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Circulating adrenomedullin mRNA is decreased in women destined to develop term preeclampsia☆

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ABSTRACT

Preeclampsia is a pregnancy complication associated with elevated placental secretion of anti-angiogenic factors, maternal endothelial dysfunction and end-organ injury. Adrenomedullin (ADM) is a pro-angiogenic peptide hormone which regulates blood pressure and vascular integrity. It is highly expressed in both the placenta and vascular endothelial cells. We performed a nested case-control study, selected from a large prospective cohort of over 2000 participants. Circulating *ADM* mRNA was reduced at both 28 (n = 39 vs 248 controls, p = 0.005) and 36 weeks' of pregnancy (n = 39 vs 205 controls, p < 0.0001) in those destined to develop term preeclampsia. It was also significantly reduced in the circulation of women with established early-onset preeclampsia (n = 34 vs 21 controls, p = 0.01). ADM mRNA (n = 34 vs 12 controls) and protein (n = 53 vs 17 controls) were significantly decreased in placental tissue from women with early-onset preeclampsia (p = 0.02, p = 0.0002 respectively), suggesting the placenta is a possible source of the reduced circulating mRNA. Functional studies in primary endothelial cells revealed significantly reduced *ADM* mRNA expression when cells were exposed to cytotrophoblast conditioned media (derived from normotensive pregnancies, p < 0.0001) or TNFα (p < 0.0001), suggesting another possible source of reduced circulating *ADM* mRNA is the endothelium.

Circulating *ADM* mRNA, but not protein, is reduced 10–12 weeks before the diagnosis of term preeclampsia. It may be of endothelial or placental origin. Whole blood mRNA is a rich source of potential biomarker discovery in the prediction of preeclampsia.

1. Introduction

Preeclampsia affects 2–8% of pregnancies worldwide each year [1] and is characterised by significant maternal endothelial dysfunction including hypertension and end organ involvement. The maternal endothelial dysfunction occurs as a result of high levels of antiangiogenic factors released from the placenta [2], e.g. soluble fms-like tyrosine kinase 1 (sFlt-1-1) [3] and soluble endoglin (sEng) [4], and reduced levels of proangiogenic molecules such as placental growth factor (PlGF).

Adrenomedullin (ADM) is a proangiogenic peptide hormone that is a potent vasodilator. It is highly expressed in the placenta relative to other human tissues, where it is speculated to play a role in trophoblast implantation and angiogenesis [5]. ADM is also expressed in endothelial cells and vascular smooth muscle cells and thus plays an important role in vascular homeostasis [6]. ADM is first secreted as a preprohormone and peripherally cleaved to its active form. Cleavage produces the biologically inactive by-product, pro-ADM (pro-ADM) and active ADM in a 1:1 ratio. This is important as active ADM has a very short half-life (22 min) and is rapidly removed from the circulation. Many studies have chosen to measure the more stable pro-ADM protein as a proxy for ADM [7]. As a result of its role in maintaining cardiovascular homeostasis, pro-ADM is being used to predict prognosis in patients suffering from septic shock [8], highlighting its potential as a biomarker of cardiovascular associated diseases. Thus, it is plausible that either pro-ADM or ADM might be altered either preceding or

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Table 1
Demographics of patients analysed at 36 weeks' prior to diagnosis of preeclampsia compared with control. There was no significant difference in age, incidence of gestational diabetes, parity, or gestational age at delivery. A significant difference between the two groups can be seen in birthweight and BMI.

		Controls (n = 205)	Preeclampsia (n = 39)	*P*
Age (mean)		33.0	32.3	0.34
GDM		22% (42)	22% (8)	0.83
Smoker		6% (12)	5% (2)	> 0.99
Parity (Primip)		63% (121)	70% (27)	0.46
Gestational age at delivery (weeks/mean)		39.5	39.11	0.08
Birthweight (Mean)		3464	3297	< 0.0001
Mode of delivery	Normal Vaginal	45% (92)	30% (13)	0.1
	Instrumental	19% (39)	18% (7)	
	Caesarean Section	36% (74)	46% (18)	
BMI (mean)		25	31	< 0.0001

following development of preeclampsia.

