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Fergus P. Mccarthy, Adedamola Adetoba, Carolyn Gill, Kate Bramham, Maria Bertolaccini, Graham J. Burton, Guillermina Girardi, Paul T. Seed, Lucilla Poston, Lucy C. Chappell

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Title

Urinary congophilia in women with hypertensive disorders of pregnancy and pre-existing proteinuria or hypertension.

Authors

Fergus P MCCARTHY, Adedamola ADETOBA, Carolyn GILL, Kate BRAMHAM, Maria BERTOLACCINI, Graham J BURTON, Guillermina GIRARDI, Paul T SEED, Lucilla POSTON, Lucy C CHAPPELL

Author details

Fergus P MCCARTHY, PhD Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom.

Adedamola ADETOBA, BSc. Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom.

Carolyn GILL, PhD. Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom.

Kate BRAMHAM, PhD. Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom.

Maria BERTOLACCINI, PhD. Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom.

Graham BURTON, PhD, Centre for Trophoblast Research, University of Cambridge, Cambridge, United Kingdom.

Guillermina GIRARDI, PhD. Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom.

Paul SEED, Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom.

Lucilla POSTON, PhD. Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom.

Lucy C CHAPPELL, PhD. Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom.

Declaration of conflicts of interest

Corresponding author

Fergus P McCarthy, Women's Health Academic Centre King's College London, St Thomas' Hospital, London, United Kingdom

Email: fergus.mccarthy@kcl.ac.uk

Telephone: +44 20 7188 3639

Mobile: +44 7578363678

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Condensation: Urinary congophilia is present in women with preeclampsia, women with chronic kidney disease and non-pregnant women with lupus nephritis.

Short version of the article title: Congophilia, preeclampsia and renal disease.

Figure to be included in print issue: Figure 1

Abstract

Background

Congophilia indicates the presence of amyloid protein, an aggregate of misfolded proteins, implicated in the pathophysiology of preeclampsia. Recently urinary congophilia has been proposed as a test for diagnosis and prediction of preeclampsia.

Objectives

To determine whether urine congophilia is present in a cohort of women with preeclampsia and in pregnant and non- pregnant women with renal disease.

Study design

Using a Preeclampsia, Chronic Hypertension, renal disease and Systemic lupus erythematosus cohort, urine samples were analysed from healthy pregnant controls (n=31), pregnant women with preeclampsia (n=23), gestational hypertension (n=10), chronic hypertension (n=14), chronic kidney disease; n=28), chronic kidney disease with superimposed preeclampsia (n=5) and those with chronic hypertension and superimposed preeclampsia (n=12). Samples from non-pregnant controls (n=10) and non-pregnant women with either systemic lupus erythematosus with (n=25) and without lupus nephritis (n=14) were analysed.

For each sample, protein concentration was standardised before mixing with Congo Red, spotting to nitrocellulose membrane and rinsing with methanol. The optical density of the

residual Congo Red stain was determined, Congo red retention calculated and groups compared with Mann-Whitney test or Kruskal-Wallis Analysis of Variance test as appropriate.

Results

Congophilia was increased in urine from women with preeclampsia (median Congo red retention 47% [interquartile range 22-68]) compared to healthy pregnant controls (Congo red retention 16% [13-21]; p=0.002), women with gestational hypertension (Congo red retention 20% [13-27]; p=0.008) or to women with chronic hypertension (Congo red retention 17% [12-28]; p=0.01). There were no differences in Congo red retention between pregnant women with chronic hypertension and normal pregnant controls (Congo red retention 17% [12-28] vs. 16 [13-21] respectively; p=0.72). Congophilia was present in pregnant women with chronic kidney disease (Congo red retention 32% [14-57]), being similar to values found in women with preeclampsia (p=0.22) and for women with chronic kidney disease and superimposed preeclampsia (Congo red retention 57% [29-71]; p=0.18).

Non-pregnant women with lupus nephritis had higher congophilia compared with non-pregnant female controls (Congo red retention 38 [17-73] vs. 9; [7-11%]; p<0.001) and non-pregnant women with systemic lupus erythematosus without nephritis (CRR 38 [17-73] vs. 13 [11-17%]; p=0.001). A significant positive correlation was observed between congophilia and protein:creatinine ratio (Spearman rank correlations 0.702; 95% confidence interval: 0.618 to 0.770; p<0.001).

