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Urine excretion of C3dg and sC5b-9 coincide with proteinuria and development of preeclampsia in pregnant women with type-1 diabetes

Short title: Preeclampsia and urine complement

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Abstract

Objective: Pregnant women with type-1 diabetes have an increased risk of preeclampsia with kidney injury and cardiovascular complications. Urine excretion of plasmin and soluble membrane attack complex (sC5b-9) is elevated in severe preeclampsia. We hypothesized a coupling between these events and that active plasmin promotes intratubular complement activation and membrane deposition.

Methods: Stored urine and plasma samples from pregnant women with type-1 diabetes (n = 88) collected at gestational weeks 12, 20, 28, 32, 36 and 38 were used. In the cohort, 14 women developed preeclampsia and were compared with 16 non-preeclampsia controls.

Results: Urine C3dg and sC5b-9-associated C9 neoantigen/creatinine ratios increased and were significantly higher in women who developed preeclampsia. Plasma concentrations did not change with gestation and were similar in cases and controls. Urine plasmin(ogen) correlated to urine C3dg ($r = 0.51$, $p < 0.001$) and C9 neoantigen ($r = 0.68$, $p < 0.001$); urine albumin correlated to C3dg ($r = 0.44$, $p < 0.001$) and C9 ($r = 0.59$, $p < 0.001$). Membrane-associated C3dg and C9 neoantigen was detected in urinary extracellular vesicles from patients but not controls at 36 weeks. ROC curves showed that C3dg and C9 neoantigen were inferior to albumin as predictive biomarkers for preeclampsia.

Conclusion: In preeclampsia, urinary excretion of activated complement relates significantly to albuminuria and to plasmin(ogen) but not to activation in plasma. Intratubular complement activation in preeclampsia is a post-filtration event tightly related to proteinuria/plasminogenuria and a possible mechanistic link to cellular damage and kidney injury.

Keywords: preeclampsia, proteinuria, complement system, plasminogen, type-1 diabetes

Introduction

Preeclampsia (PE) is characterized by new-onset hypertension after 20 weeks of gestation often accompanied by proteinuria in the mother [1]. PE can progress to multi-organ dysfunction, including hepatic, renal and cerebral disease, with eclampsia (maternal seizures) if the fetus and placenta are not delivered [2]. It occurs in 4-5% of all pregnancies and the incidence is increased 2-7-fold in patients with type 1 diabetes [3,4]. A nation-wide Danish study found that the presence of albuminuria in early pregnancy was associated with a fourfold risk of PE [5]. It is well established in PE that the risk for cardiovascular disease, chronic hypertension, chronic kidney disease (CKD) and end stage kidney disease (ESKD) is increased also later in life [6-9].

Increasing evidence suggest that complement is involved in the pathogenesis of PE [10-12]. As part of the innate immunity, the complement system consists of more than 30 proteins activated in cascading fashion by the classic, lectin or alternative pathways [13]. The three pathways converge at the level of C3 conversion. Increased C3 activation subsequently leads to C5 activation and generation of the membrane attack complex (MAC, C5b-9)[14] and the anaphylatoxins C3a and C5a, powerful chemoattractant molecules, that promote immune-cell migration and phagocytosis [15-17]. In PE, we and others have shown active plasmin in urine up to 100-fold above normal pregnancy which is likely generated from the zymogen plasminogen in tubular fluid by urokinase (uPA) [18-21]. Urine soluble (s)C5b-9 is elevated in manifest PE in cross sectional study design [11,22,23] and increases between gestational age (GA) 25-28 weeks to delivery [24]. Urine C5a is elevated in PE [22], and both urine C5a and sC5b-9 correlate to kidney injury marker 1 (KIM-1) in severe PE, linking proteinuria and complement activation with proximal tubular injury [25]. We have previously shown C3dg and

C5b-9 deposition in urine extracellular vesicles (uEV) from proximal tubular cells in kidney transplant recipients with proteinuria [26]. In vitro, active plasmin can directly cleave complement factors C3 and C5 and produce functional C3a and C5a fragments [27-29]. Further cleavage and inactivation of C3b is also mediated by plasmin [29,30], but although plasmin cleaves C3b at slightly different sites than factor I, the fragments produced closely resemble native C3c and C3dg both in structure and biological function [31]. Evidence of plasmin-mediated downstream activation is conflicting and there are no studies in urine. One study found that plasmin generated C5b-9 in rabbit erythrocyte hemolytic assay independent of convertase activity [28]. A different study reported that plasmin inhibited C5b-9 formation and hemolysis in sheep erythrocytes [30]. We hypothesized that in PE 1) complement precursors are aberrantly filtered together with plasminogen from plasma coinciding with proteinuria and 2) that active plasmin in the tubular fluid is associated with complement activation and inflammation.

Methods

Study population

Stored urine and plasma samples from a published, prospective, observational study in pregnant women with pregestational type 1 diabetes were used [32]. The original study included 88 pregnant women with type 1 diabetes at gestational age (GA) 12 weeks and followed them with consecutive clinical evaluations, and blood and urine samples at GA 20, 28, 32, 36 and 38 weeks. Patients were included between August 2013 and January 2015 at two centers: Department of Gynecology and Obstetrics at Aarhus University Hospital and Odense University Hospital. Inclusion criteria were singleton pregnancies, age ≥ 18 years and pregestational type-1 diabetes. Patients with pregestational hypertension or other relevant comorbidities (e.g., systemic inflammatory diseases or preexisting kidney diseases) were

excluded. PE was defined in accordance with national and American guidelines in 2013 [32,33]. In the present study, all women who developed PE during the follow-up period (n = 14), and controls with a complete set of urine samples from inclusion to delivery, blood pressures < 140/90 mmHg and no proteinuria on dipstick (n = 16) were included. Controls were not matched in any other way. Patient characteristics for the control subgroup and PE patients are presented in Table 1. Patient characteristics of the full cohort is published previously [32]. The original study was approved by the regional research ethics committee in Region of Central Denmark (Project ID: 1-10-72-1-13) with amendment by the research ethics committee in Region of Southern Denmark (March 2020, Project-ID: S-20200006), by the Danish Data Protection agency (ID: 1-16-02-69-13) and was registered at www.Clinicaltrials.gov, identification number NCT01821053. The study adhered to the Declaration of Helsinki, and all participants gave written informed consent.

