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Is urinary excretion of plasminogen associated with development of pre-eclampsia? An observational, explorative case–control study

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ABSTRACT

Objectives Pre-eclampsia (PE) is characterised by renal glomerular endotheliosis and injury to the glomerular filtration barrier with proteinuria. Patients with PE display aberrant filtration of the plasma proenzyme plasminogen which is activated, in the tubular fluid, to plasmin. Plasmin may activate the epithelial sodium channel and cause impaired sodium excretion and contribute to hypertension. An explorative study was conducted to test the association between urinary total plasminogen/plasmin and the development of PE. A positive association was hypothesised.

Design An observational, explorative, nested case–control study of healthy pregnant women.

Settings A Danish County hospital. Samples were collected between 2001 and 2004.

Participants 1631 healthy pregnant women participated. Urine samples were collected longitudinally six times during pregnancy. 30 developed PE (cases) and were compared with 146 randomly selected healthy pregnant women (controls).

Primary outcome The association between total plasminogen/plasmin excreted in the urine and PE development is expressed by ORs. Total urinary excretion of plasminogen/plasmin was defined by the urine plasminogen-plasmin/creatinine ratio.

Secondary outcome The association between urine (u)-albumin/creatinine ratio, u-aldosterone/creatinine ratio and PE development is expressed by ORs. The correlation between urinary (u-) plasmin and u-aldosterone concentration is expressed as a correlation coefficient.

Results The development of PE in late pregnancy was associated with increased levels of the urine plasminogen-plasmin/creatinine ratio (OR=2.35; 95% CI: 1.12 to 4.93; p<0.05). U-aldosterone/creatinine ratio did not predict PE at any time. U-albumin/creatinine ratio was positively associated with the development of PE from gestational week 33 (OR=14.04; 95% CI: 2.56 to 76.97; p<0.01) and in week 33–35 (OR=14.15; 95% CI: 3.44 to 58.09; p<0.001) and after gestational week 36, respectively.

Conclusion Aberrant filtration of plasminogen may contribute to the pathophysiological features of impaired sodium excretion and hypertension associated with PE late in pregnancy. However, increased urinary albumin levels reveal stronger associations with PE development compared with urinary plasminogen levels.

Strengths and limitations of this study

- The study was observational and does not allow conclusions on causality.
- The proposed mechanism/hypothesis is new with regards to pre-eclampsia pathophysiology.
- Samples used had experienced some freeze-thaw cycles which could have affected the quality and thus absolute levels.
- The hypothesis was investigated by measuring total immunoreactive plasminogen with no ability to discriminate between zymogen, active form, plasmin–antiplasmin complexes and degradation products and not the relevant parameter of plasmin activity.
- The study was single centre and the vast majority of participants were of Caucasian ethnicity.

INTRODUCTION

Pre-eclampsia (PE) affects 3%–5% of all pregnancies and is a leading cause of prematurity and fetal growth restriction.[1] The pathophysiological mechanisms that underlie manifestations of PE are not fully elucidated. Several subtypes of PE may even exist.[2] To date, the only treatment available is to terminate the pregnancy. However, in many cases, patients can be stabilised for hours or even days by symptomatic treatment with antihypertensive therapy. An imbalance between prostacyclin and thromboxane has been discovered and high-risk patients benefit from prophylactic aspirin.[3] Thus, prediction of PE is important as aspirin/acetylsalicylic acid can be advised to high-risk patients and termination of pregnancy can be delayed if symptomatic treatment is started timely. Several prediction models have been tested and one of the best models to date involves a combination

of patient history, blood pressure measurements, flow measurements in the uterine artery and measurements of pregnancy-associated plasma protein A and placental growth factor (PlGF).[4] Models involving the biomarkers soluble fms-like tyrosine kinase-1 (sFlt-1)/PlGF have also demonstrated promising results.[5] In the present study, the focus was on the involvement of the kidneys in the pathophysiology.