Conclusion

This study confirms that women with preeclampsia and chronic kidney disease without preeclampsia have elevated urine congophilia compared to healthy pregnant women. Non-pregnant women with lupus nephritis also have elevated urine congophilia compared with healthy controls. An elevated Congo Red retention may not be able to differentiate between these conditions and further research is required to explore the use of congophilia in clinical practice.

Key words; amyloid; Congo red; chronic kidney disease; preeclampsia; renal disease; unfolded protein response; urine congophilia.

Introduction

Preeclampsia, a disease in pregnancy characterised by the development of hypertension and multi-organ manifestations including proteinuria, is a leading cause of maternal mortality, accounting for 17-24% of all maternal deaths in low income settings.[1] Current theories suggest that preeclampsia arises from impaired placentation (trophoblast invasion of the maternal uterine spiral arteries), which in turn leads to placental hypoxia and ischaemia, and stimulation of sustained endoplasmic reticulum and oxidative stress.[2-5] It has been proposed that this pathophysiological cascade generates the characteristic systemic symptoms of the maternal disease.[6] Endoplasmic reticulum stress in the placenta, as in other cell types, leads to upregulation of the unfolded protein response pathway.[7-9] The unfolded protein response is a common cellular defence mechanism that promotes removal of unfolded or misfolded proteins to prevent potentially toxic accumulation. Activation of placental unfolded protein response has been shown to occur in early onset preeclampsia but not in late onset preeclampsia or normotensive controls.[5]

Congo red, initially developed as a textile dye, has been used most commonly to identify amyloid in tissue sections by demonstration of green birefringence under crossed polarisers,[10] including identification of amyloid beta deposits at post-mortem in brain tissue from patients with Alzheimer's disease.[11] As a result of these associations, the presence of Congo red staining itself is now thought to represent protein misfolding due to its propensity to detect proteins with amyloid-like characteristics.[12-14]

Previous work demonstrated the presence of urine congophilia using the Congo red 'dot' test, and the authors proposed that it carries diagnostic and prognostic potential for preeclampsia.[15] This Congo red assay is now being investigated as an innovative mobile health solution in countries with limited resources, as a diagnostic and prognostic tool for preeclampsia.[16]

The aim of this study was to determine whether urine congophilia is present in a cohort of women with preeclampsia and in pregnant and non-pregnant women with renal disease

Materials and Methods

We conducted a retrospective analysis of samples collected as part of a prospective study. Samples were obtained from participants recruited to a multicentre preeclampsia, chronic hypertension, renal disease and SLE cohort.[20] A pragmatic approach was adopted and all samples available for analysis within the cohort were selected and analysed and all data presented. The groups examined are defined in Table 1 and consisted of healthy pregnant controls (n=31), pregnant women with preeclampsia (n=23), gestational hypertension (n=10), chronic hypertension (n=14), chronic kidney disease (CKD; n=28), CKD with superimposed preeclampsia (n=5) and those with chronic hypertension and superimposed preeclampsia (n=12). Exclusion criteria were <18 years old or >50 years old, an inability or unwillingness to give informed consent, known HIV, Hepatitis B or C positive or a multi-fetal pregnancy.

Three additional groups of non-pregnant women were assessed for urinary congophilia, including healthy controls (n=10), women with systemic lupus erythematosus (SLE; n=25) and women with lupus nephritis (n=14). The patients were identified through the Registry of

Connective tissue diseases (10/H0405/35) at St Thomas' Hospital. The National Health Service (NHS) National Research Ethics Service approved the collection and utilization of samples for research purposes. SLE was defined using American College of Rheumatology criteria for the classification of SLE with and without kidney involvement (category III, IV and V according to the International Society of Nephrology/ Renal Pathology Society glomerulonephritis classification).[21,22]

Midstream urine samples were collected into sterile containers, centrifuged at 1400g (10 min), and stored in multiple aliquots at -80℃ within 3 h of collection. Total protein concentration was quantified using the Pierce Bicinchonic Acid (BCA) Assay Kit (Life Technologies) according to manufacturer's instructions, each sample being tested in triplicate. Standards were made according to manufacturers' instructions to cover a range of 20 - 2000 μg/ml and also added in triplicate. Protein;creatinine ratio calculations were derived from total urinary protein quantified using the benzethonium chloride method (Roche Diagnostics) (intra-assay precision: 1-2%, inter-assay precision: 0.9-1.6%) and urinary creatinine concentrations measured with the enzymatic creatinine method (Roche Diagnostics) (urine: intra-assay coefficient of variation 0.8% and inter-assay coefficient of variation 2.1%; serum: intra-assay coefficient of variation 0.9%, and inter-assay coefficient of variation 1.1%).