Enzyme linked immunosorbent assay (ELISA)

C3dg and sC5b- associated C9 neoantigen was measured using established ELISAs using neoepitope specific monoclonal antibodies rat α-huC3dg IgM 15-39-06 [34], and α-huC9 neoantigen WU13-15 (Hycult Biotech, Uden, Netherlands)[35], as described previously [26,36]. Samples were stored at -80˚C, thawed on ice and centrifuged prior to analysis (plasma 1,000 g; urine 10,000 g). Ethylenediaminetetraacetic acid (EDTA)- treated plasma was used but urine samples were not protease inhibited.

Isolation of uEVs

uEVs were isolated by from protease inhibited spot urine by ultracentrifugation at 220,000 g for 100 minutes using an L-70 Beckman Ultracentrifuge (Beckman Coulter Inc., Brea, CA, USA) and a type 45Ti rotor, as described here [37]. One cOmplete™ tab (Roche, Basel, Switzerland)

per 50 ml urine was added directly after voiding and samples were stored at -80˚C. uEVs were normalized by relative dilution in PBS to urine creatinine concentration 1 µg/ml.

Immunoblotting

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on Bolt™ 4 – 12% Bis-Tris Plus gels with MES running buffer using the NuPAGE system (Invitrogen, Carlsbad, CA, USA) and blotted onto Trans Blot Turbo membranes (Bio-Rad Laboratories, Hercules, CA, USA) [26]. Membranes were blocked with PBS with 0.05% tween20 (PBStw) with 3% no-fat skim-milk for 1 h and incubated in PBStw with mouse monoclonal antibodies: α-HuC9 WU13-15 (0,25 µg/ml, Hycult) [35], α-CD63 MX-49.129.5 (1:500, sc-5275, Santa Cruz Biotechnology, Dallas, TX) or α-AQP1 (1:1,000, sc-25287, Santa Cruz); rabbit polyclonal: anti-plasmin(ogen) (1:1,000, 154560, Abcam, Cambridge, United Kingdom) or α-tumor susceptibility gene 101 protein (TSG101, 1:1,000, ab30871, Abcam) or sheep α-HuTamm Horsfall glycoprotein (THP, 1:4,000, Biotrend Chemikalien GmbH, Cologne, Germany). Secondary antibodies were HRP-conjugated goat anti-mouse (P0447, 1:2,000, Agilent-DAKO, Santa Clara, CA, USA), goat anti-rabbit (P0448, 1:2,000, Agilent-DAKO), rabbit anti-sheep (P0163, 1:2,000, DAKO, Glostrup, Denmark). Biotinylated α-huC3dg IgM 15-39-06 [34] was used in combination with HRP-conjugated streptavidin (1:3,000, Invitrogen).

Biochemical analyses

Standard clinical biochemical analyses were performed at the Department of Biochemistry and Clinical Pharmacology, Aarhus University Hospital, Aarhus, Denmark, and total plasmin(ogen) (plasmin, plasminogen, and immunogenic fragments) was measured using Human Plasminogen Total Antigen Kit (IHPLGKT-TOT; Innovative Research, Novi, MI, USA) [32]. Estimated glomerular filtration rate (eGFR) was calculated from plasma creatinine by the CKD-EPI formula without correction for race.

Statistical analyses

Controls and PE patients were compared by mixed model two-way analysis of variance (TWANOVA) with repeated measures, and with Tukey's and Šidak's post hoc tests. Students t-test or Mann-Whitney's test was used to compare patient characteristics. Correlations were calculated by the Spearman method. Fractional excretions (FE) were compared to 1 by One-sample Wilcoxon signed-rank test. Normality was tested and data was log-transformed if data were only log-normally distributed. Prism 8 (GraphPad Software, San Diego, CA) was used and $p < 0.05$ was considered statistically significant.

EV-TRACK

We have submitted all relevant data of our experiments to the EV-TRACK knowledgebase (EV-TRACK ID: EV220074)[38].

Results

Patient characteristics

The women who developed PE had a higher BMI and a longer diabetes duration (Table 1). Systolic blood pressure was higher at 12 weeks in the PE group (mean difference 9.6 ± SD 3.2 mmHg), but none had blood pressures above 140/90 mmHg. The control group also had a significantly longer pregnancy (Table 1). At baseline, groups did not differ in kidney function or grade of albuminuria (Table 1), but at the last measurement point before delivery ACR was below detection level in the majority of controls (1 [1 – 3] mg/g) while it was increased significantly in patients that developed PE (171 [12.5 – 626] mg/g, $p < 0.001$). Blood pressure (mean ± SD) at the time of diagnosis of PE was 147 ± 7 mmHg (systolic) and 92 ± 9 mmHg (diastolic).

Urine excretion of activated complement increase during development of PE

The increase in C3dg and sC5b-9-associated C9 neoantigen urine excretion followed disease development and level of albumin excretion and was most prominent closest to delivery. Urine C3dg/creatinine ratios were significantly higher in the PE group ($p < 0.004$, Fig 1A) and there was also a significant effect of gestation alone ($p = 0.001$). Similarly, urine C9 neoantigen/creatinine ratios were higher in PE patients ($p = 0.014$, Fig 1B) and GA had an isolated effect ($p < 0.001$). Plasma levels of C3dg and C9 neoantigen did not change significantly with GA. There were no differences between controls and PE patients in C3dg plasma levels throughout pregnancy, but C9 neoantigen plasma concentrations were significantly higher in PE patients ($p = 0.019$) (Fig. 1C-D). C3dg/creatinine exceeded 10 U/mmol in six patients and occurrence preceded the PE diagnosis with an average 25 ± 27 days. C9 neoantigen/creatinine ratio exceeded 80 U/mmol in 7 patients and preceded diagnosis with 37 ± 45 days.