Patients with PE typically display proteinuria.[6] In established PE, patients excrete 10–100 times more plasminogen in urine compared with healthy pregnant women and there is a positive correlation between urinary plasminogen and urinary albumin concentrations which suggests aberrant filtration from the plasma of this abundant zymogen.[7–9] In urine, activated plasmin potentially impairs renal sodium excretion through effects on tubular sodium (Na)-transport proteins. Plasmin is able to activate the epithelial sodium channel (ENaC) in the collecting duct of the kidneys proteolytically[10 11] by releasing a 43-amino acid inhibitory peptide tract from the exodomain of the ENaC gamma subunit. This promotes channel open probability.[11 12]

This mechanism is interesting in regard to PE, as aberrant activation of ENaC causes sodium retention, extracellular fluid expansion and salt-sensitive hypertension which are present to variable degrees in established PE.[13 14] Other characteristics of PE are increased systemic vascular resistance, lower cardiac output, lower glomerular filtration ratio and a suppressed renin–angiotensin–aldosterone system (RAAS).[14–21] Despite these pathophysiological features have been known for years, there is no plausible explanation for the paradoxical suppression of RAAS when the intravascular circulating volume is reduced, blood pressure is high and extracellular volume often is increased in PE. The findings of aberrant filtration of plasmin and activation of ENaC combined with the avid sodium retention in PE led us to hypothesise that plasmin is the link between renal sodium retention and secondary RAAS suppression in established PE.

It was hypothesised that the amount of total immunoreactive plasminogen excreted in the urine could be associated with PE development and as a secondary parameter, that urinary (u-) plasminogen related negatively to the u-aldosterone concentration, an integrated measure of aldosterone secretion. The association between urinary plasmin and PE development was compared with urinary albumin concentrations which is an established sensitive marker for PE. The association between urinary plasmin and PE development was also investigated with the aim to determine whether plasmin could be used as a predictor of PE.

To shed light on these hypotheses, we conducted this explorative, observational, longitudinal, nested case–control study and analysed consecutive urine samples from a published cohort of pregnant women from a Danish Region throughout pregnancy and compared PE cases with controls.[22 23]

MATERIAL AND METHODS

Urine samples were collected in years 2001–2004 at a single centre, Randers County Hospital, in connection with an approved research project.[22 23] Urine samples were collected longitudinally prospectively six times through pregnancy in a non-selected cohort of all pregnant women (n=1631) of whom 32 developed PE (2%). Samples were stored at −80°C.

In the present study, samples from 30 cases that developed PE were available for analysis. Two patients with PE were excluded from the original cohort due to missing urine samples.

A total of 146 healthy pregnant controls were analysed from the cohort, corresponding to approximately 5 controls per case. Controls were selected as the two healthy pregnant women entering the study before and the three healthy, pregnant women entering the study after each woman developing PE. Samples were collected in gestational week <19, 20–24, 25–29, 30–32, 33–35 and ≥36.[22] If more than one urine sample was collected in the time interval, the latest sample was used for analysis. No data obtained from urine samples after week 38 were used in this study due to missing values.

'Baseline' values are those registered and obtained ≤19 weeks of gestation.

The patients who developed PE are referred to as the 'PE group' and the group that stayed normotensive throughout pregnancy is referred to as the 'control group'.

PE was defined as de novo hypertension >140/90 after gestational week 20 combined with proteinuria, defined as >300 mg/L or ≥2+ on a urine dip stick. Both were measured with at least 4-hour interval.[23] All participating women had a type II routine ultrasound scan around the 18–19th week of gestation to rule out macroscopic abnormalities of the fetus and confirm the gestational age.[22] Women with pre-existing hypertension, diabetes, renal disease or inflammatory bowel disease were excluded.

The time interval between the >36 weeks tests and the diagnosis of PE was maximum 3 days.

Plasminogen/plasmin in urine was analysed by ELISA Kit Human Plasminogen Total Antigen (IHPLGKT-TOT, Innovative Research, Novi, Michigan, USA), a sandwich ELISA developed for plasma, serum and urine. Urine (100 µL) was added to a plate coated with capture antibody for human plasminogen, polyclonal antihuman plasminogen primary antibody was added, which binds to the captured protein. Then secondary antibody conjugated to horseradish peroxidase was added, allowing for detection with substrate developing colour readable at 450 nm. The assay procedure was performed as described by the manufacturer. Human EDTA-plasma pool, diluted 1:10.000 was used as an internal standard. Between-assay coefficient of variation with a urine pool sample was ~20%. The manufacturer reports 11%. The sensitivity of the kit was not tested in the house but is reportedly 0.07 ng/mL with a measuring range of 0.5–500 ng/mL.