Congo red retention (CRR) was calculated following the method described by Buhimschi *et al.*[15] The total protein concentration of each sample was standardised to 6.6mg/ml total protein, achieved either by dilution or by concentration in a vacuum centrifuge (Concentrator Plus, Eppendorf, Germany). Following protein standardisation, 100μl of sample was added to 2μl of

Congo Red dye (Sigma). Samples were incubated at room temperature (60min) and 5 μl then spotted in triplicate onto an unsupported nitrocellulose membrane (Nitrocellulose Membrane, 0.2μm, Bio-Rad Laboratories). After drying in air and washing with deionised water (3 min), Congo red was imaged (GelLogic 2200 Pro), using white light illumination and a single exposure of 1 sec (114m field of view; f-stop 13.2). The membrane was then washed in increasing concentrations of methanol (50% methanol: 3 minutes, 70% methanol: 1 minute, 90% methanol until the red in the blank samples disappeared completely (~10 minutes)). A second image was then captured. Analysis was performed with Image J software. The background of each image (obtained from the blank control) was subtracted and the image inverted on the black and white axis to measure Congo red retention (rather than clearance). A standard area of interest was used to obtain a value for the 'mean grey value (GV)', the measure of staining density, for each spot. CRR was calculated by dividing the Grey Value of the spot from the second image by the Grey Value of the same spot in the first image and expressed as a percentage. The CRR was calculated as an average of the triplicates. The CRR index was performed masked to study group. Results were independently double-read

Statistical analysis was performed using GraphPad Prism (Version 6). Results are presented as median with interquartile ranges (non- parametric distribution). Data sets were compared with Mann-Whitney test, Kruskal-Wallis Analysis of Variance (ANOVA) or Chi square as appropriate. Correlation analysis between congophilia and protein:creatinine ratios (PCR) was performed using the Spearman rank correlation.

Results

Baseline characteristics and outcomes for pregnant and non-pregnant study participants are described in Tables 2, 3 and 4. No differences in congophilia were observed between normal pregnant controls and those with gestational hypertension (CRR median 16% [IQR 13-21] vs. 20% [13-27]; p=0.48) or pregnant women with chronic hypertension (CRR 17% [12-28] vs. 16 [13-21]; p=0.72; Figure 1). Congophilia was increased in urine from women with preeclampsia (CRR 47% [22-68]) compared to healthy pregnant controls (CRR 16% [13-21]; p=0.002), women with gestational hypertension (CRR 20% [13-27]; p=0.008) or to women with chronic hypertension without superimposed preeclampsia (CRR 17% [12-28]; p=0.01).

Congophilia also was present in pregnant women with CKD without superimposed preeclampsia (CRR 32% [14-57]), at comparable CRR values to women with preeclampsia (p=0.22) and was not significantly higher in women with CKD with superimposed preeclampsia (CRR 57% [29-71]; p=0.18).

Non-pregnant women with lupus nephritis had significantly higher CRR values compared with healthy non-pregnant controls (CRR 38% [17-73] vs. 9%; [7-11]; p<0.001) and women with SLE without nephritis (13 [11-17]; p=0.001; Figure 2)

Protein:creatinine ratios were also assessed in 174 women (excluding healthy controls). A significant positive correlation was observed between congophilia and protein:creatinine ratios (Spearman rank correlations 0.702; 95% confidence interval: 0.618 to 0.770; p<0.001: Figure 3).

Discussion

This study has confirmed the previously reported presence of urinary congophilia, assessed by CRR, in women with preeclampsia but demonstrates that it is also evident in pregnant and non-pregnant women with other conditions associated with renal impairment. We have also reported a strong correlation between the magnitude of congophilia and protein:creatinine ratios.

Strengths of this study include accurate phenotyping of participants with urine samples collected under standardised conditions and processed consistently to determine CRR. Post-hoc power calculations, demonstrated that this study had sufficient power to detect a mean difference of 20% Congo red retention between normal pregnant women and women with preeclampsia (90% power) or CKD (94% power), preeclamptic women and women with chronic kidney disease (79% power) and between non pregnant lupus nephritis cases and non-pregnant controls (89% power). There was inadequate power to detect differences in other subgroups. In our study the group examining women with preeclampsia included those with term and pre-term preeclampsia. Therefore, these results may not be generalizable to women with preterm preeclampsia (<34 weeks gestation). Additional studies on larger cohorts would allow examination of differences between sub groups.