Relation between urine complement and eGFR

eGFR did not relate significantly to C3dg/creatinine ratio (Fig. 2A) and only a trend towards a negative inverse relationship was observed for C9 neoantigen/creatinine ratio ($r = - 0.23$, $p = 0.084$) (Fig. 2B). Plasma creatinine was higher in PE patients by mixed effects analysis ($p = 0.004$) and increased significantly from GA 12 weeks in both the control and the PE group during pregnancy (Fig. 2C).

C3dg and C9 neoantigen relate to albumin and plasminogen in urine

Urine C3dg and C9 neoantigen related directly and significantly to urine albumin and plasminogen. Urine [albumin] correlated positively to [u-C3dg] (Fig. 3A) and [u-C9 neoantigen] (Fig. 3B, Spearman test), and log-log curve fit slopes were significantly different from 0; C3dg, 0.49 (95%CI 0.26 – 0.76), $R^2 = 0.21$; C9 neoantigen, 0.47 (95%CI 0.25 – 0.77), $R^2 = 0.22$.

Correlations to urine plasminogen were significant and slightly stronger than for albumin (Fig. 3C-D). Log-log curve fit were also better for plasmin(ogen) and slopes were significantly different from 0; C3dg, 0.64 (95%CI 0.44 – 0.89), R^2 = 0.38; C9 neoantigen, 0.70 (95%CI 0.48 – 1.0), R^2 = 0.45.

Fractional excretion (FE) of complement was similar to albumin

The FE was calculated for C3dg and C9 neoantigen and compared to the FE of albumin for the timepoint closest to delivery for PE patients ($FE_{complement}$ / $FE_{albumin}$ ratio). The FE ratio was significantly lower for C3dg at the last measurement before parturition (p = 0.03, one-sample Wilcoxon test). The FE ratio for C9 neoantigen vs. albumin was not different at any timepoint (ratio = 1).

Complement deposition was detected in uEVs in week 36 but not week 12 in PE patients

In three PE patients and two controls, it was possible to collect larger volume of spot urine with immediate addition of a protease inhibitor cocktail to allow for optimal preservation of uEV proteins after ultracentrifugation and comparison of uEV-associated proteins by immunoblotting. C3dg was not detected in uEVs from PE patients or controls at inclusion at 12 weeks but appeared in one of three PE patient at 36 weeks (Fig. 4A). C9 neoantigen was detected in one PE patient and one control at 12 weeks. At 36 weeks C9 neoantigen was present in all three PE patients with increased intensity compared to 12 weeks, whereas the signal was absent in controls, including the sample that was positive at 12 weeks (Fig. 4A). A band corresponding to plasmin was detected only in uEVs from PE patients at 36 weeks (Fig 4B). The uEV markers CD63 and TSG101 were present in all samples (10/10) and the water channel AQP1, a proximal tubular signature protein, only in two PE patients and one control at

12 weeks but in all five samples at 36 weeks (Fig 4C). THP was present in all samples with varying intensity (Fig 4C).

Urine C3dg and C9 neoantigen are inferior to albumin as predictive biomarkers

Receiver operating curves (ROC) were calculated for urine C3dg/creatinine and C9 neoantigen/creatinine ratios and ACR at week 34 and week 36 combined (Fig. 5). The area under the curve (AUC) ± standard error (SE) was for ACR 0.89 ± 0.05 ($p < 0.0001$), C3dg 0.55 ± 0.08, ($p = 0.55$) and sC5b-9 0.56 ± 0.08 ($p = 0.40$).

Discussion

The present study demonstrates in patients with type-1 diabetes that the urine excretion of complement activation products C3dg and sC5b-9 associated C9 neoantigen increases in relation to development of PE and excretion relates significantly in time and abundance with albumin and plasminogen. Conversely, changes in concentrations of C3dg and C9 neoantigen in plasma throughout pregnancy did not relate to PE incidence, urine excretion or eGFR, although C9 plasma levels were higher in the patients that developed PE. In PE, renal tubular complement activation follows the glomerular barrier defect. This notion was corroborated by demonstration of complement activation split products associated with uEVs from patients with manifest PE versus pregnant controls. Complement is deposited on tubular apical membranes and/or podocytes in preeclampsia. C3dg and C9 neoantigen urine excretions were not superior to albumin as predictive biomarkers of PE.

Complement is a potential contributor in the pathophysiological manifestations of PE and regulation of the immune system is essential for placental function and pregnancy [12,39-42]. C3 activation and dysregulation in early pregnancy and C5 activation and C5b-9 formation in late pregnancy play a role in disease development [40,42-45]. Urine excretion of sC5b-9

[11,22,23] and C5a [22] is increased in cross-sectional studies and sC5b-9 increases with gestation in PE [24]. The present data corroborate that C9 neoantigen urine excretion is increased in PE, but show in addition that this coincides with albuminuria, and that it is likely due to glomerular or luminal tubular activation and not to onset of activation in plasma. We found no significant changes in the C3 activation split product C3dg or in C9 neoantigen in plasma over the course of pregnancy. In healthy pregnancy, C3 and C3dg plasma levels increase in second and third trimester [46] while plasma C3dg is not altered in manifest PE, consistent with the present findings. Interpretation of longitudinal observational data in pregnancy is, however, complicated by the physiological plasma expansion (and subsequent hemodilution) in normal pregnancy, and that this plasma expansion is reduced in PE [47]. Surprisingly, we observe a rise in plasma creatinine in both groups which is in contrast to normal pregnancy [48]. This highlights the importance of disease-matched controls and suggests an underlying stress on the kidney function during pregnancy in patients with type 1 diabetes.