Aldosterone was analysed on ELISA (MS E-5200, Labor Diagnostika Nord GmbH & Co. KG, Germany), a competitive binding ELISA. Urine was diluted 1:50 with Urine Diluent (Labor Diagnostika) and 50 µL was incubated with aldosterone–horseradish peroxidase conjugate for 1 hour. as described by the manufacturer. Competition occurs between the unlabelled antigen present in the sample and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the plate. Plasma samples were in range. Accuracy was confirmed for urine by running a dilution series. Intra-assay variation was tested two times with n=10 repetitive determinations each time and was 5.6% and 7.1%, respectively. On every plate ran the same sample of human EDTA-plasma pool aliquot as an internal standard. On the first set of plates (n=21), between-assay coefficient of variation was ~9.4% and in the second set with a new pool sample (n=19 plates) CV was 9.7%. In-lab control of cross-reactivity was not performed but according to the manufacturer, the ELISA had reportedly no cross-reactivity with progesterone and cortisol which can be a challenge.

Statistical analyses were performed using STATA V.12 statistical package.

The sample size for the current study was not defined pre hoc since the hypothesis was stated post hoc. With regard to evaluating the predictive value of plasminogen/plasmin, the sample size was based on results from a similar study which evaluated u-albumin as a predictor for PE in a cohort of women with diabetes.[24] In that study, 136 participants were enrolled which yielded highly significant prediction by u-albumin. As plasminogen relates directly to albumin in the urine,[7 25] a total number of 176 participants were considered adequate. Continuous data were tested for normal distribution. Variables in table 1 (age, BP systolic, BP diastolic and gestational age) were normally distributed and student's unpaired *t* test was used for comparisons of continuous data between the two groups. In table 1, data are presented as absolute numbers (±SD). If SD differed significantly and appeared proportional to the mean, natural log-transformed data were used for calculations. The variables, u-plasminogen/creatinine ratio, u-albumin/creatinine ratio and u-aldosterone/creatinine ratio, were not normally distributed and the results were then presented in semi-logarithmic diagrams.

For categorical data comparisons between the two groups were performed by the Fisher exact test. The predictive values of the variables were derived from logistic regression analysis and expressed as ORs. For continuous variables, the OR represented the risk associated with a 10-fold increase of the variable. Variables, not normally distributed, were compared between groups by the Wilcoxon rank sum test. Correlations are expressed as correlation coefficients (*r*) and evaluated statistically by linear regression analysis. To evaluate prognostic qualities of u-plasminogen/plasmin excretion, we used receiver operating characteristics (ROC) curves. P values <0.05 were considered significant. The original project was approved by the local ethics committee (Region of Central Denmark. Project ID: 20010153), and the present sub-study was approved in 2015 (Region of Central Denmark. Project ID: 1-10-72-19-15).

Patient and public involvement

All women were informed about the original study by the involved medical staff and gave written, informed consent. The study conformed to the Declaration of Helsinki.

In this present sub-study, all data were passed on anonymously according to rules set out by the Danish ethical committee.

Patients were not involved in the design of the present study. According to Danish rules and regulations, patients

Table 1 Baseline and delivery characteristics

	PE	Control	P value
Number of patients (n)	30	146	
Age (years)*	30.1±3.2	28.9±4.2	0.12
Parity†	1.4±0.1	1.7±0.1	0.03
BP systolic, baseline (mm Hg)‡	120±15	110±11	<0.001
BP diastolic, baseline (mm Hg)‡	75±7	68±3	<0.001
Gestational age at delivery (days) §	264±12	278±11	<0.001
Birth weight (grams)¶	3278.1±798.8	3656.7±537.1	<0.01
Caesarean sections **	50.0%	11.64%	<0.001

Values are mean values±SD Data were normally distributed and Student unpaired *t* test was used for analysis.
*Age: PE, n=29;. controls, n=141.
†Parity: PE, n=28; controls, n=139.
‡BP at first visit: PE, n=20; controls, n=112.
§Gestational age: PE, n=26.
¶Birth weight: PE, n=28; controls, n=145.
**Caesarean sections: PE, n=28.
BP, blood pressure; n, number of patients; PE, pre-eclampsia.

were contacted neither before study initiation nor afterwards due to the respect of their anonymity.

Data availability statement

All original, clinical data have been handed over from third parties (and published elsewhere in other settings[22 23]) and due to cooperation agreements, and Danish ethical regulations, data are not publicly available, however data regarding urine plasminogen/plasmin, creatinine, aldosterone and albumin measurements are accessible if requested (lihn@clin.au.dk).

