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Death associated protein kinase 1 (DAPK-1) is increased in preeclampsia

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ABSTRACT

Introduction: Death associated protein kinase-1 (DAPK-1) is highly expressed in the placenta relative to all other human tissues. We examine whether it is differentially expressed with preeclampsia.

Methods: We examined samples from a large prospective collection of plasma from 2002 women. We split the samples into two cohorts: Cohort 1 (n = 1000) and Cohort 2 (n = 1002). We first measured circulating DAPK-1 at 36 weeks' gestation in a nested case-control group (from Cohort 1) of 39 women who developed preeclampsia and 98 controls. We then validated our findings by measuring circulating levels in all samples from both cohorts. We also measured DAPK-1 in the circulation and placentas of women who were diagnosed with preterm preeclampsia or delivered a growth restricted infant at < 34 weeks' gestation.

Results: In the case-control study, circulating DAPK-1 was significantly increased in women destined to develop preeclampsia ($p < 0.01$). We validated this by measuring circulating levels in Cohorts 1 and 2. Again, circulating DAPK-1 was significantly higher ($p < 0.001$) among women destined to develop preeclampsia (Cohort 1, Area under the receiver operator characteristic curve (AUC) = 0.66; Cohort 2 AUC = 0.67). Circulating DAPK-1 was also significantly elevated in women with established preterm preeclampsia. Placental DAPK-1 mRNA and protein expression were elevated in women with established preeclampsia.

Discussion: DAPK-1 is a novel placenta-enriched molecule that is elevated in the circulation of women preceding the diagnosis of preeclampsia and is likely to be secreted from the placenta.

1. Introduction

Preeclampsia – affecting 3–8% of pregnancies - is one of the most serious complications of pregnancy, responsible for 60,000 maternal deaths annually [1]. It is a multi-system disorder affecting maternal vessels (causing hypertension and endothelial dysfunction), kidneys, liver, haematological system, brain (causing seizures) and the fetus (growth restriction) [1]. Most preeclampsia occurs late in pregnancy (term preeclampsia), and there are no treatments to arrest disease progression except delivery. Delivery removes the placenta, the source of circulating pathogenic factors causing the maternal disease. While delivery at term is safe for the baby, preeclampsia (at any gestation) carries the potential for serious maternal adverse outcomes including stroke, seizures, and clotting dysfunction. Identifying those at higher risk earlier in the disease course could offer clinicians an opportunity to offer alternative management to reduce the risk of these serious complications [2,3], meaning there is value in early detection.

We have previously proposed it may be worthwhile further interrogating placenta-enriched molecules [4]. Investigation of these molecules, that are highly expressed in the placenta relative to other human cells or tissues, may identify novel factors that contribute to disease pathogenesis or may be dysregulated in the circulation of women preceding disease diagnosis [4]. These molecules can be readily identified bioinformatically and we hypothesise that given that they are deliberately expressed in the placenta at such high levels, some may be differentially expressed when there is placental pathology.

Death Associated Protein Kinase-1 (DAPK-1) is one such placenta-enriched molecule, with online repository bioGPS suggesting it is around 3.5x more highly expressed in placenta than any other human cell or tissue [5] and found within the syncytiotrophoblast layer of the placenta (www.proteinatlas.org). Interestingly, DAPK-1 is widely reported as a regulator of cell death and autophagy [6]. Programmed cell

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death or apoptosis is a highly regulated response to extrinsic (death receptor ligands) or intrinsic (DNA damage) stimuli. Apoptosis consists of a series of steps from activation, propagation, to commitment and execution which are carried out by signal transduction mechanisms. It is postulated that DAPK-1 is possibly involved prior to the commitment steps of the signal transduction mechanism [6]. DAPK-1 has also been implicated as a critical regulator of autophagy. Autophagy is also a highly regulated quality control mechanism that occurs in the cytoplasm to degrade long-lived proteins and scavenge damaged organelles and misfolded proteins [7]. Autophagy is enhanced under cellular stress including oxidative stress and hypoxia. Indeed there have been numerous studies assessing autophagy in preeclamptic placentas [8,9], with oxidative stress and hypoxia believed to be key contributors to the pathogenesis of the disease [9–14].

Although DAPK-1 is most highly expressed in the placenta relative to other human tissues [15], there is very little literature assessing its role in placental dysfunction. One study [16] identified DAPK-1 as an autophagy molecule significantly down-regulated in first-trimester placental explants exposed to inflammatory stimuli. They also assessed DAPK mRNA and protein expression in preeclamptic placenta but they only examined very few placental samples (6 preeclamptic placentas and 5 controls) and concluded it was down-regulated in preeclampsia. Given DAPK-1 is highly expressed in placenta, localised to the placental surface and known to be a regulator of autophagy (reported as increased in preeclampsia), in this study we were interested in assessing whether DAPK-1 protein is measurable within the maternal bloodstream, and whether it is dysregulated in preeclampsia.