The women who developed PE varied with respect to the presence of albuminuria. Some exhibited very rapid onset of PE and proteinuria and delivered before the next protocolled timepoint, while some patients were diagnosed based on new onset hypertension and end organ damage without proteinuria. Our data demonstrate that it was those patients who developed albuminuria that excreted activated complement. A striking observation here was that in urine, complement associated directly and closely with albumin and plasminogen. Coagulopathy and systemic inflammation are typical features in PE [40], and crosstalk between complement and coagulation system components may be important for successful pregnancy [27,41]. We found that plasmin(ogen) related significantly and directly with C3dg and C9 neoantigen in urine and that the relation was stronger than for albumin. The coincidence of active plasmin and complement activation split products in tubular fluid and colocalization in

uEVs is compatible with a pathophysiological causal link but this awaits mechanistic studies. A cartoon showing tentative interaction between plasmin and complement is shown in Figure 5B. Complement regulators circulate and are present in placenta to prevent illicit activation [12]. Plasminogen activator inhibitor type 2 (PAI2/SERPINB2) is produced by the trophoblasts during pregnancy which inhibits plasminogen activation by uPA and tissue-type plasminogen activator (tPA), and thus reduces fibrinolytic activity [49]. Lower levels of PAI2 are associated with PE [49,50] which could explain why plasminogen urine activation and excretion is increased 100-fold in PE [18,19].

The longitudinal-observational data presented here, indicate that the increased excretion of sC5b-9 in PE is related to the filtration barrier defect and intratubular complement activation and not filtration of activation products from plasma [11,22-24]. We do observe higher levels of plasma C9 neoantigen in PE patients already from early pregnancy and higher levels of C5b-9 in PE have been reported by others [11,51,52]. However, we observe no increase over the course of pregnancy, and it is possible that the difference can be ascribed to shorter diabetes duration, lower BMI, and lower blood pressure at baseline in the present group of controls.

sC5b-9 is a macromolecule with an average size of 1000 kDa. Intact sC5b-9 would have a lower passage across the glomerular filtration barrier than albumin in PE and the presence of C9 neoantigen both in crude urine and associated to uEVs strongly suggest post-filtration assembly and exposure of the neoantigen. Accordingly, the concept of post-filtration generation relates to surface binding/deposition and subsequent activation. Proteinuria relates directly to podocin, nephrin and synaptopodin mRNAs in pelleted urine samples from patients with PE, indicating that the loss/detachment of podocytes to the urine relates to the glomerular barrier defect [53]. In accordance, the present data show that the glomerular barrier defect relates to complement activation and deposition in uEVs. Podocytes shed uEVs

and express complement receptor 1 (CR1) that can activate C3 and bind C3, C3b and iC3b [14,54-56], but isolation by ultracentrifugation cannot differentiate between podocyte and tubular uEVs. It is probable that some of the complement we detect in uEVs is associated with podocyte uEVs, but we have shown in kidney transplant recipients with proteinuria that complement is also found on uEVs originating specifically from the proximal tubules [26].

Urine sC5b-9 differentiates between hypertensive controls and PE with severe features (AUC = 0.70) but not PE without severe features [22]. The patients in the present study had a mix of both mild and severe PE and sampling was per protocol at fixed timepoints. PE can accelerate rapidly and samples from the acute phase are often lacking. It is therefore not surprising that urine C3dg and sC5b-9 could not predict PE incidence and were inferior to ACR as diagnostic biomarkers for PE. On the other hand, acute kidney injury is common in PE [57] and increased future risk for kidney disease after PE is well established [7-9]. Complement activation could contribute to such detrimental effects since C3dg and C5b-9 were associated with the proximal tubular water channel AQP1 in uEVs from patients with PE. This is in accord with findings that complement activation relates to urine KIM-1 that originates from proximal tubules. KIM-1 excretion is significantly elevated in PE [25,58]. Urine levels of the proximal tubular injury marker N-acetyl-beta-D-glucosaminidase (NAG) is also higher in PE [59,60]. We previously demonstrated iC3b/C3dg and C5b-9 in proximal tubular uEVs by sodium glucose transporter 2 (SGLT2) co-immunoprecipitation and by enrichment by Lotus Tetragonolobus lectin-affinity in kidney transplant recipients with albuminuria [26].

Strengths and limitations: The current study included only 16 of 74 non-PE controls from the original study as many had one or two missing samples, urine dipsticks with proteinuria or elevated blood pressures without developing preeclampsia. This could have introduced bias as these controls might be healthier and more adherent than others. The original study was

designed to investigate plasminogen excretion but not complement activation and urine samples used for ELISA were not protease inhibited, contrary to the samples saved for uEV analysis. A strength of the present study is the longitudinal consecutive sampling which allows direct comparison between changes in plasma and urine. Moreover, the novel monoclonal C3dg antibody applied here is highly specific [34,61] and allows unprecedented accuracy in detection and quantification of C3 end activation in vivo in humans. Also, C3dg and C9 neoantigen are very stable across multiple freeze-thaw cycles, at least in protease inhibited urine [26].

Conclusion

In patients with type 1 diabetes and high-risk pregnancy, development of PE coincides with tubular luminal activation of complement and plasminogenuria with no changes in complement activation in plasma. In manifest PE, complement activation split products and active plasmin is deposited within apical membranes contained in uEVs which demonstrates membrane attack secondary to proteinuria. In perspective, complement activation might contribute to kidney injury and inflammation from the apical side of the tubules. Complement inhibitors reaching the tubular lumen might have therapeutic effect

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Author contributions

GLI, LHN, CB, KM, YP, BLJ conceptualized and designed research, GI performed the experiments, LHN, DMJ, LLTA, JSJ, PGO performed the original study and collected the biobank, GI and BLJ drafted the manuscript. GLI, LHN, YP, DMJ, LLTA, KM, CB, JSJ, PGO and BLJ revised the manuscript. All authors approved of the final version of the manuscript.