Our group has previously shown that *ADM* mRNA transcripts are detectable within the circulation [9]. In that study, we demonstrated *ADM* mRNA was detectable in maternal circulation in those with severe preterm preeclampsia and in the placentas from the same patients. We showed no difference in this very small cohort. Here we measured maternal circulating *ADM* mRNA in those destined to develop term preeclampsia. Given ADM is highly expressed in both the placenta and vasculature, the tissue of origin of circulating *ADM* mRNA could be either. We measured placental *ADM* mRNA expression in preeclamptic placentas and controls (suggesting possible placental origin), and we undertook functional studies to elucidate whether endothelial ADM is changed in response to endothelial dysfunction (suggesting possible endothelial origin).

2. Methods

Preeclampsia can be divided into two categories: early onset preeclampsia, the pathogenesis of which is thought to be poor placentation and occurs before 34 weeks, and late onset preeclampsia which occurs after 34 weeks with maternal endothelial cell dysfunction implicated as the cause [10]. Furthermore, preeclampsia can be referred to as term or preterm [11], referring to before or after 37 weeks' gestation which is considered full term. Of course, there is a degree of overlap between these classification systems. In our studies involving the FLAG cohort, we have used the label 'term preeclampsia' as our patients developed preeclampsia after 37 weeks' gestation, but this also refers to late-onset preeclampsia by definition.

2.1. Study participants

The Fetal Longitudinal Assessment of Growth (FLAG) study was carried out at the Mercy Hospital for Women, a tertiary maternity hospital in Melbourne with approximately 6000 births annually. The FLAG study, designed to identify biomarkers to detect small for gestational age (SGA) fetuses, included prospective collection of 2015 blood samples from pregnant women at 28 and 36 weeks' gestation (collected February 2015-May 2016). 3.9% of participants developed preeclampsia which is consistent with the expected population incidence [1].

For measurement of ADM mRNA we undertook 3 separate analyses from the FLAG study. The first analysis was a case/control cohort at 36 weeks. The second analysis was a case/control cohort at 28 weeks and the final analysis was validation of changes in the entire first 1004 women at 36 weeks.

We initially performed a nested case-control study using blood samples from the first 1004 FLAG participants. At 36 weeks we chose controls at a 2:1 ratio with those participants who went on to deliver an SGA infant; our analysis for preeclampsia was a sub-study in which we utilised these same controls. At 36 weeks' we therefore analysed 205 controls. Twelve were excluded due to technical processing error (e.g. poor cDNA synthesis). We had available to us 40 patients who went on to develop preeclampsia and 1 was excluded due to technical processing error.

We next went on to assess the case control cohort at 28 weeks. At 28 weeks, we had samples available from 39 women who went on to develop term preeclampsia. 27/39 were the same women that we measured ADM mRNA levels in at 36 weeks. 7 women from which we had a 28 week blood, did not give a 36 week sample. The additional 5 women that were included at 36 weeks gave a 36 week blood, but not a 28 week blood sample.

When we went on to validate our results in the entire first 1004, we again had available to us 40 women who went on to develop term preeclampsia, of which 39 were included in the analysis. 4 of the women included in the case control study did not have RNA remaining when we ran the validation study – however we had an addition 4 patients available to us – thus samples from 39 women were included in the validation study, who subsequently developed preeclampsia.

This study was approved by the Mercy Health Research Ethics Committee, number R14/12 and written informed consent was obtained from all participants.

2.2. Participant cohort: the (FLAG) study

Women were screened for eligibility and invited to participate at their oral glucose tolerance test, offered around 28 weeks' gestation to diagnose Gestational Diabetes Mellitus (GDM). English-speaking women aged over 18 years, carrying a well-dated singleton pregnancy with normal mid-trimester morphology ultrasound were eligible to participate. Whole blood was collected in a PAXgene RNA tube at the time of enrolment, when the glucose tolerance test was taken (at 27^{+0}–29^{+0} weeks) and at 35^{+0}–37^{+0} weeks' gestation inclusive.

Maternal characteristics and pregnancy outcomes were obtained by review of each participant's medical record, investigation results and hospital database entry. The maternal characteristics and clinical outcomes for participants at 36 weeks are summarised in Table 1. Thirty-nine then went on to develop preeclampsia after 36 weeks, their demographics and pregnancy outcome data are presented in Supplementary Table 1. We also analysed blood samples collected at 28 weeks' gestation – the maternal characteristics of this cohort are presented in Supplementary Table 2. To validate our date, we also analysed the expression of *ADM* mRNA among the first 1004 FLAG patient samples, comparing those destined to develop preeclampsia to the entire cohort as controls, including those with other placental pathologies such as SGA to validate our findings from the case control cohort. The maternal characteristics of this cohort are presented in Supplementary Table 3.

