patterns of clusterin in blood prior to the development of these pregnancy complications could be utilized to develop discriminatory biomarkers.

Acknowledgments

We sincerely thank the women on behalf of the SCOPE consortium who donated blood used in this study, the SCOPE Project Manager, Rennae Taylor for her enormous support coordinating the clinical study, the SCOPE Data Manager, Eliza Chan for her statistical support, the SCOPE research midwives for their dedicated effort recruiting the women and the SCOPE laboratory technicians for their meticulous handling and storage of the specimens.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was funded by the New Zealand Foundation for Research, Science and Technology and by the Health Research Council of New Zealand.

References

1. McCormick MC. The contribution of low birth weight to infant mortality and childhood morbidity. *N Engl J Med*. 1985; 312(2): 82-90.
2. McCowan LME, Roberts CT, Dekker GA, et al; SCOPE consortium. Risk factors for small-for-gestational-age infants by customized birthweight centiles: data from an international prospective cohort study. *Br J Obstet Gynaecol*. 2010; 117(13): 1599-1607.
3. Groom KM, North RA, Poppe KK, Sadler L, McCowan LM. The association between customized small for gestational age infants and pre-eclampsia or gestational hypertension varies with gestation at delivery. *Br J Obstet Gynaecol*. 2007; 114(4): 478-484.
4. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science*. 2005; 308(5728): 1592-1594.
5. Ness RB, Sibai BM. Shared and disparate components of the pathophysiologies of fetal growth restriction and preeclampsia. *Am J Obstet Gynecol*. 2006; 195(1): 40-49.
6. Shankar R, Gude N, Cullinane F, Brennecke S, Purcell AW, Moses EK. An emerging role for comprehensive proteome analysis in human pregnancy research. *Reproduction*. 2005; 129(6): 685-696.
7. Blumenstein M, McMaster MT, Black MA, et al. A proteomic approach identifies early pregnancy biomarkers for preeclampsia: novel linkages between a predisposition to preeclampsia and cardiovascular disease. *Proteomics*. 2009; 9(11): 2929-2945.
8. Watanabe H, Hamada H, Yamada N, et al. Proteome analysis reveals elevated serum levels of clusterin in patients with preeclampsia. *Proteomics*. 2004; 4(2): 537-543.
9. Blankley RT, Gaskell SJ, Whetton AD, Dive C, Baker PN, Myers JE. A proof-of-principle gel-free proteomics strategy for the identification of predictive biomarkers for the onset of pre-eclampsia. *Br J Obstet Gynaecol*. 2009; 116(11): 1473-1480.
10. Auer J, Camoin L, Guillonneau F, et al. Serum profile in preeclampsia and intra-uterine growth restriction revealed by iTRAQ technology. *J Proteomics*. 2010; 73(5): 1004-1017.
11. Jenne DE, Tschopp J. Clusterin: the intriguing guises of a widely expressed glycoprotein. *Trends Biochem Sci*. 1992; 17(4): 154-159.
12. McCowan L, North R, Taylor R. ACTRN12607000551493. Australian New Zealand Clinical Trials Registry, 2007. www.anzctr.org.au/trialSearch.aspx.
13. Gardosi J, Mongelli M, Wilcox M, Chang A. An adjustable fetal weight standard. *Ultrasound Obstet Gynecol*. 1995; 6(3): 168-174.
14. North RA, McCowan LM, Dekker GA, et al. Clinical risk prediction for pre-eclampsia in nulliparous women: development of model in international prospective cohort. *BMJ*. 2011; 342: d1875.
15. Shin JK, Han KA, Kang MY, et al. Expression of clusterin in normal and preeclamptic placentas. *J Obstet Gynaecol Res*. 2008; 34(4): 473-479.
16. Witte DP, Aronow BJ, Stauderman ML, et al. Platelet activation releases megakaryocyte-synthesized apolipoprotein J, a highly abundant protein in atheromatous lesions. *Am J Pathol*. 1993; 143(3): 763-773.
17. Chen M, Yuan Z, Shan K. Association of apolipoprotein J gene 866C–>T polymorphism with preeclampsia and essential hypertension. *Gynecol Obstet Invest*. 2005; 60(3): 133-138.
18. Bartl MM, Luckenbach T, Bergner O, Ullrich O, Koch-Brandt C. Multiple receptors mediate apoJ-dependent clearance of cellular debris into nonprofessional phagocytes. *Exp Cell Res*. 2001; 271(1): 130-141.
19. Santilli G, Aronow BJ, Sala A. Essential requirement of apolipoprotein J (clusterin) signaling for IkappaB expression and regulation of NF-kappaB activity. *J Biol Chem*. 2003; 278(40): 38214-38219.
20. Schwarz M, Spath L, Lux CA, et al. Potential protective role of apoprotein J (clusterin) in atherogenesis: binding to enzymatically modified low-density lipoprotein reduces fatty acid-mediated cytotoxicity. *Thromb Haemost*. 2008; 100(1): 110-118.
21. Sivamurthy N, Stone DH, Logerfo FW, Quist WC. Apolipoprotein J inhibits the migration, adhesion, and proliferation of vascular smooth muscle cells. *J Vasc Surg*. 2001; 34(4): 716-723.
22. Trougakos IP, Djeu JY, Gonos ES, Boothman DA. Advances and challenges in basic and translational research on clusterin. *Cancer Res*. 2009; 69(2): 403-406.
23. Kapron JT, Hilliard GM, Lakins JN, et al. Identification and characterization of glycosylation sites in human serum clusterin. *Protein Sci*. 1997; 6(10): 2120-2133.

24. Cochrane DR, Wang Z, Muramaki M, Gleave ME, Nelson CC. Differential regulation of clusterin and its isoforms by androgens in prostate cells. *J Biol Chem*. 2007; 282(4): 2278-2287.
25. Hellebrekers DM, Melotte V, Vire E, et al. Identification of epigenetically silenced genes in tumor endothelial cells. *Cancer Res*. 2007; 67(9): 4138-4148.
26. Jackson JK, Gleave ME, Gleave J, Burt HM. The inhibition of angiogenesis by antisense oligonucleotides to clusterin. *Angiogenesis*. 2005; 8(3): 229-238.
27. Stewart EM, Aquilina JA, Easterbrook-Smith SB, et al. Effects of glycosylation on the structure and function of the extracellular chaperone clusterin. *Biochemistry*. 2007; 46(5): 1412-1422.
28. Arkwright PD, Redman CW, Williams PJ, Dwek RA, Rademacher TW. Syncytiotrophoblast membrane protein glycosylation patterns in normal human pregnancy and changes with gestational age and parturition. *Placenta*. 1991; 12(6): 637-651.
29. Karamessinis PM, Malamitsi-Puchner A, Boutsikou T, et al. Marked defects in the expression and glycosylation of alpha2-HS glycoprotein/fetuin-A in plasma from neonates with intrauterine growth restriction: proteomics screening and potential clinical implications. *Mol Cell Proteomics*. 2008; 7(3): 591-599.
30. Rodriguez-Pineiro AM, de la Cadena MP, Lopez-Saco A, Rodriguez-Berrocal FJ. Differential expression of serum clusterin isoforms in colorectal cancer. *Mol Cell Proteomics*. 2006; 5(9): 1647-1657.