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Legends

Figure 1. Urine excretion of C3dg and sC5b-9 associated C9 neoantigen was significantly higher in patients that developed preeclampsia (PE) and increased with gestational age (GA) during development of PE. Graphs show consecutive measurements from week 12 and throughout pregnancy in women with T1DM that served as controls and patients that developed PE for A) urine C3dg/creatinine, B) urine C9 neoantigen (C9 neo)/creatinine, C) plasma C3dg concentration and D) plasma C9 neoantigen concentration. Data are presented as geometric mean with geometric SD (controls, ○ and dashed line, GA 12 - 36 weeks n = 15 - 16, GA 38 weeks n = 8; PE patients: □ and full line, GA 12 to 32 weeks: n = 12 - 14, GA 36 weeks, n = 8; GA 38 weeks, n = 1). Individual patients are shown in gray (controls: dashed line; PE patients: full line). Differences between groups were analyzed by mixed model two-way analysis of variance with repeated measures; p represents differences between controls and PE patients.

Figure 2. Estimated glomerular filtration rate (eGFR) in PE patients does not correlate to A) C3dg or B) C9 neoantigen urine/creatinine ratio. All measurements in patients with PE from gestational age (GA) 12 weeks to parturition (n = 57) are included. Semi-logarithmic curves were fitted for eGFR and C3dg, slope -3.3 (95%CI -7.5 to 0.87) and C9 neoantigen, slope -4.0 (95%CI -7.0 to -1.0). C) Plasma creatinine concentration increased in patients both groups, both in those that developed PE and in controls over the course of pregnancy and was significantly higher in PE patients (repeated measures mixed model two-way analysis of variance p = 0.004). Data shown as mean ± SD for controls (○ and dashed line; GA 12 - 36 weeks n = 15-16, GA 38 weeks n = 8) and PE patients (□ and full line; GA 12 to 32 weeks: n = 13-14, GA 36 weeks, n = 8; GA 38 weeks n = 2). Šidak's post hoc test between groups: #p<0.01, Tukey´s post-hoc test from baseline at 12 weeks: **p < 0.01, ****p < 0.0001. Spherical distribution was assumed.

Figure 3. Urine concentrations of C3dg and sC5b-9-associated C9 neoantigen correlated significantly to albumin and plasminogen concentrations by Spearman correlation. Graphs show urine albumin concentration vs. A) C3dg and B) C9 neoantigen and urine plasminogen concentration vs. C) C3dg and D) C9 neoantigen concentrations. Detection level for C3dg was set to 0.01 U/ml and for C9 neoantigen 0.1 U/ml. Urine albumin concentrations below detection level was defined as 1 mg/L. Log-log curves were fitted by least square fit.

Figure 4. Urine extracellular vesicles were isolated by ultracentrifugation at 220.000 g for 100 minutes from three patients that developed pre-eclampsia (PE) and two pregnant women with normal pregnancies that served as controls (C) at gestational age (GA) 12 and 36 weeks. A) Western blots show C3dg (free 37 kDa, protein bound ~53 kDa) and C9 neoantigen (61 kDa) in human plasma diluted 1:100 (P), patients with preeclampsia (PE) and controls (C); B) plasmin (63 kDa) was associated with uEVs from patients that developed PE at 36 weeks but not at 12 weeks. C) The uEV markers CD63 (migration at 30 – 60 kDa), TSG101 (migrating at ~50 kDa) were present in all samples; the proximal tubular transmembrane water channel aquaporin 1 (AQP1 ~28 kDa) was present in 3 samples at 12 weeks and in all samples at 36 weeks. THP was also detected in all samples. Sample concentrations were normalized by relative dilution in PBS to urine creatinine 1 μg/ml.

Figure 5. Receiver operating characteristics (ROC) curves calculated from the last two measurements before delivery in preeclampsia (PE) patients and controls. A) Albumin/creatinine ratio (ACR, full drawn line) had an area under the curve (AUC) ± SE = 0.89 ± 0.05, $p < 0.0001$. C3dg/creatinine ratio (dashed line, AUC = 0.55 ± 0.08, $p = 0.55$) and sC5b-9 associated C9 neoantigen (dotted line, AUC = 0.56 ± 0.08, $p = 0.40$) did not distinguish controls from cases and were inferior to ACR. Measurements in patients serving as controls, n = 32; measurements in patients that developed preeclampsia, n = 25. B) Schematic overview

showing potential interaction of plasmin with the complement cascade, adapted and modified after *Amara et al. J Immunol 2010* [27]: Plasmin generates C3a and C5a in plasma but promotes degradation of C3b to C3c-like and C3dg-like fragments [31], and might both promote and inhibit the terminal pathway and C5b-9 formation [28,30]. Full drawn line with arrows: pathway of activation, dashed lines with arrow: proteolytic activation, full drawn line with bar: inhibition. uPA, urokinase type plasminogen activator.