RESULTS

Baseline characteristics

There was no difference in maternal age and parity, at inclusion between the two groups (table 1). Of the original cohort of 1631 women, 32 developed PE corresponding to 2% of the cohort. Two patients with PE and one control had PE in a previous pregnancy. The PE group displayed a significantly higher blood pressure at inclusion compared with the group that stayed normotensive throughout the pregnancy (table 1).

Neonatal outcome

Significantly more women in the PE group delivered by caesarean sections (50% in the PE group compared with 12% in the control group (P<0.001)). There was a significant difference (P<0.001) in the mean gestational age at delivery between the two groups (264 days (~38 weeks) (CI; 260 to 269) in the PE group compared with 278 days (~40 weeks) (CI; 277 to 280) in the control group respectively) and there was a significant difference (P<0.01) in birth weight between the two groups (PE group: 3278 g; control group: 3657 g).

Urine plasminogen-plasmin/creatinine ratio, aldosterone and albumin/creatinine ratio and prediction of PE

In gestational week ≥36, there was a statistically significant positive association between the value of urine plasminogen-plasmin/creatinine ratio (U-plg/crea) and development of PE with an OR=2.35 (95% CI: 1.12 to 4.93; p<0.05) and there was a significant difference in U-plg/crea levels between groups (PE group: 7898 pg/mL; control group: 3086 pg/mL; p<0.05).

There was no association between u-plg/crea ratio and development of PE at any earlier gestational week (figure 1A). Due to the significant association at gestational week ≥36, a ROC area under the curve (AUC) was calculated for that gestational period. The ROC area was 0.66 (95% CI: 0.49 to 0.83) (figure 2).

The u-aldosterone/creatinine ratio was without significant differences between groups and was not associated with PE development at any time interval measured during pregnancy.

There was a statistically significant association between u-albumin/creatinine ratio and PE development from gestational week 33 and throughout pregnancy

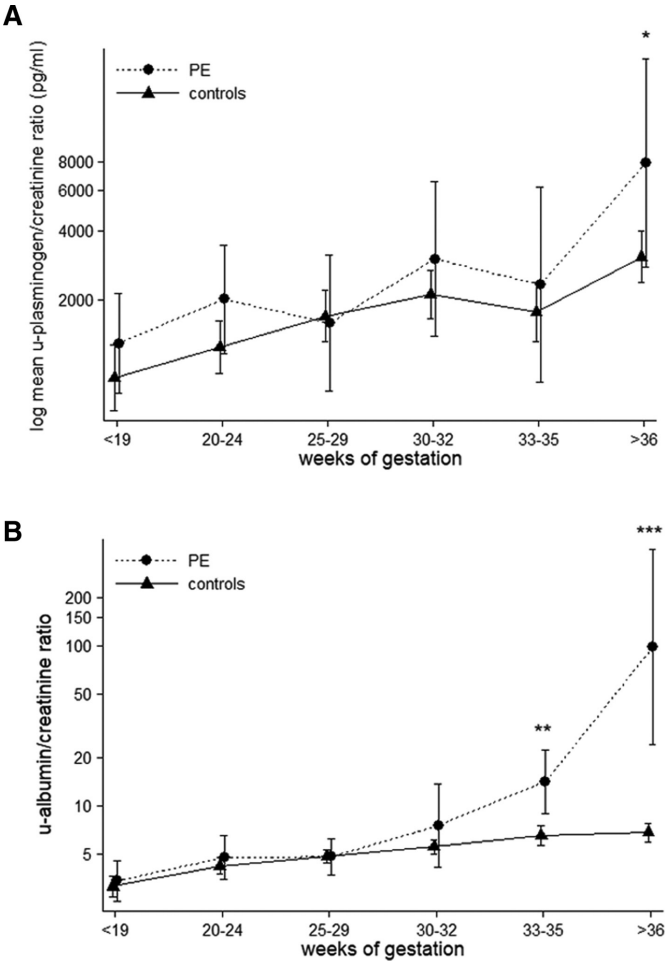

Figure 1 (A) Concentrations of urine total plasminogen-plasmin/creatinine ratio measured in the PE group and the control group in gestational weeks<19, 20–20, 25–29, 30–32, 33–35 and ≥36. Mean value in gestational week ≥36 in the PE group: 7898 pg/mL and in the control group: 3086 pg/mL. PE=group of pregnant women developing PE; controls=group of pregnant women not developing PE. *P<0.05 (p-value <0.05 was considered significant). (b) Concentrations of urine albumin/creatinine ratio measured in the PE group and the control group in gestational weeks <19, 20–20, 25–29, 30–32, 33–35 and ≥36. **P<0.01 ***p<0.001 (p value <0.05 was considered significant). Data were log-transformed to obtain normal distribution and mean values and confidence intervals are presented in the figure. PE, pre-eclampsia.