2. Materials and methods

2.1. The Fetal Longitudinal Assessment of Growth (FLAG) study

The FLAG study was undertaken at Mercy Hospital for Women, a tertiary referral hospital in Melbourne, Australia. It involved the prospective recruitment of pregnant participants where we obtained over 2000 blood samples at both 28 (27^{+0}–29^{+0} days) and 36 (35^{+0}–37^{+0}) weeks' gestation. It was designed to identify biomarkers for pregnancy complications such as preeclampsia and fetal growth restriction. Women were screened for eligibility and invited to participate at their oral glucose tolerance test, universally offered to non-diabetic pregnant women around 28 weeks' gestation to test for gestational diabetes mellitus. English-speaking women aged over 18 years, with a singleton pregnancy and normal mid-trimester fetal morphology examination were eligible to participate. Whole blood was collected in a 9 ml ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was stored at −80 °C until the time of sample analysis. 4.2% of women who gave 36 week blood samples later developed preeclampsia.

The FLAG study samples were divided into two consecutive collected cohorts of approximately 1000 samples each, Cohorts 1 and 2.

A case control-cohort was selected from Cohort 1 for initial biomarker discovery (where our DAPK-1 results are being presented in this report). After identifying differential levels of circulating DAPK-1 in the case-control sample set, we then measured DAPK-1 in all samples in both Cohort 1 (initial validation) and Cohort 2 (independent validation).

The FLAG study was approved by the Mercy Health Research Ethics Committee (Ethics Approval Number R14/12) and written informed consent was obtained from all participants.

2.2. Outcomes and definitions of cases

Maternal characteristics and pregnancy outcomes were obtained from review of each participant's medical record, investigation results and hospital database entry, by a single clinician blinded to the DAPK-1 Assay results. Preeclampsia was defined according to the guidelines published by the American College of Obstetricians and Gynecologists [17]. Fetal growth restriction (FGR) was defined as birthweight < 10th centile. Most cases examined here would likely have true growth restriction given we only examined those who required preterm delivered for fetal indications, suggesting there was underlying placental pathology.

Table 1
Maternal characteristics for case-control cohort from first 1000 FLAG participants. Data presented as mean (standard deviation) if normally distributed data, as median [interquartile range] if not normally distributed data, and as number (%) if categorical. BMI = Body Mass Index; GDM = Gestational Diabetes Mellitus. Note: some percentages do not sum to 100% due to rounding to one decimal place.

	Preeclampsia N = 39	Controls N = 98	*P*
Age	33.1 (4.7)	32.4 (3.9)	0.32
Booking BMI	26.8 [23.1–31.3]	23.7 [21.7–28.3]	0.04
Nulliparous	28 (71.8%)	50 (51.0%)	0.04
Smoking status			
- Current smoking	2 (5.1%)	6 (6.1%)	0.91
- Ex-smoker	10 (25.6%)	22 (22.4%)	
- Never smoked	27 (69.2%)	70 (71.4%)	
Gestational Diabetes	9 (23.1%)	21 (21.4%)	0.82
Onset of labour			
- Spontaneous	13 (33.3%)	42 (42.9%)	0.37
- Induced	19 (48.7%)	46 (46.9%)	
- No labour	7 (17.9%)	10 (10.2%)	
Mode of delivery			
- Physiological vaginal	11 (28.2%)	49 (50.0%)	0.009
- Instrumental delivery	7 (17.9%)	23 (23.5%)	
- Caesarean section	21 (53.8%)	26 (26.5%)	
Gestation at delivery (weeks^{+days})	39^{+0} (1^{+4})	39^{+6} (1^{+1})	0.002
Birthweight (g)	3308 (640.4)	3540 (433.1)	0.02
Birthweight centile	38.1 [18.8–69.5]	45.6 [28.6–67.7]	0.21

2.3. Measurement of DAPK-1 in plasma samples

DAPK-1 protein was measured in sample cohorts using the Human death-associated protein kinase 1 ELISA kit (Mybiosource.com) according to the manufacturer's instructions. We initially measured circulating DAPK-1 concentrations at 36 weeks' gestation in a nested case-control cohort selected from Cohort 1 (n = 98 controls, n = 39 PE; Table 1). These cohorts were used as part of our discovery studies. We next re-measured DAPK-1 in all samples from Cohort 1 (961 controls and 39 who developed preeclampsia, Table 2) and then validated our findings in Cohort 2 which was an independent cohort (955 controls and 47 who developed preeclampsia; Table 3).

We also measured DAPK-1 in plasma samples collected from another separate cohort of women who had established preterm preeclampsia and delivered at < 34 weeks' gestation (Table 4). Controls for this cohort were pregnant women where plasma samples were collected around the same gestation, but who then progressed to an uncomplicated delivery of a healthy neonate of normal birthweight at term.