Buhimschi *et al* reported elevated congophilia in women with superimposed preeclampsia, the values being similar to those observed in women with severe preeclampsia. Congophilia was observed in our study in women with CKD with and without superimposed preeclampsia. It is possible that congophilia identified in women with superimposed preeclampsia by Buhimschi

included those with undiagnosed chronic kidney disease. Buhimschi *et al* also included women with pre-existing nephropathy (n=9) in their study, but this group was amalgamated with cases presenting with intrauterine growth restriction (n=20), liver and/or kidney failure of unknown aetiology (n=4) and anti-phospholipid syndrome (n=4). In this heterogeneous group 22% (8/37) had CRR levels above the 15% threshold used to define a "non–reassuring" value. The inclusion of a large number of normotensive participants with low proteinuria may have resulted in an underestimation of congophilia in women with renal disease, when merged within the heterogeneous group. Other limitations of our study include differences in baseline characteristics across groups including BMI, age, ethnicity and parity which may influence congophilia.

In keeping with our findings of a significant correlation between urinary congophilia and protein:creatinine ratio, Buhimschi *et al* demonstrated a significant correlation between 24 h urine collection and urinary congophilia. In our study, the association was examined across all study groups. Due to limited numbers, the relationship was not addressed between CRR and protein:creatinine ratios in individual study groups.

Placental endoplasmic reticulum stress is considered central to the pathophysiology of preeclampsia. Further research is required to determine the source of these misfolded proteins and to examine whether they are associated with ER stress as has been demonstrated in other pathology [7-9] The median gestational age at delivery was 38 weeks, indicating that the majority fell into the late-onset sub-type of the syndrome. Activation of unfolded protein response pathways is no different in placentas from such patients compared to normotensive

age-matched controls, and the normal ultrastructural appearance of the endoplasmic reticulum indicates no accumulation of misfolded proteins in the syncytiotrophoblast.[5]

In clinical practice particularly in low to middle income countries, chronic kidney disease is prevalent and is estimated to affect up to one in ten women of reproductive age, making confirmation of superimposed preeclampsia challenging.[23-27] These figures are likely to increase in parallel with the increasing prevalence of hypertensive disease and diabetes in these countries. Endoplasmic reticulum stress and the unfolded protein response can be activated by many processes, all of which have been implicated in the pathogenesis of preeclampsia.[31-33] In addition, preeclampsia shares many common aetiological and pathological features with other diseases in which the unfolded protein response pathway has been implicated via endoplasmic reticulum stress, including cardiovascular disease,[34-36] renal disease,[17,18] cerebrovascular disease,[37-41] stroke, metabolic disorders, cancer, inflammatory disease, diabetes mellitus and neurodegenerative disease.[42]

This study confirms that women with preeclampsia and chronic kidney disease without preeclampsia have elevated urine congophilia compared to healthy pregnant women. Non-pregnant women with lupus nephritis also have elevated urine congophilia compared with healthy controls. An elevated Congo Red retention may not be able to differentiate between these conditions and further research is required to explore the use of congophilia in clinical practice.

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TABLE 1: DEFINITIONS USED FOR CLASSIFICATION OF WOMEN

Definition	Criteria
Healthy control women	• No risk factors for preeclampsia • No history of preeclampsia, hypertension, diabetes, renal disease, connective tissue disease or anti-phospholipid antibody syndrome • Systolic blood pressure <140mmHg • Diastolic blood pressure <90mmHg • No protein on dipstick analysis of midstream urine • Not in labour
Gestational Hypertension	• Previously normotensive • Two recordings of systolic blood pressure ≥140mmHg or diastolic blood pressure ≥ 90mmHg greater than 4 hours apart • After 20 weeks' gestation • Not in labour
Preeclampsia	• Gestational Hypertension AND • Proteinuria of >300mg protein over 24 hours, (or protein:creatinine ratio of >30mg/mmol);
Superimposed preeclampsia on chronic hypertension; *Hypertension*	• New onset of proteinuria >300mg protein over 24 hours, (or protein:creatinine ratio of >30mg/mmol); OR Additional features – severe persistent right upper quadrant pain or epigastric pain unresponsive to mediation or alanine transaminase <