Preeclampsia was diagnosed according to The American College of Obstetricians and Gynecologists' Taskforce on Hypertension in Pregnancy definition [12] new onset hypertension (blood pressure ≥140 mmHg systolic, or ≥90 mmHg diastolic on two occasions ≥four hours apart after 20 weeks' gestation); plus one of new-onset:

proteinuria, thrombocytopaenia, renal insufficiency, impaired liver function, pulmonary oedema or cerebral symptoms.

2.3. Participant cohort: established preeclampsia

Blood was collected from women presenting to the Mercy Hospital for Women who gave written informed consent. From preterm pregnancies not complicated by preeclampsia we obtained blood from 21 women. In those complicated by severe early-onset preeclampsia (< 34 weeks' gestation) we obtained 34 blood samples, with preeclampsia diagnosed as above. Preterm control samples were gestation matched and selected from women who went on to deliver healthy babies at term. We also obtained blood samples from 8 term controls with no evidence of infection or hypertensive disorders and 10 term samples from patients with established preeclampsia. All patients delivered by caesarean section. Patient characteristics are shown in Supplementary Tables 4 and 5 respectively.

Human Ethics approval was obtained for this study from the Mercy Health Human Research Ethics Committee number R11/34. All women provided informed written consent.

2.4. Placental tissue collection

Placental tissue was obtained immediately following delivery. Placental samples were taken from multiple sites according to CoLab recommendations [13]. Maternal and fetal surfaces were removed and the sample washed in sterile phosphate-buffered saline (PBS) before being placed in RNA later and then stored at −80 °C.

We obtained preterm placentas (< 34 weeks gestation) from 12 pregnancies not complicated by preeclampsia and 34 placentas from those with severe early onset disease for RNA analysis. We utilised an expanded cohort of placentas for western blot protein analysis; 17 control placentas from normotensive pregnancies and 53 from patients with severe early onset preterm preeclampsia. Patient characteristics are shown in Supplementary Table 6 (for mRNA analysis) and 7 (for protein analysis).

2.5. Isolation of primary human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated as previously described [14]. Cells were cultured in M199 media (Life Technologies) containing 10% fetal calf serum, 1% antibiotic-antimycotic (Life Technologies) and 1% endothelial cell growth factor (Sigma) and 1% heparin and used between passages 2 to 4. Primary HUVECS were maintained in a humidified incubator with room concentration O_2 at 5% CO_2.

2.6. Isolation of primary human cytotrophoblast

Human cytotrophoblasts were isolated from term, caesarean section placentas as previously described [15]. Primary cytotrophoblasts were cultured in DMEM high Glutamax (Life Technologies) containing 10% FCS and 1% antibiotic-antimycotic on fibronectin (10 mg/mL; BD Biosciences, New South Wales, Victoria) coated wells. Cells were plated and allowed to attach over 12–18 h before washing with dPBS (Life Technologies) to remove cellular debris. Primary cytotrophoblasts were maintained in a humidified incubator at 8% O_2 (to simulate placental normoxia) or 1% O_2 (to simulate placental hypoxia) and 5% CO_2 for 24 h.

2.7. Isolation of placental explants

Placental explants were collected from term placentas. Maternal and fetal surfaces were removed before small (1 mm^3) explants of villous tissue were dissected. 3 villous explants were utilized per well in triplicate wells. Placental explants were maintained in a humidified incubator at 8% O_2 (normoxia) or 1% O_2 (hypoxia) and 5% CO_2 for 48 h for analysis of ADM.

2.8. RNA extraction

PAxgene blood RNA tubes were incubated for at least 2 h at room temperature after blood collection. Tubes were centrifuged for 10 mins at 3000–5000*g*. Supernatant was decanted and pellet resuspended in RNase free water before another centrifuge for 10 min at 3000–5000**g*. The pellet was dissolved in buffer BM1. The remaining steps for RNA extraction were performed in the QIAcube (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA was stored at −80 °C.

RNA was extracted from primary HUVECs, placental tissue or primary cytotrophoblasts using an RNeasy mini kit (Qiagen, Valencia, CA) and quantified using the Nanodrop ND 1000 spectrophotometer (NanoDrop technologies Inc, Wilmington, DE).

2.9. cDNA and RT-PCR

RNA was converted to cDNA using Applied Biosystems high capacity cDNA reverse transcriptase kit (Life Technologies).