2.4. Placental tissue collection

Women presenting to the Mercy Hospital for Women gave informed written consent for placental tissue collection. Human Ethics approval was obtained for this study from the Mercy Health Human Research Ethics Committee (R11/34). We measured DAPK-1 expression in placentas from pregnancies complicated by preterm preeclampsia or fetal growth restriction, delivered at < 34 weeks' gestation and in gestation-matched control placentas from pregnancies not affected by preeclampsia or fetal growth restriction. Indications for preterm birth in the preterm control cohort were preterm labour, vasa praevia or antepartum haemorrhage. Controls did not have any evidence of infection

Table 2
Maternal characteristics and pregnancy outcomes for cohort 1 - FLAG participants who provided a 36 week blood sample. Data presented as mean (standard deviation) if normally distributed data, as median [interquartile range] if not normally distributed data, and as number (%) if categorical. BMI = Body Mass Index; GDM = Gestational Diabetes Mellitus; SGA = small-for-gestational-age (customised birthweight < 10th centile). Note: some percentages do not sum to 100% due to rounding to one decimal place.

	Preeclampsia N = 39 (3.9%)	Controls N = 961 (96.1%)	*P*
Age	33.1 (4.7)	32.6 (4.3)	0.44
Booking BMI	26.8 [23.1–31.3]	24.0 [21.6–27.4]	0.005
Nulliparous	28 (71.8%)	469 (48.8%)	0.005
Smoking status			
- Current smoking	2 (5.1%)	30 (3.1%)	0.70
- Ex-smoker	10 (25.6%)	219 (22.8%)	
- Never smoked	27 (69.2%)	712 (74.1%)	
Gestational Age	9 (23.1%)	129 (13.4%)	0.10
Small for gestational Age	7 (17.9%)	99 (10.3%)	0.18
Onset of labour			
- Spontaneous	13 (33.3%)	468 (48.7%)	0.12
- Induced	19 (48.7%)	327 (34.0%)	
- No labour	7 (17.9%)	166 (17.3%)	
Mode of delivery			
- Physiological vaginal	11 (28.2%)	483 (50.3%)	0.01
- Instrumental delivery	7 (17.9%)	173 (18.0%)	
- Caesarean section	21 (53.8%)	305 (31.7%)	
Gestation at delivery (weeks^{+days})	39^{+2} [37^{+6}-40^{+3}]	39^{+4} [38^{+5}-40^{+3}]	0.08
Birthweight (g)	3308 (640.4)	3451 (489.9)	0.08
Birthweight centile	38.1 [18.8–69.5]	44.6 [23.7–72.1]	0.28

Table 3
Maternal characteristics and pregnancy outcomes for cohort 2 - FLAG participants who provided a 36 week blood sample. Data presented as mean (standard deviation) if normally distributed data, as median [interquartile range] if not normally distributed data, and as number (%) if categorical. BMI = Body Mass Index; GDM = Gestational Diabetes Mellitus; SGA = small-for-gestational-age (customised birthweight < 10th centile). Note: some percentages do not sum to 100% due to rounding to one decimal place.

	Preeclampsia N = 47 (4.7%)	Controls N = 955 (95.3%)	*P*
Age	31.7 (3.9)	32.5 (4.1)	0.25
Booking BMI	24.0 [21.5–29.4]	24.4 [22.0–27.9]	0.91
Nulliparous	32 (68.1%)	411 (43.0%)	< 0.001
Smoking status			
- Current smoking	3 (6.4%)	28 (2.9%)	0.37
- Ex-smoker	9 (19.1%)	219 (22.9%)	
- Never smoked	35 (74.5%)	708 (74.1%)	
GDM	9 (19.1%)	114 (11.9%)	0.17
SGA	7 (14.9%)	98 (10.3%)	0.33
Onset of labour			
- Spontaneous	10 (21.3%)	439 (46.0%)	< 0.001
- Induced	28 (59.6%)	326 (34.1%)	
- No labour	9 (19.1%)	190 (19.9%)	
Mode of delivery			
- Physiological vaginal	15 (31.9%)	476 (49.8%)	0.009
- Instrumental delivery	6 (12.8%)	159 (16.6%)	
- Caesarean section	26 (55.3%)	320 (33.5%)	
Gestation at delivery (weeks^{+days})	39^{+0} (1^{+2})	39^{+3} (1^{+1})	0.02
Birthweight (g)	3350 [2930–3680]	3400 [3110–3710]	0.29
Birthweight centile	43.0 [21.0–70.9	42.4 [20.9–67.6]	0.83

(on histopathological examination of the placentas) or hypertensive disease. All participants where placental specimens were obtained were delivered by caesarean section. Patient characteristics are outlined in Tables 5 and 6.

Placental tissue was obtained immediately following delivery. Maternal and fetal surfaces were removed and the samples were washed in ice-cold sterile phosphate-buffered saline (PBS). Samples for protein

Table 4
Maternal clinical characteristics for plasma blood samples < 34 weeks.