already present	71U/l or platelet count <100,000/µl or pulmonary oedema or new onset cerebral or visual disturbance
Superimposed preeclampsia *Proteinuria already present*	• Two recordings of systolic blood pressure ≥140mmHg or diastolic blood pressure ≥ 90mmHg greater than 4 hours apart OR • Additional features as listed above
Superimposed preeclampsia *Hypertension and proteinuria already present*	• Development of severe hypertension (Systolic blood pressure ≥160mmHg or diastolic blood pressure ≥110mmHg) AND • Greater than two fold increase in proteinuria above 300mg protein over 24 hours, (or protein:creatinine ratio of >30 mg/mmol); OR Additional features as listed above
Chronic Hypertension	• Primary or secondary causes of hypertension
Chronic Kidney Disease	• According to Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines pre-pregnancy OR • Persistent proteinuria (>30mg/mmol (protein:creatinine ratio) before 20 weeks' gestation OR • Any recorded serum creatinine >70µmol before 20 weeks' gestation without risk factors for acute kidney injury.

Table 2: Participant characteristics at sampling in the pregnant groups. Values are presented as n (%) or median (interquartile ranges).

Variable	Healthy pregnant controls (n=31)	Preeclampsia (n=23)	Chronic kidney disease (CKD) (n=28)	Gestational hypertension (n=10)	Chronic hypertension (n=14)	Superimposed preeclampsia on chronic hypertension (n=12)	Superimposed preeclampsia on CKD (n=5)	P-Value
Age (years)	32 (30–35)	29 (27-34)	34 (29-38)	30 (29-31)	32 (29-40)	37 (34-42)	24 (22-32)	0.007
Ethnicity (n;%) White European	21 (68)	6 (26)	15 (54)	5 (50)	7 (50)	3 (25)	2 (40)	0.050
BMI (kg/m^2)	24 (22-26)	30 (24-34)	24 (23-30)	32 (22-39)	29 (26-34)	32 (26-37)	33 (27-36)	0.002
Nulliparous (n;%)	21 (68)	15 (65)	13 (47)	7 (70)	6 (43)	4 (33)	4 (80)	0.170
Gestational age at sampling	34 (33-37)	35 (34-36)	30 (21-34)	37 (35-39)	29 (27-32)	35 (34-37)	33 (32-35)	0.018

(weeks)							
Systolic Blood Pressure at booking (mmHg)	110 (100-119)	118 (111-124)	120 (116-126)	123(116-130)	120 (114-135)	134 (129- 140)	121 (112- 131)	<0.0001
Diastolic Blood pressure at booking (mmHg)	66 (60-72)	74 (69-82)	78 (70-88)	72 (63-82)	80 (70- 85)	91 (87- 99)	74 (70-87)	<0.0001
Systolic Blood Pressure at sampling (mmHg)	123 (113-136)	140 (133-151)	118 (114-134)	138 (126-140)	132 (125-135)	145 (141- 155)	138 (136- 156)	<0.0001
Diastolic Blood	75 (67-89)	87 (76- 89)	78 (70-84)	87 (76- 90)	89 (82- 92)	92 (85- 100)	86 (84- 88)	<0.0001

pressure at sampling (mmHg)								
Protein:creatinine ratio (mmol/ml)	10 (0-14)	62 (35-132)	57 (28-112)	14 (13-22)	11 (5-14)	27 (17-44)	76 (62 -261)	<0.0001
Congo red retention (%)	16 (13-21)	47 (22- 68)	32 (14- 57)	20 (13-27)	17 (12- 28)	20 (13- 49)	57 (29- 71)	0.001

Table 3: Birth outcomes in the pregnant groups. Values are presented as n (%) or median (interquartile ranges).