(OR=14.04; 95% CI: 2.56 to 76.97; p<0.01) in week 33–35 and (OR=14.15; 95% CI: 3.44 to 58.09; p<0.001) after 36 weeks of gestation (figure 1B). From week 33, there was a significant difference in the u-albumin/creatinine ratio between groups (in gestational week ≥36: PE group: 2248 mg/g; control group: 9 mg/g) (p<0.01). The ROC area was 0.79 (95% CI: 0.63 to 0.96) at gestational week ≥36. (figure 2)

Correlations

There was a significant positive correlation between u-albumin and u-plasminogen/plasmin in gestational week 20–24 and in gestational week ≥36 (the correlation

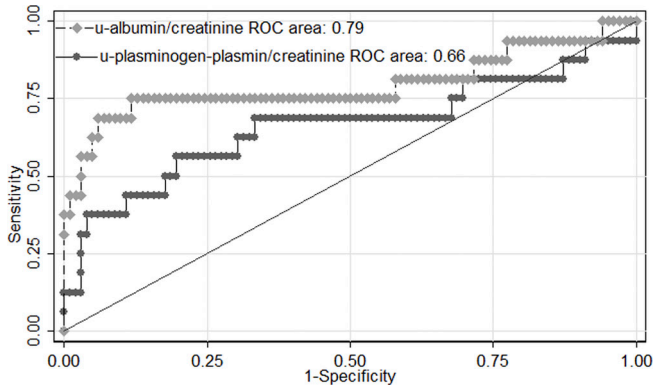

Figure 2 ROC curves comparing the AUC for urine total plasminogen-plasmin/creatinine ratio and urine albumin/creatinine ratio in both groups joined together in gestational week ≥36. ROC AUC for urine total plasminogen-plasmin/creatinine ratio=0.66 (95% CI: 0.49 to 0.83) is illustrated by a black dotted line. ROC AUC urine albumin/creatinine ratio=0.79 (95% CI: 0.63 to 0.96) is illustrated by a light grey dotted line. Data were log-transformed. AUC, area under the curve; ROC, receiver operating characteristics.

coefficient, r=0.24, p<0.01; and r=0.26, p<0.01, respectively) when the groups were analysed together (figure 3).

The correlation between u-plasminogen and u-aldosterone was calculated using ratios corrected for creatinine. A minor positive, but significant, correlation was found throughout the pregnancy in both groups without any difference between groups.

DISCUSSION

Urine samples from a longitudinal observational study of healthy pregnant women, originally designed to identify urine and plasma biomarkers for PE, were analysed to test the hypothesis that total plasminogen/plasmin excreted in the urine would be positively associated with the development of PE. We found that urinary plasmin

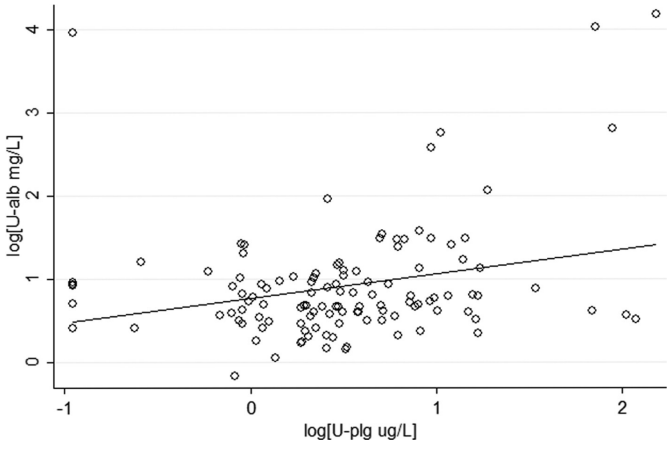

Figure 3 The figure shows the correlation between U-alb and U-plg at gestational week ≥36 in the two groups in total. Data were log-transformed and a linear regression analysis was used for evaluation. The correlation coefficient *r*=0.26 (p<0.01). U-alb, urine concentrations of albumin; U-plg, urine concentration of plasminogen/plasmin.

only revealed significant increased ORs in pregnancy after gestational week 36, whereas the albumin/creatinine ratio revealed significant increased ORs already from gestational week 33 and throughout the pregnancy. This indicates that u-plasminogen/plasmin levels do not reveal stronger associations with PE development compared with for example, urinary albumin levels in pregnancy.