Gene expression of ADM (*ADM*), Vascular Cell Adhesion molecule 1 (*VCAM-1*), endothelin 1 (*ET-1*), *YWHAZ, Topoisomerase-1 (TOP-1), cytochrome-c1 (Cyc-1), GUSB and B2M* (Life Technologies) were quantified by real time PCR (RT-PCR) on the CFX 384 (Bio-Rad, Hercules, CA) using FAM-labeled Taqman universal PCR master mix and its specific primer/probe set (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). *GUSB, YWHAZ and B2M* were used as housekeepers for PAXgene whole blood analyses. *YWHAZ* was used as a housekeeper for mRNA analyses on cells. *Top-1 and CYC-1* were used as housekeepers for placental tissue. Where multiple housekeepers were used, we took an average of Cq values. Data was analysed using the ΔΔCT method of analysis.

2.10. Western blot analysis

20 μg of placental lysates (n = 17 PT and n = 53 preeclampsia) were separated on 10% polyacrylamide gels with wet transfer to PVDF membranes (Millipore, Billerica, MA). Membranes were incubated overnight with a primary antibody targeting ADM (Rabbit anti-human *ADM*, ABCAM, 1:500) or GAPDH (1:5000, Cell Signaling Technology, Danvers, MA, USA) and visualized using an enhanced chemiluminescence detection system (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ChemiDoc XRS (BioRad, Hercules, CA, USA). Relative densitometry was determined in all samples using Image Lab (BioRad).

2.11. Endothelial dysfunction assays

Primary HUVECs were seeded into 24-well plates for 24 h before media was removed and replaced with M199 containing 10% FCS, 1% anti-anti, 1% heparin, 1% ECGS, and 50% cytotrophoblast conditioned media, or 50% DMEM, 1% antibiotic–antimycotic, 10% FCS (that had not been exposed to cytotrophoblasts) for 24 h. Media was removed and cell lysates were collected for RNA extraction, cDNA conversion and RT-PCR for *ADM*, and *VCAM*.

Alternatively, endothelial dysfunction was induced with 1 ng/ml TNFα. Vehicle control or TNFα was applied to HUVECs for 24 h before cell lysates were collected for RNA extraction. cDNA conversion and RT-PCR for *ADM*, *ET1* and *VCAM1*. We used n = 3 replicates.

2.12. ELISA

Pro-ADM, ADM, sFlt-1, sENG and ET-1 was measured in conditioned cell media using the Human Pro-ADM ELISA kit (Resolving Images), Human ADM ELISA kit (mybiosource.com), Human sFlt-1 ELISA kit

(RnD Systems, Minneapolis, USA) Human sENG ELISA kit (RnD Systems) and the Human Endothelin-1 ELISA kit (In Vitro Technologies) according to manufacturer's instructions.

2.13. siRNA knockdown of ADM

siRNA targeting *ADM* was administered to primary HUVECs at 20 nM or a matching concentration of control non-targeted (negative) siRNA (Qiagen, Limburg, Netherlands) using RNAiMAX (ThermoFisher). Primary HUVECs were cultured for 72 h with media changed at 24 h and 48 h post transfection. Media was collected for ELISA and cell lysates for mRNA expression as described above. We used n = 3 replicates.

2.14. Statistical analysis

Triplicate technical replicates were performed for in vitro experiments, with a minimum of three independent biological replicates performed for each in vitro study. Data was tested for normal distribution and statistically analysed as appropriate. When three or more groups were compared a 1-way ANOVA (for parametric data) or Kruskal-Wallis test (for non-parametric data) was used. When two groups were compared a student's T-test or Mann-Whitney test was used. All data is expressed as mean ± SEM. P values < 0.05 were considered significant. Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA).

3. Results

3.1. Circulating ADM mRNA is reduced among women destined to develop term preeclampsia, and in those with established disease

To determine whether circulating *ADM* is altered preceding disease, *ADM* mRNA was measured in samples collected as part of the Fetal Longitudinal Assessment of Growth (FLAG) study. The FLAG study took blood samples from > 2000 women at 28 and 36 weeks of gestation. We identified 39 of the first 1004 participants who went on to develop late-onset preeclampsia and selected these in a case-control analysis. Controls were matched to a separate cohort of women who delivered babies which were small for gestational age. Circulating *ADM* mRNA in those destined to develop preeclampsia at 36 weeks' gestation was significantly lower than controls in the case control cohort (Fig. 1A, $p < 0.0001$). The receiver operating characteristic (ROC) curve showed an area under the curve (AUC) of 0.72 (Fig. 1B). Circulating *ADM* mRNA at 28 weeks' gestation was also significantly decreased (Fig. 1C, $p = 0.005$). The ROC curve indicated an AUC of 0.64 (Fig. 1D). Thus, term preeclampsia is associated with significantly decreased circulating *ADM* mRNA, up to 10–12 weeks preceding clinical diagnosis.