Variable	Healthy pregnant controls (n=31)	Preeclampsia (n=23)	Chronic kidney disease (CKD) (n=28)	Gestational hypertension (n=10)	Chronic hypertension (n=14)	Superimposed preeclampsia on chronic hypertension (n=12)	Superimposed preeclampsia on CKD (n=5)	P value
Gestational age at delivery	40 (39-41)	38 (35-38)	38 (37-39)	40 (38-40)	40 (39- 40)	37 (37- 37)	34 (33- 37)	<0.0001
Mode of delivery (% Caesarean section)	6 (19)	14 (50)	17 (61)	4 (40)	5 (36)	9 (75)	5 (100)	0.0006
Birthweight	3600 (3210-3885)	2400 (2100-2900)	2925 (2500-3200)	3310 (2973-3773)	3350 (3120-3960)	2750 (2473-3105)	2050 (1562-2725)	<0.0001
Neonatal unit admission	0 (0)	7 (30)	1 (4)	1 (10)	1 (7)	1 (8)	1 (20)	0.959

Table 4: Participant characteristics from non-pregnant groups.

Variable	Non-pregnant controls (n=10)	Non-pregnant SLE (n=14)	Non-pregnant lupus nephritis (n=25)	P Value
Age (years; range)	34 (28- 38)	38 (35-45)	36 (30-43)	0.27
Ethnicity (n; %) White European	7 (67)	9 (64)	13 (48)	0.402
Protein:creatinine ratio (mmol/ml)	Not measured	9 (1-15)	51 (17- 123)	0.004
Congo red retention (%)	9 (7- 11)	13 (11- 17)	38 (17- 73)	<0.001

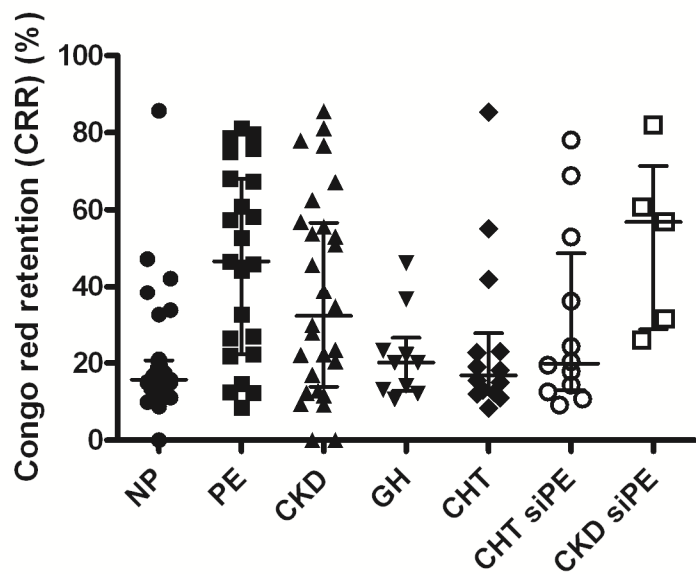Figure 1: Congo red retention index in pregnant participants