	Controls (n = 19)	Preeclampsia (n = 27)
Maternal Age (years)		
Mean (SD)	31.4 (4.4)	32.4 (17.4)
Gestation at blood collection (weeks)		
Median (range)	27.6 (28.3–30.4)	29.7 (27.5.- 31.4)
Gestation at Delivery (weeks)****		
Median (range)	40.0 (38.9–40.4).	29.7 (27.5–31.4)
BMI (kg/m^2)		
Median (IQR)	25.0 (22.0–28.4)	28.5 (25.6–34.5)
Nulliparous		
Number (%)**	6 (31.6)	19 (70.4)
Smoking status		
Current smoker	4 (21.1)	1 (3.7)
Ex-smoker	0 (0)	2 (7.4)
Never smoked	15 (78.9)	22 (81.4)
SBP at Delivery (mmHg)		
Median (IQR)****	125 (120–130)	175 (169–183)
DBP at Delivery (mmHg)		
Median (IQR)****	75 (70–80)	105 (100–110)
Birth weight (g)		
Median (range)	3560 (3180–3690)	1330 (866–1628)
Male		
Number (%)	8 (42.1)	9 (33.3)
Highest protein creatinine ratio (g/mmoL) §		
Median (range)	N/A	0.26 (0.15–0.62)
Liver function abnormalities		
Number (%)	N/A	17 (63.0)
Thrombocytopenia < 150 (x10^9/L)		
Number (%)	N/A	11 (40.7)

BMI data available for 23/27 PE women. Smoking data unavailable for 2/27 PE women. *p < 0.05, **p < 0.01, ****p < 0.0001 § Reference range < 0.03.

Table 5
Maternal clinical characteristics of placental mRNA samples < 34 weeks.

	Controls (n = 13)	Preeclampsia (n = 45)	FGR (n = 11)
Maternal Age (years)			
Mean (SD)	30.3 (6.3)	31.4 (5.5)	30.3 (7.2)
Gestation at Delivery (weeks)			
Median (range)	30.7 (30–31.7)	28.6 (30.4.- 31.9)	31.6 (30.9–32.6)
BMI (kg/m^2)			
Median (range)	30 (26.0–35.3)	27 (25–37.3)	24.2 (19.5–30.0)
Nulliparous			
Number (%)	5 (38.5)	30 (66.7)	8 (72.3)
Smoking status*			
Current smoker	1 (7.7)	2 (4.4)	2 (18,2)
Ex-smoker	2 (15.4)	2 (4.4)	4 (36.4)
Never smoked	10 (76.9)	36 (80.0)	5 (45.5)
SBP at Delivery (mmHg)			
Median (IQR)****	130 (112–130)	175 (165–180)	120 (116–132)
DBP at Delivery (mmHg)			
Median (IQR)****	75 (70–80)	100 (95–110)	80 (70–82.5)
Birth weight (g)			
Median (IQR)*	1687 (1350–1886)	1329 (1001–1470)	1000 (958–1214)
Birthweight < 10th centile for gestation****			
Number (%)	0 (0)	0 (0)	11 (100)
Male			
Number (%)	9 (69.2)	24 (53.3)	7 (63.4)
Highest protein creatinine ratio (g/mmoL) §			
Median (range)	N/A	0.23 (0.1–0.63)	N/A
Liver function abnormalities			
Number (%)	N/A	27 (60.0)	N/A
Thrombocytopenia < 150 (x10^9/L)			
Number (%)	N/A	15 (33.3)	N/A

*p < 0.05, ****p < 0.0001 § Reference range < 0.03. BMI data available for 9/13 controls and 36/45 women with PE. Smoking data unavailable for 5/45 women with PE.

Table 6
Maternal clinical characteristics for placental proteins < 34 weeks **p < 0.01, ***p < 0.001, ****p < 0.0001 § Reference range < 0.03. BMI data available for 18/23 controls, and 54/68 PE women. Smoking unavailable for 8/65 PE women.

	Controls (n = 23)	Preeclampsia (n = 68)	FGR (n = 13)
Maternal Age (years)			
Mean (SD)	31.1 (6.7)	31.3 (5.6)	29.8 (6.9)
Gestation at Delivery (weeks)			
Median (range)	30.0 (28.7–31.9)	30.4 (28.5–31.9)	31.4 (30.4–32.3)
BMI (kg/m^2)			
Median (range)	27.3 (24.3–29.8)	27.6 (25.0–36.1)	23 (19–29)
Nulliparous			
Number (%)***	7 (10.3)	50 (73.5)	10 (76.9)
Smoking status**			
Current smoker	1 (4.3)	3 (4.4)	2 (15.4)
Ex-smoker	5 (21.7)	2 (2.9)	5 (38.5)
Never smoked	17 (73.9)	55 (80.9)	6 (46.2)
SBP at Delivery (mmHg)			
Median (IQR)****	120 (113–130)	173 (160–180)	120 (117–130)
DBP at Delivery (mmHg)			
Median (IQR)****	70 (70–80)	100 (100–110)	80 (70–83)
Birth weight (g)			
Median (IQR)**	1540 (1226–1823)	1200 (866–1445)	999 (870–1126)
Birthweight < 10th centile for gestation			
Number (%)****	0 (0)	17 (25.0)	13 (100)
Male			
Number (%)	11 (47.8)	34 (50.0)	8 (61.5)
Highest protein creatinine ratio (g/mmoL) §			
Median (range)	N/A	22 (0.08–0.57)	N/A
Liver function abnormalities			
Number (%)	N/A	40 (58.8)	N/A
Thrombocytopenia < 150 (x10^9/L)			
Number (%)	N/A	21 (30.9)	N/A

extraction were frozen within 15 min of delivery and stored at −80 °C, and samples for RNA or protein collected in RNA Later™ stabilisation solution. Placenta was also fixed in 10% buffered formalin for histology.