To validate the finding from the case control set that *ADM* mRNA is depressed at 36 weeks' gestation, we re-measured levels in the entire first 1004 patient samples from the FLAG cohort (Supplementary Table 3). Importantly, this reduces the incidence of preeclampsia from being an enriched cohort (case, control design) to that seen in the general population. In doing this, we assigned all women without preeclampsia into the control group, including those with small for gestational age pregnancies without preeclampsia (those with preeclampsia and a small for gestational age infant at birth were placed in the preeclampsia cohort) We found that *ADM* mRNA was consistently significantly decreased (Fig. 1E, $p = 0.017$, Fig. 1F AUC = 0.62) in patients who developed preeclampsia (n = 39) compared with controls (n = 965). This confirms that *ADM* mRNA is reduced preceding the diagnosis of late onset preeclampsia.

We next measured circulating *ADM* mRNA in whole blood among an independent cohort − 34 women with established early-onset preeclampsia (delivered < 34 weeks' gestation), compared with gestation matched blood from 21 normotensive healthy control women who subsequently delivered at term. The clinical characteristics of the participants in this cohort are shown in Supplementary Table 4. Circulating *ADM* mRNA concentrations were significantly reduced among participants with preterm preeclampsia, compared to gestation matched controls ($p = 0.011$; Fig. 2A). Similarly, circulating *ADM* mRNA was significantly reduced in the blood of women from a third cohort established term preeclampsia (n = 8) relative to gestation matched controls (n = 10, $p = 0.035$ Fig. 2B). The clinical characteristics of the participants in this cohort are shown in Supplementary Table 5. Thus, we have shown in three independent cohorts that circulating *ADM* mRNA concentrations are consistently decreased in association with preeclampsia.

3.2. Circulating ADM and pro-ADM protein are increased in patients with established preeclampsia but not preceding disease diagnosis

Given our finding that circulating *ADM* mRNA was reduced in established preeclampsia, we next measured circulating ADM protein. Interestingly, circulating ADM protein was significantly increased in patients with established early onset preeclampsia (Fig. 2C, $p = 0.014$; intra-assay %CV = 0–4.3) i.e. the opposite direction circulating *ADM* mRNA concentrations. Given ADM has a short half-life and pro-ADM is more stable, we also measured pro-ADM in the same patients. Consistent with the trends seen with ADM protein, we found circulating pro-ADM was significantly increased in patients with established early onset preeclampsia (Fig. 2D, $p < 0.0001$, intra-assay %CV = 0.2–8.7). Subsequently we measured pro-ADM preceding diagnosis (samples taken at 36 weeks in the FLAG cohort) and found no significant difference in circulating pro-ADM in patients destined to develop preeclampsia compared to controls ($p = 0.95$, Fig. 2E; intra-assay %CV = 0.1–32.3).

3.3. ADM mRNA and Pro-ADM protein are significantly reduced in preeclamptic placentas

ADM is known to be highly expressed in placenta [9]. As such, *ADM* mRNA and protein expression could be dysregulated in preeclamptic placentas and this could be a tissue of origin of circulating *ADM* mRNA. Subsequently we measured *ADM* mRNA (Patient data detailed in Supplementary Table 6) and protein expression (Patient data detailed in Supplementary Table 7) in preterm preeclamptic placentas. Placental *ADM* mRNA was significantly reduced (Fig. 3A, $p = 0.02$) in patients with severe early onset preeclampsia (n = 34) compared with gestation matched controls (n = 12). We next measured pro-ADM placental protein expression in a larger cohort of preterm preeclamptic (n = 53) and gestation matched control placentas (n = 17) and also found significantly decreased pro-ADM protein expression (Fig. 3B and 3C, $p = 0.0002$). Thus, placental *ADM* mRNA and protein levels are reduced in preterm preeclamptic placentas.