NP normal pregnant controls; n=31; PE preeclampsia; n=23; CKD chronic kidney disease; n=28; GH Gestational hypertension; n=10; CHT chronic hypertension; n=14; CHT siPE chronic hypertension with superimposed preeclampsia; n=12; CKD siPE chronic kidney disease with superimposed preeclampsia; n=5.. Congophilia was increased in urine from women with preeclampsia (CRR 47% [22-68]) compared to healthy pregnant controls (CRR 16% [13-21]; p=0.002), Congophilia also was present in pregnant women with CKD without superimposed preeclampsia (CRR 32% [14-57])
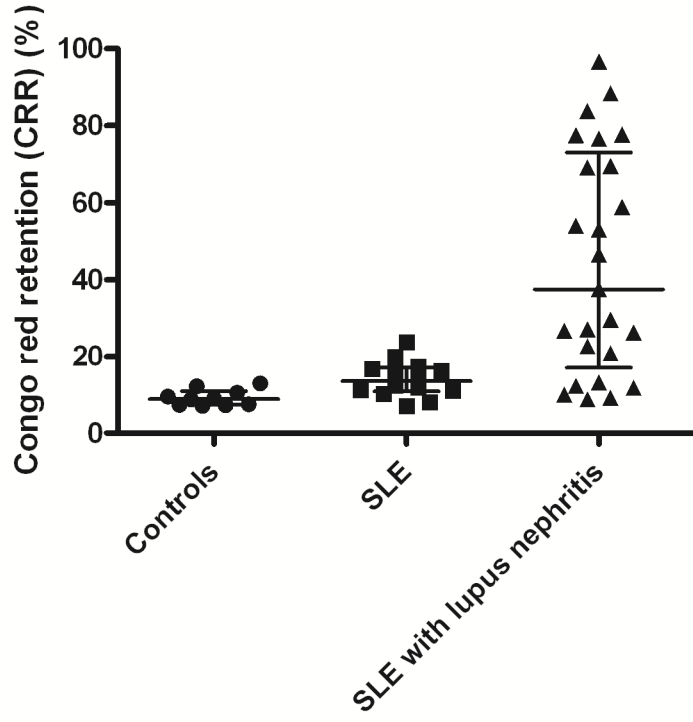
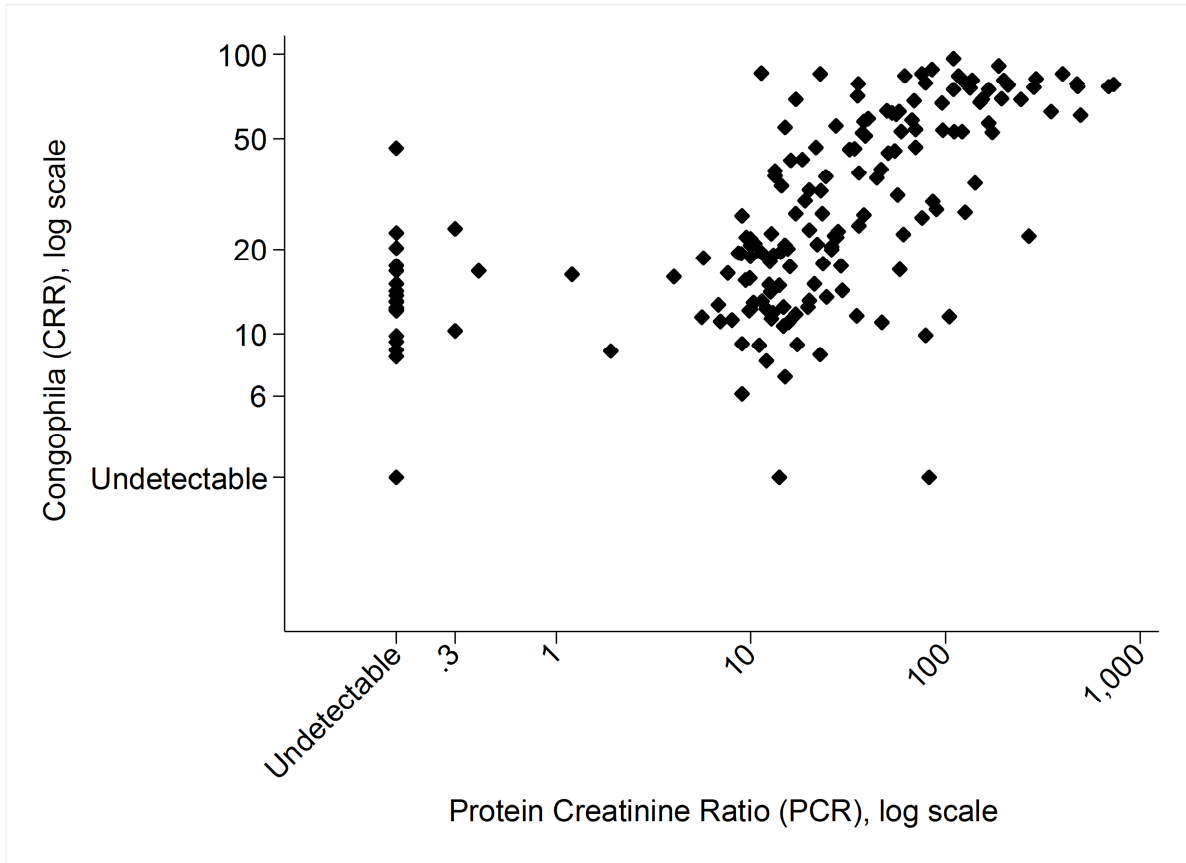Figure 2: Congo red retention index in non- pregnant women

Non-pregnant controls (n=10), non-pregnant women with systemic lupus erythematosus (SLE) without lupus nephritis (n=14), non-pregnant women with SLE with lupus nephritis (n=25). Non-pregnant women with lupus nephritis had significantly higher CRR values compared with healthy non-pregnant controls (CRR 38% [17-73] vs. 9%; [7-11]; p<0.001) and women with SLE without nephritis (13 [11-17]; p=0.001)

Figure 3: Quantitative relationship of urine congophilia with protein:creatinine ratio.

A significant positive correlation was observed between congophilia and protein:creatinine ratios p<0.001).