2.5. RT-PCR to measure human DAPK-1 mRNA expression

RNA was extracted from 20 to 30 mg of RNAlater™ preserved frozen human placental samples by homogenization or from cytotrophoblast using an RNeasy mini-kit (Qiagen). 1 μg of RNA was converted to cDNA using Applied Biosystems high capacity cDNA Reverse Transcriptase Kit (Life Technologies, Carlsbad, CA, USA). Taqman gene expression assays (Life Technologies) for human DAPK-1, TOP1 and CYC1, were used. For comparisons between human placental samples, data was normalised to the geometric mean of two housekeepers; TOP1 and CYC1. RT-PCR was performed on the CFX 384 (Biorad, Hercules, CA, USA) using FAM-labeled Taqman universal PCR mastermix (Life Technologies) with the following run conditions: 50 °C for 2 min; 95 °C for 10 min, 95 °C for 15 s, 60 °C for 1 min (40 cycles).

2.6. Western blot analysis to measure human placental DAPK-1 protein expression

20 μg of placental lysates were separated on 10% SDS-polyacrylamide gels with wet transfer to PVDF membranes (Millipore, Billerica, MA). Membranes were blotted overnight with an antibody targeting DAPK-1 (Rabbit anti-human DAPK-1 Sigma), an anti-GAPDH-HRP (1:5000, Cell Signaling Technology, Danvers, MA, USA) or an anti-b-actin antibody (1:10,000, Sigma) and visualized using the Amersham ECLTM Prime Western blotting detection reagent (VWR International) and ChemiDoc XRS (BioRad, Hercules, CA, USA). Relative densitometry was determined in all samples using Image Lab (BioRad). For housekeeping proteins, an average of the two proteins was utilised for normalisation.

2.7. Statistical analysis

Maternal characteristics and birth outcome data were compared for all women who developed preeclampsia, had established preeclampsia or delivered an FGR baby, against controls using unpaired *t*-test (Mann-Whitney) or rank-sum test for continuous data, according to distribution; and Fisher's exact test or Chi square test for categorical data. Overall discrimination of DAPK-1 was assessed with area under the receiver operator characteristic (ROC) curve analysis. Placental and circulating data was initially assessed for normality before an unpaired *t*-test was used (Mann-Whitney). Statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Circulating DAPK-1 is increased at 36 weeks' gestation prior to the diagnosis of preeclampsia

We measured plasma DAPK-1 in the circulation of participants who enrolled in the Fetal Longitudinal Assessment of Growth (FLAG) study. DAPK-1 was initially measured as part of our discovery studies in a case-control cohort at 36 weeks' gestation. We found that DAPK-1 was significantly increased in the women who subsequently developed preeclampsia relative to controls (Fig. 1A controls n = 98, preeclampsia n = 39).

We next sought to re-measure DAPK-1 in all 1000 samples from Cohort 1 to determine whether circulating DAPK-1 concentrations were still increased among those who developed preeclampsia when the incidence of disease is the same as that of the population (instead of being inflated, as seen in a case-control analysis). DAPK-1 was undetectable in 19 of the control women, leaving 942 in the control group and 39 who developed preeclampsia. We confirmed DAPK-1 was significantly increased in the women who subsequently developed preeclampsia relative to those who did not, with an area under the receiver operator characteristic curve (AUC) of 0.66 (Fig. 1B and C).

To confirm the observation that those destined to develop preeclampsia have high circulating concentrations of DAPK-1 we measured circulating DAPK-1 concentrations at 36 weeks' gestation in Cohort 2. Cohort 2 were consecutively obtained samples from participants in the FLAG study and are an independent sample set from Cohort 1 (which was what we used to discover the association between DAPK-1 and preeclampsia). Of the 1002 samples in cohort 2, DAPK-1 was undetectable in 28 of the control women which we removed from our analysis. This left 927 controls and 47 who developed preeclampsia. DAPK-1 was significantly increased in the women who subsequently developed preeclampsia in cohort 2 compared to controls with an AUC of 0.67 (Fig. 1D and E). Finally, given the field's interest in sex-differences, we analysed DAPK-1 in the controls from cohort-1 and cohort 2 based on the gender of the infant at delivery (Figure F,G). We found no significant differences between genders in cohort 1 and a modest but significant difference in cohort 2. Together this data suggests that gender differences are unlikely to contribute significantly to circulating DAPK-1 levels. In summary, we have identified and validated the observation that increased circulating DAPK-1 precedes the clinical diagnosis of preeclampsia.