3.4. Placental ADM is increased by hypoxia

It is known that ADM is regulated by hypoxia and that the preeclamptic placenta may be affected by hypoxia. Thus we measured its expression in primary cytotrophoblasts and placental explants exposed to hypoxia. When term placental explants were exposed to hypoxia, we found no significant change in pro-ADM protein production (Supplementary Fig. 1A, $p = 0.7$), however there was a significant increase in *ADM* mRNA in the explant tissues (Supplementary Fig. 1B, $p = 0.005$). In primary cytotrophoblast cells the presence of hypoxia induced a significant increase in pro-ADM protein production (Supplementary Fig. 1C, $p = 0.033$) but no change in *ADM* mRNA expression (Supplementary Fig. 1D, $p = 0.41$). These findings are somewhat consistent with ADM being a hypoxia-responsive molecule, however are not consistent with our findings in preeclamptic placentas

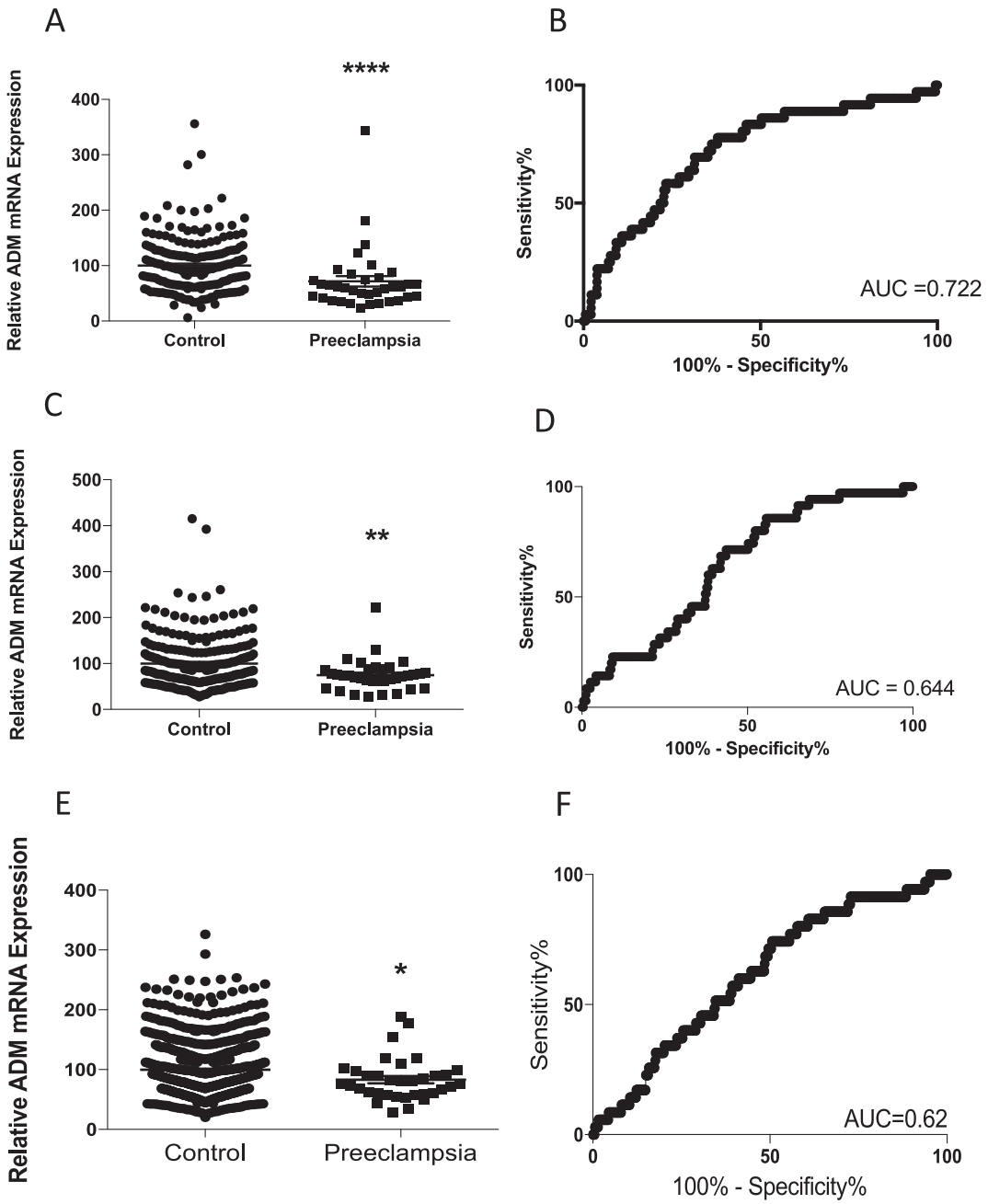

Fig. 1. *ADM* mRNA expression is reduced in maternal whole blood in patients destined to develop preeclampsia. A, *ADM mRNA* was significantly reduced at 36 weeks ($p < 0.0001$) in patients destined to develop preeclampsia (n = 39) compared to those who did not go onto develop the disease (n = 205). B, ROC curve at 36 weeks shows AUC 0.72. C *ADM* mRNA was also significantly reduced at 28 weeks ($p = 0.005$) in patients destined to develop preeclampsia (n = 39) compared to controls (n = 248). D, ROC curve shows area under the curve 0.64 at 28 weeks. E, *ADM* mRNA was significantly reduced ($p = 0.017$) at 36 weeks' gestation in women who subsequently developed preeclampsia at a later gestation