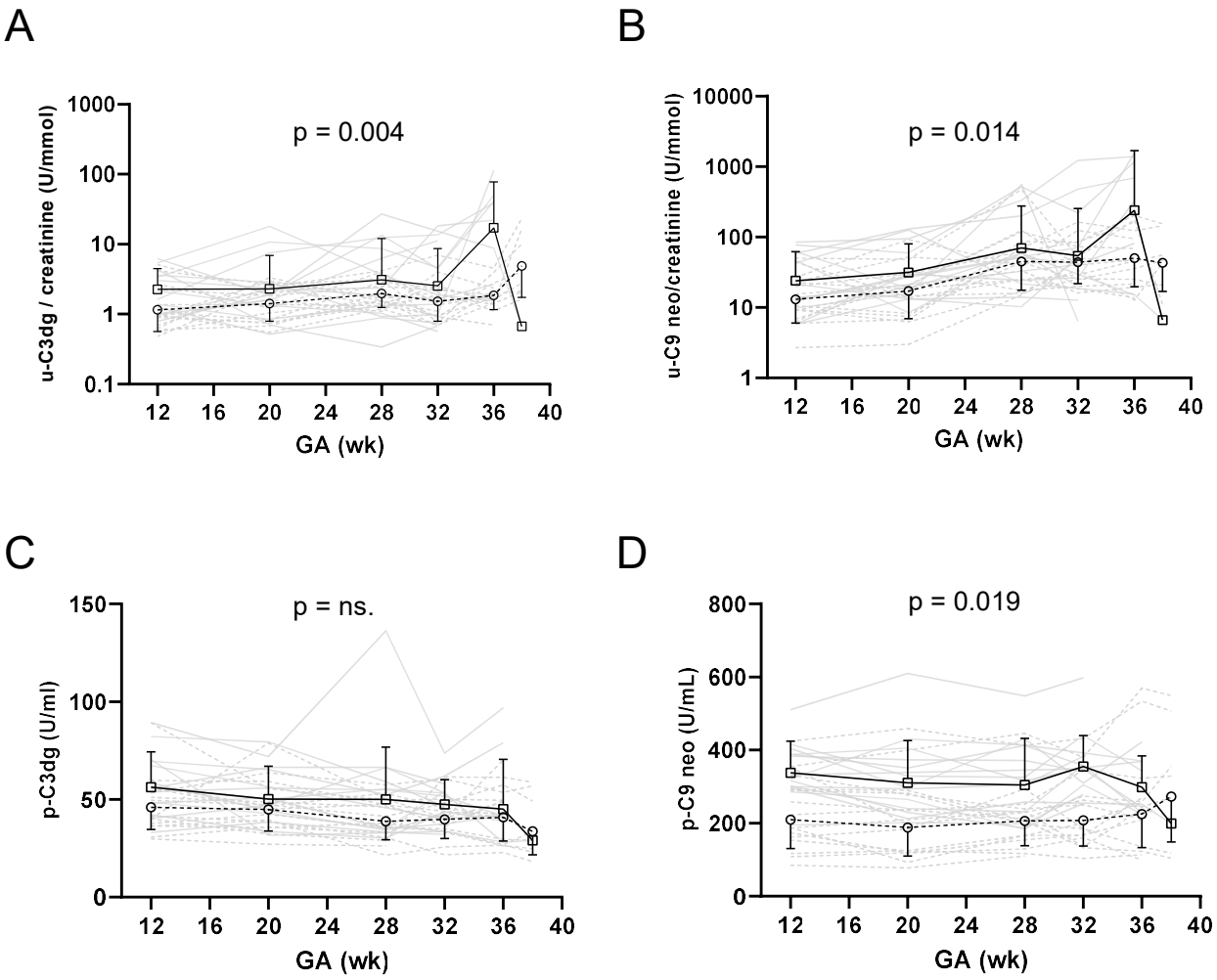
A
p = 0.004
u-C3dg / creatinine (U/mmol)
1000
100
10
1
0.1
12 16 20 24 28 32 36 40
GA (wk)
B
p = 0.014
u-C9 neo/creatinine (U/mmol)
10000
1000
100
10
1
12 16 20 24 28 32 36 40
GA (wk)
C
p = ns.
p-C3dg (U/ml)
150
100
50
0
12 16 20 24 28 32 36 40
GA (wk)
D
p = 0.019
p-C9 neo (U/mL)
800
600
400
200
0
12 16 20 24 28 32 36 40
GA (wk)

Figure 1

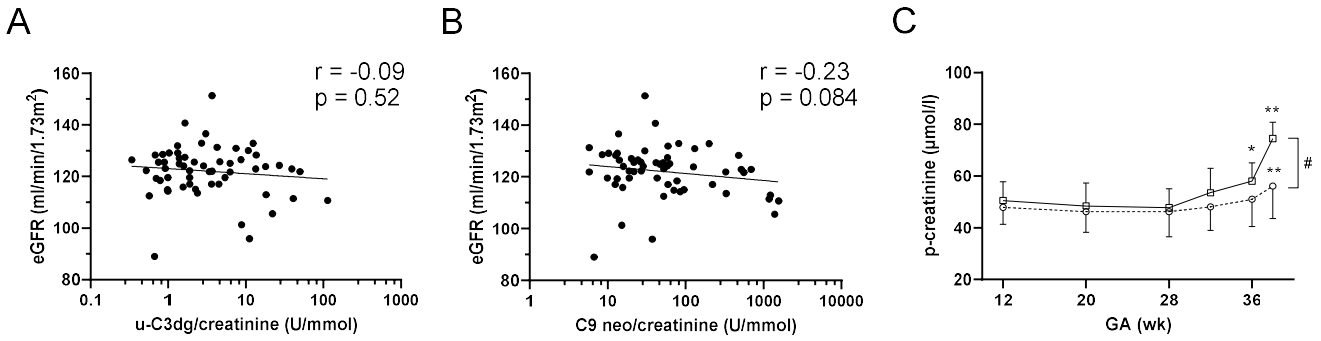
A
B
C
r = -0.09
p = 0.52
r = -0.23
p = 0.084
eGFR (ml/min/1.73m²)
u-C3dg/creatinine (U/mmol)
C9 neo/creatinine (U/mmol)
p-creatinine (µmol/l)
GA (wk)
*
**
**
#