3.2. Circulating DAPK-1 is increased in women diagnosed with preterm preeclampsia

We next set out to determine whether circulating DAPK-1 was associated with preterm preeclampsia. We measured circulating levels among women who delivered at < 34 weeks with preterm

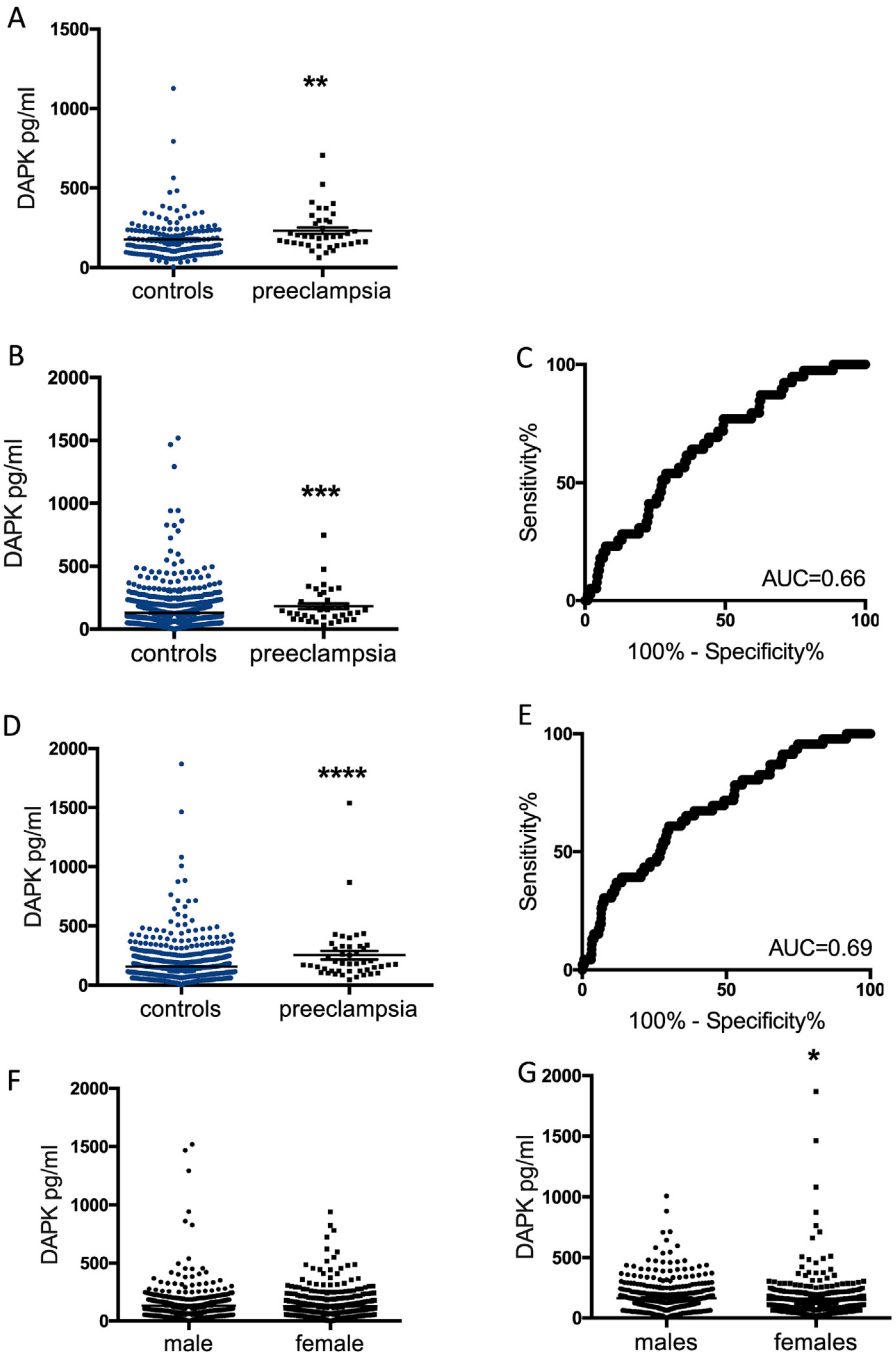

Fig. 1. DAPK-1 is increased in the circulation of women at 36 weeks' preceding the diagnosis of preeclampsia. Circulating DAPK-1 was initially measured in a case control cohort consisting of n = 39 women who later developed preeclampsia and n = 98 controls. In this cohort, DAPK-1 was significantly elevated in the preeclamptic group **(A)**. We subsequently re-measured DAPK-1 in cohort 1. **(B)** DAPK-1 was undetectable in 19 control women. We confirmed it was significantly elevated in those destined to develop preeclampsia (n = 39) compared to the remaining control women (n = 942), with an AUC ROC of 0.66 **(C)**. To validate this find we measured circulating DAPK-1 in cohort 2. DAPK-1 was undetectable in 28 control women from cohort-2, but significantly elevated in the circulation of women destined to develop preeclampsia **(D)** with an AUC ROC of 0.69 **(E)**. To determine whether there were any sex differences in circulating DAPK1, we split the cohort into the 1st **(F)** and 2nd 1000 **(G)** and assessed levels in female and male pregnancies. No significant difference was identified in the first 1000 and a modest increase in pregnancies carrying a female infant was observed in the 2nd 1000. Individual dots represent individual patients, data show as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