in the first 1000 FLAG samples, compared to those with normotensive controls (preeclampsia, n = 39; controls, n = 965) which included patients with other placental disorders e.g. SGA as part of the control group. F, ROC curve shows an area under the curve of 0.62. Data expressed as mean ± SEM. Each individual symbol represents an individual patient.

where ADM was significantly reduced at the mRNA and protein levels.

3.5. ADM is decreased with endothelial dysfunction in HUVECs

Preeclampsia is characterised by widespread maternal endothelial cell dysfunction caused by excessive placental secretion of circulating antiangiogenic factors [16]. Given ADM is highly expressed in endothelial cells, it is plausible that this may be the tissue of origin of circulating ADM mRNA, and that endothelial dysfunction seen in preeclampsia reduces its secretion. To examine this possibility, we investigated whether endothelial dysfunction is associated with changes in ADM expression.

First, we exposed primary HUVECs to cytotrophoblast conditioned media (which is likely to contain placental secreted factors including sFlt-1-1 and sEng) or control media. We demonstrated a significant increase in *VCAM-1* (Fig. 4A, $p = 0.005$, VCAM-1 is a marker of endothelial dysfunction), and significantly reduced *ADM* mRNA expression (Fig. 4B, $p < 0.0001$). Similarly, when we exposed primary HUVECs to Tumour Necrosis Factor-α (TNFα is known to be involved in endothelial dysfunction [17] and elevated in the circulation of preeclamptic patients [18]) we found significantly increased *VCAM-1* expression (Fig. 4C, $p < 0.0001$) and this was concurrent with