Figure 2

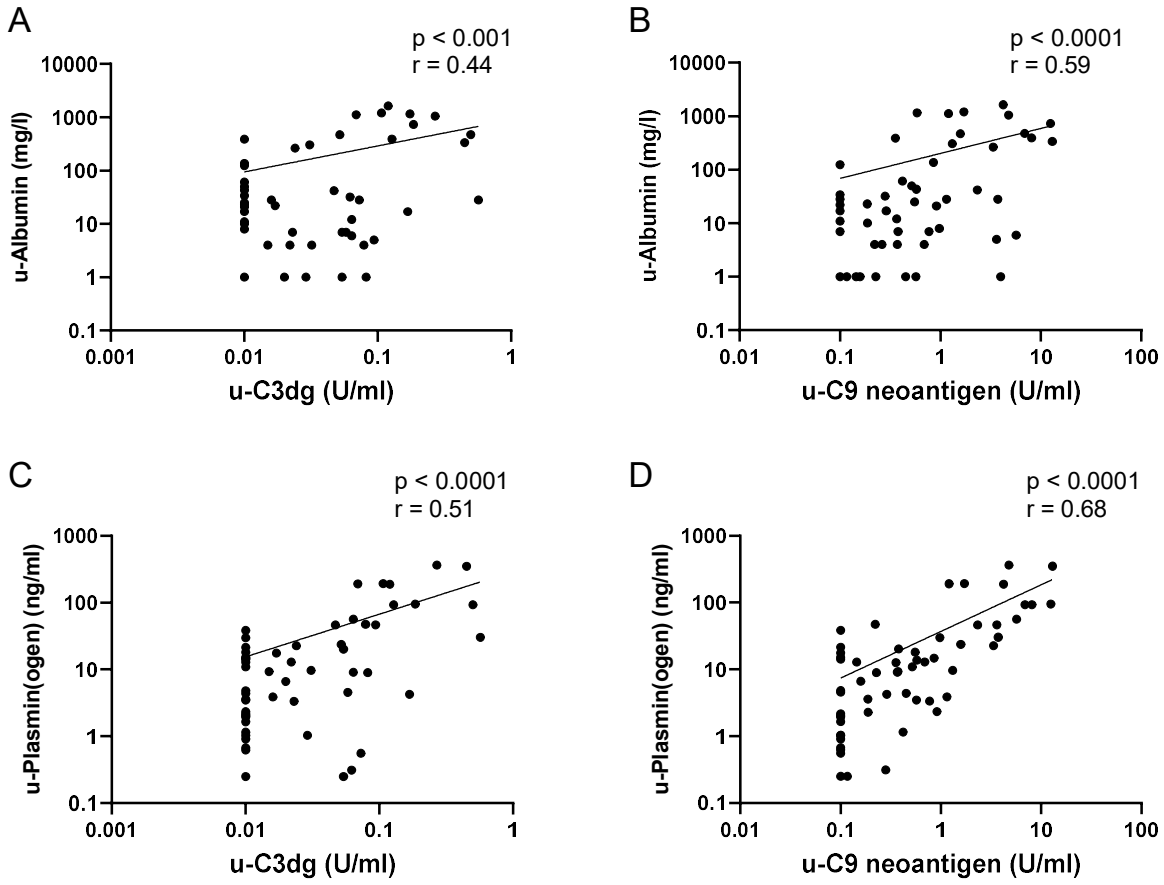
A
p < 0.001
r = 0.44
u-Albumin (mg/l)
10000
1000
100
10
1
0.1
0.001
0.01
0.1
1
u-C3dg (U/ml)
B
p < 0.0001
r = 0.59
u-Albumin (mg/l)
10000
1000
100
10
1
0.1
0.01
0.1
1
10
100
u-C9 neoantigen (U/ml)
C
p < 0.0001
r = 0.51
u-Plasmin(ogen) (ng/ml)
1000
100
10
1
0.1
0.001
0.01
0.1
1
u-C3dg (U/ml)
D
p < 0.0001
r = 0.68
u-Plasmin(ogen) (ng/ml)
1000
100
10
1
0.1
0.01
0.1
1
10
100
u-C9 neoantigen (U/ml)