The significant relationship between u-albumin and plasminogen and the predictive value of total plasminogen/plasmin late in pregnancy could be in accordance with aberrant filtration and a potential role for the pathophysiological hallmarks in established PE of impaired sodium excretion and suppressed RAAS.

Plasmin correlated positively with u-albumin in gestational week 20–24 and again in pregnancy week later than 36. This extends and confirms the findings by Buhl *et al* of a positive relation late in pre-eclamptic pregnancy.[7] It is also in good agreement with the concept that plasminogen is filtered from plasma across the injured barrier into the tubular fluid. Plasminogen is then converted to plasmin, likely by urokinase (uPA), with subsequent potential proteolytic activation of ENaC, increased sodium retention and contribution to oedema and hypertension.[11] Cleaved ENaC has been shown in human kidneys in patients with proteinuria.[26] Due to negative feedback the sodium retention may suppress RAAS which is observed also in other proteinuric diseases such as diabetic nephropathy and nephrotic syndrome.[27]

However, in the present study, u-plasmin did not relate negatively to u-aldosterone concentration. This is of interest as studies have indicated that aldosterone level is important for placental development in rodents and humans.[28 29] Buhl *et al* found a negative correlation between u-plg/crea ratio and u-aldosterone/crea ratio in a group of patients with PE late in pregnancy.[7] We can only speculate why a similar relation was not found in the present study as it is well established that renin, angiotensin II (ANGII) and aldosterone are suppressed in plasma in PE pregnancy. Plasma was not available from the present cohort and urine aldosterone is an integrated surrogate measure for aldosterone secretion. Among the cases, there could have been significant drop-out of the more severe patients with PE before late pregnancy stages where this is most pronounced.

A range of PE predictors in late pregnancy, such as clinical symptoms, blood pressure, serum urate levels and liver enzymes are already being used and recently, the sFlt-1 to PlGF ratio has been evaluated as a promising predictor of PE[30] and from the present study, it does not seem like total urinary plasminogen/plasmin concentration is a superior PE predictor, but it might benefit from validation in an independent cohort.

The sample size of this study was estimated to be sufficient. However, the samples used had been stored for a prolonged time and had experienced some freeze-thaw cycles which could have affected the quality. The applied ELISA kit detected all species of plasmin: plasminogen,

plasmin and plasmin–antiplasmin complexes and likely also immunogenic degradation fragments and it could not distinguish between inactive pro-forms and active forms.

Because all species of plasmin were measured and because we did not have the opportunity to measure 24-hour NaCl excretion and cleaved ENaC in the urine, the presented hypothesis was explored indirectly. Moreover, as an observational study, causality can only be supported not demonstrated. Study samples were stored for a prolonged and variable time due to 3 years of inclusion. The present study has to be considered as explorative.

In summary, it was found that u-plasmin and u-aldosterone concentrations did not demonstrate a stronger association with PE development compared with already established early predictors of incident PE in a cohort of healthy pregnant women. However, late in pregnancy, total plasminogen was positively associated with PE development and it is concluded that active plasmin could contribute to the pathophysiological features of impaired sodium excretion and hypertension late in PE. There remains an unmet need to identify new predictors relating more closely to the pathophysiology of PE.

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Contributors LHN: set up the study and was the project coordinator and wrote the first draft of the manuscript. CK: collected the samples and most of baseline data; revised the manuscript critically and approved the final version to be submitted for publication. EV: analysed and organised all samples in 2001–2004; revised the manuscript critically and approved the final version to be submitted for publication. GK: analysed and organised the samples for this specific study; organised data, revised the manuscript critically and approved the final version to be submitted for publication. BLJ: supervisor of LHN; involved substantially in the process of drafting the protocol, design of the study, established the assays and contributed to the manuscript writing process. UBK: supervisor of CK and participated with substantial contributions collecting the samples and the clinical data; revised the work critically and approved the final version to be submitted for publication. PGO: main supervisor of LHN; involved in the process of drafting the protocol and the design; revised the work critically and approved the final version to be submitted for publication. All authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Competing interests None declared.

Patient consent for publication Not required.

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Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement The original investigator group who collected the samples in 2001–2004 has additional confidential unpublished data that are not relevant for this project. Data relevant for this project and presented in this article are not allowed to be shared with a third part in compliance with Danish ethical and authority regulations.

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