preeclampsia. We indeed confirmed that DAPK-1 was significantly increased in the circulation of women with preterm preeclampsia (Fig. 2A) relative to women who delivered healthy babies at term (gestation at blood sampling was matched between groups).

3.3. Placental DAPK-1 is increased in women with established preterm preeclampsia

Given circulating DAPK-1 is increased in association with both preterm and term preeclampsia, and that DAPK-1 is highly expressed in the placenta, it is plausible that the origin of circulating DAPK-1 is the placenta. To examine whether this may be possible, we next assessed the expression of DAPK-1 in the placentas from women with preterm preeclampsia, or fetal growth restriction (defined as a birthweight < 10th centile and had an iatrogenic preterm birth for fetal indications) who delivered at < 34 weeks' gestation.

Consistent with our findings in the circulation, we observed that DAPK-1 mRNA was significantly increased in preeclamptic placentas but not in placentas from cases of fetal growth restriction (Fig. 2B).

We next measured DAPK-1 protein via Western blot and densitometric analysis. We indeed confirmed that DAPK-1 was significantly increased at the protein level in preterm preeclamptic placentas. Interestingly, we also found DAPK-1 protein expression was significantly increased in placentas from pregnancies affected by preterm fetal growth restriction (Fig. 2C,D).

4. Discussion

Preeclampsia is a serious complication of pregnancy for which the pathogenesis is still quite poorly understood. In this study we report the

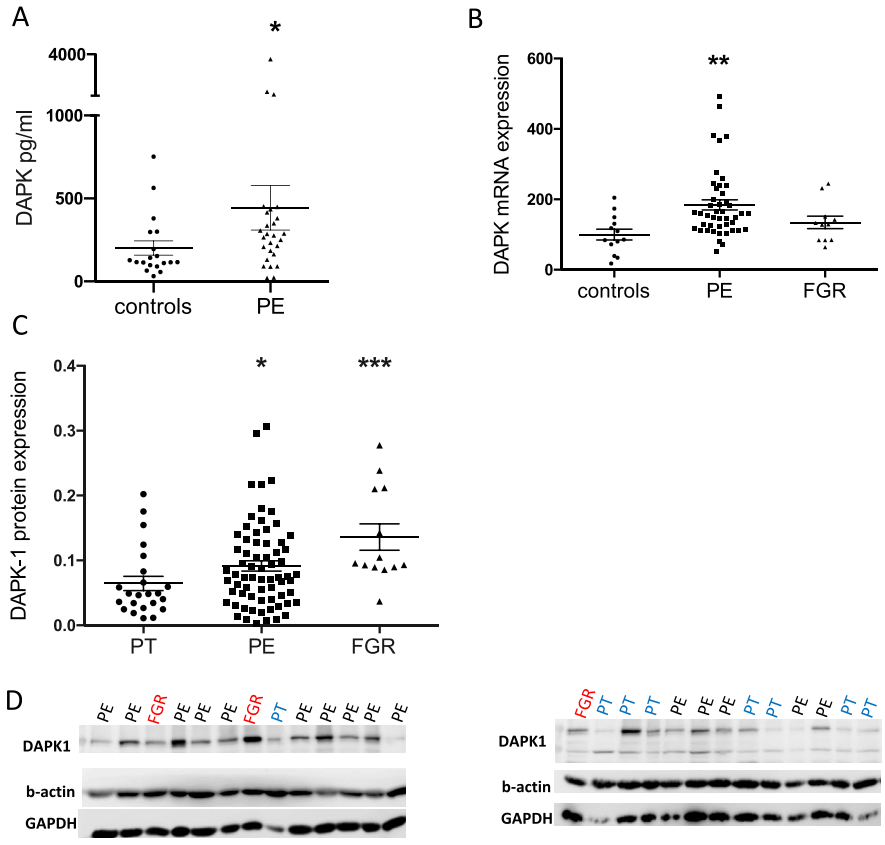

Fig. 2. DAPK-1 is increased in the circulation and placenta of women with early onset pre-eclampsia. Circulating DAPK-1 was measured in a group of women who delivered at < 34 weeks' gestation with preeclampsia (n = 19) relative to women who delivered normal healthy babies at term (n = 27; gestation at blood collection was matched to PE women) **(A).** Circulating DAPK-1 was significantly higher in women with preeclampsia relative to healthy controls. We also measured DAPK-1 mRNA **(B)** and protein expression **(C)** in placentas from women with preeclampsia or FGR relative to pre-term controls. DAPK-1 mRNA expression was significantly elevated in the preeclamptic cohort (n = 45) relative to pre-term controls (n = 13), with no significant changes in the FGR cohort (n = 11) identified. DAPK-1 protein measured via Western blot was also significantly elevated in the PE cohort (n = 68) relative to preterm controls (n = 23) and also significantly elevated in the FGR cohort (n = 13). A representative Western blot is shown in D. Individual dots represent individual patients, data show as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