Figure 3

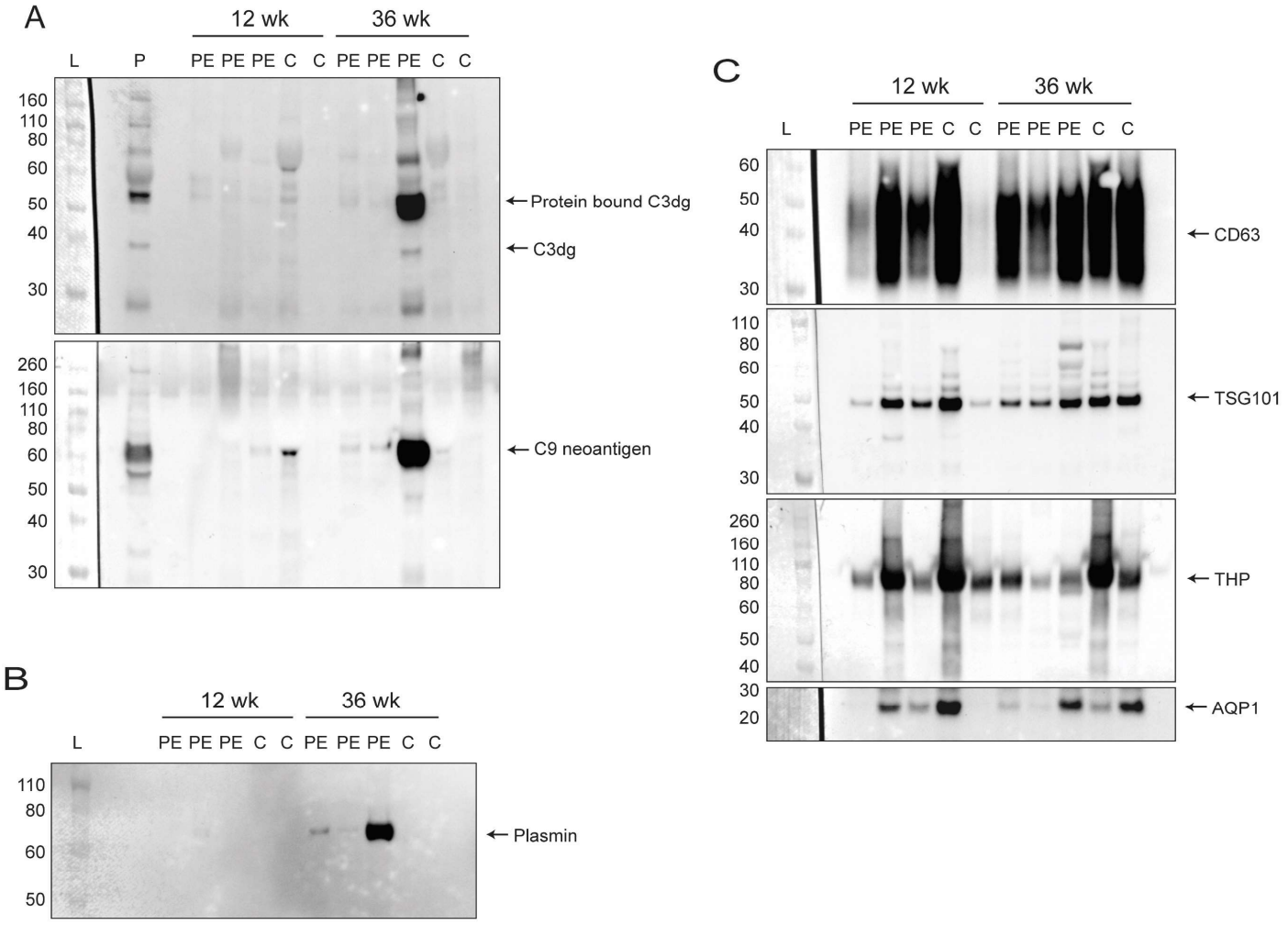
A
12 wk
36 wk
L
P
PE PE PE C C PE PE PE C C
160
110
80
60
50
40
30
← Protein bound C3dg
← C3dg
260
160
110
80
60
50
40
30
← C9 neoantigen
B
12 wk
36 wk
L
PE PE PE C C PE PE PE C C
110
80
60
50
← Plasmin
C
12 wk
36 wk
L
PE PE PE C C PE PE PE C C
60
50
40
30
← CD63
110
80
60
50
40
30
← TSG101
260
160
110
80
60
50
40
← THP
30
20
← AQP1

Figure 4

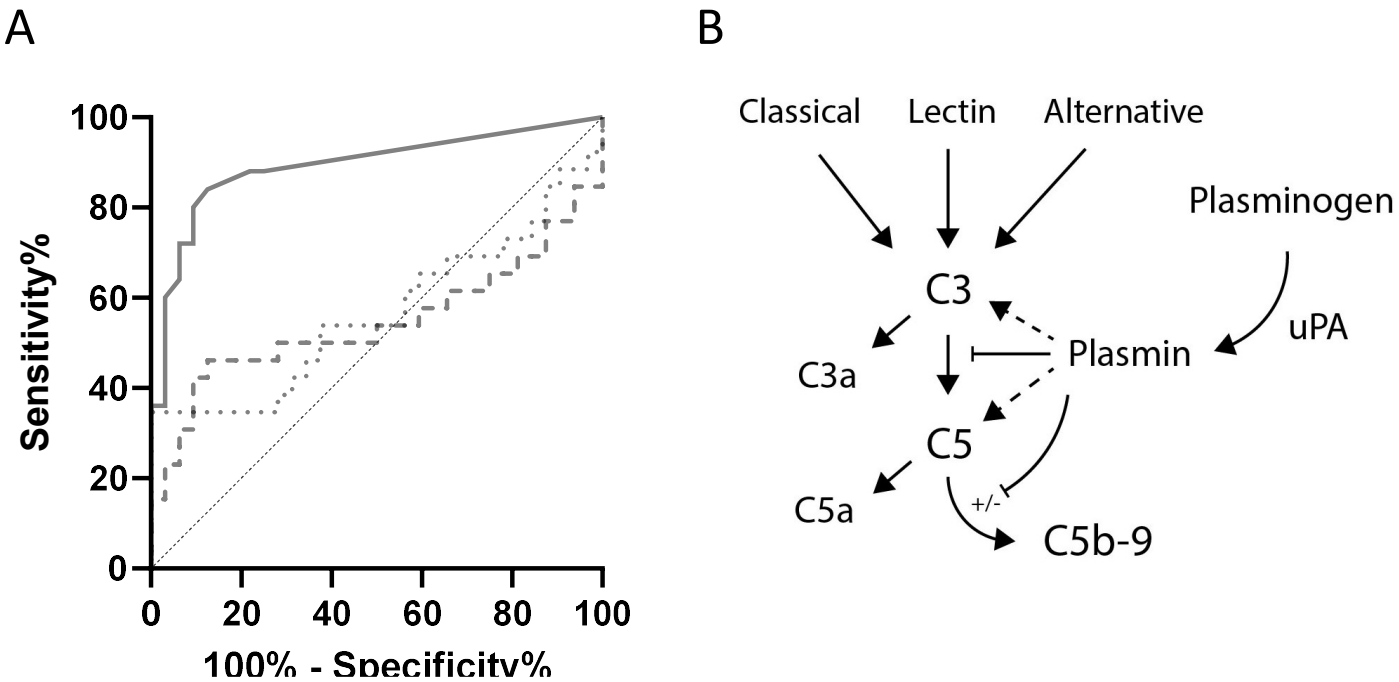
A
B
100
80
60
40
20
0
Sensitivity%
0
20
40
60
80
100
100% - Specificity%
Classical
Lectin
Alternative
Plasminogen
C3
uPA
C3a
Plasmin
C5
C5a
+/-
C5b-9

Figure 5