novel finding that DAPK-1, a molecule that is highly expressed in the placenta relative to other human tissues, is raised in association with preeclampsia. We robustly demonstrated in a large cohort that DAPK-1 is increased in the circulation of women preceding their diagnosis of preeclampsia at term. We also showed that the mRNA and protein expression of DAPK-1 is elevated in the placentas of pregnancies affected by preterm preeclampsia, suggesting that the source of circulating levels may be placental.

As its name suggests, DAPK-1's primary function is regulating cell death (for review see Ref. [18]). DAPK-1 was originally discovered as a gene whose protein product was necessary for interferon-γ induced cell death of He–La cells [19], and it is now well established that its death-promoting properties can be induced by various stimuli beyond interferon-γ. Indeed, studies have linked DAPK-1 to both caspase-dependent and caspase-independent cell death [20–22]. It participates in a wide variety of cellular events including apoptosis, autophagy, membrane blebbing and stress-fibre formation. Additional evidence supporting its role in cell death was obtained from studies showing that reduced DAPK-1 expression can lead to increased cell survival in long-term clonal viability assays [19]. DAPK family members also function as tumour suppressors and are specifically down-regulated in many types of cancer [23–26] with loss of DAPK in cancer usually due to hypermethylation of the DAPK gene [24].

To date, there has been little study of DAPK-1 expression or its role in placental biology, specifically preeclampsia. One study that measured DAPK-1 expression concluded it was down-regulated following treatment of first trimester explants with TNFα and interleukin-6 and that it is also down-regulated in preeclamptic placentas [16]. This is the opposite to our consistent findings. We note a possible explanation is that in the prior study, authors only examined a small cohort of placentas, 5 from pregnancies affected by preterm preeclampsia, which were compared with six placentas from term pregnancies (thus samples were not gestation-matched). In contrast, we examined 1) circulating DAPK-1 levels from a large cohort; 2) circulating levels in another cohort of women with preterm preeclampsia; and 3) a cohort of placentas from cases of preterm preeclampsia. These all robustly demonstrate that DAPK-1 is consistently increased in preeclampsia. In future studies, it may also be of interest to assess umbilical-cord blood DAPK-1 expression as further evidence of its dysregulation in preeclamptic placenta.

Indeed, finding that DAPK-1, a molecule associated with apoptosis and autophagy, is increased in preeclampsia is perhaps not surprising. There are now a number of studies that suggest there is elevated apoptosis and autophagy in preeclamptic placentas [8,27–29]. To our knowledge however, this is the first report on plasma levels of DAPK-1 protein as a potential disease marker. Of note, we found circulating DAPK-1 levels were consistently increased preceding the diagnosis of clinical disease. We note that the AUCs, like all other biomarkers for preeclampsia, suggest it will not perform sufficiently well as a single blood biomarker. However, it may have merit when combined with other markers and we believe it worth studying as a potential biomarker in future studies. Interestingly, there have been reports proposing the measurement of circulating DAPK-1 as a biomarker of other diseases. It has been shown that circulating plasma mRNA or DNA for DAPK-1 is increased in cardiovascular disease [30] and that the promoter of the DAPK-1 gene has an altered methylation state and may represent an epigenetic marker for cervical cancer risk [31,32].

In conclusion, this study has identified DAPK-1 as a novel circulating marker elevated preceding preeclampsia diagnosis. We also provide data to suggest that its likely site of origin is the placenta. Although our studies provide robust evidence that circulating DAPK-1 is increased with preeclampsia, it is unlikely to be useful as a lone clinical biomarker for preeclampsia. However, it may be worthwhile examining whether it adds to the performance of other well-known markers of preeclampsia to screen for risk of developing preeclampsia, such as soluble fms-like tyrosine kinase-1 (sflt1) [33,34], soluble endoglin (sEng) [35], placental growth factor (PlGF) [34], activin A [36]

and many others. Given its known role in apoptosis and autophagy, cellular processes that occur in preeclampsia, the molecular role of DAPK-1 in the placenta merits further attention. Furthermore, it may have potential as a clinical biomarker, perhaps when combined with other circulating biomarkers, or if added with clinical risk factors.

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Declaration of competing interest

TMM, SPW, ST and TKL are listed as inventors for molecules to identify placental insufficiency unrelated to DAPK-1.

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