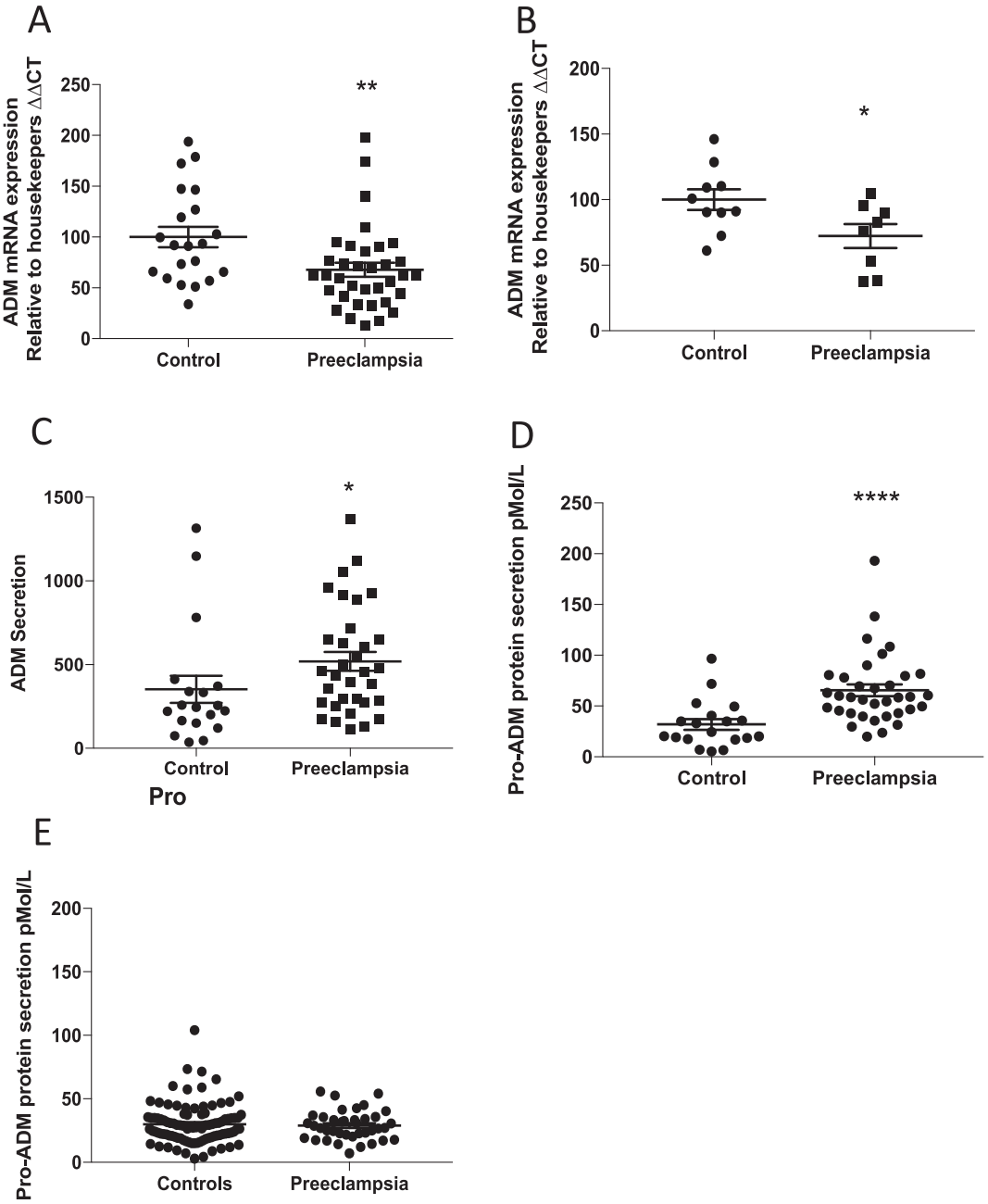

Fig. 2. *ADM* mRNA is reduced in maternal circulation in those with both established early onset and late onset preeclampsia. ADM protein is increased in maternal plasma in patients with established preeclampsia but unchanged in those destined to develop the disease. A, *ADM* mRNA expression was significantly reduced ($p = 0.011$) in patients with severe early onset preeclampsia ($n = 34$) compared to gestation matched preterm controls with no preeclampsia ($n = 21$). B, *ADM* mRNA expression was significantly reduced in the circulation of mothers with established term preeclampsia ($n = 8$) vs control ($n = 10$) ($P = 0.02$) compared to control. Data expressed as mean ± SEM. Each individual symbol represents an individual patient. C, Circulating ADM protein was significantly increased ($p = 0.014$) in patients with severe early onset preeclampsia ($n = 34$) compared to gestation matched preterm controls collected from women who delivered at term without preeclampsia ($n = 21$). D, Circulating pro-ADM was significantly increased ($p < 0.0001$) in patients with severe early onset preeclampsia ($n = 34$) compared to gestation matched preterm controls collected from women at term without preeclampsia ($n = 21$) E, Circulating pro-ADM was unchanged at 36 weeks in patients who were destined to develop preeclampsia at a later gestation ($n = 39$, $p = 0.97$) compared to those who did not develop preeclampsia ($n = 205$). Data expressed as mean ± SEM. Each individual symbol represents an individual patient.

significantly reduced *ADM* (Fig. 4D, $p < 0.0001$). There was no difference in pro-ADM protein secretion from cells treated with TNFα (Fig. 4E, $p = 0.41$). Together, this data provides evidence that endothelial *ADM* mRNA is reduced in states of endothelial dysfunction. Given ADM is highly expressed in endothelial cells, this may also be a tissue of origin of circulating *ADM mRNA*.

3.6. Silencing ADM has no effect on sFlt-1 or sEng secretion from HUVECs

The two main sources of sFlt-1 and sEng are the placenta and endothelial cells within the maternal vasculature. Therefore, we assessed whether silencing ADM using siRNA would affect sFlt-1 or sEng secretion in primary HUVECs. When we silenced ADM, there was no significant change in the secretion of either sEng (Supplementary Fig. 2A, $p = 0.55$) or sFlt-1 (Supplementary Fig. 2B, $p = 0.17$). This suggests

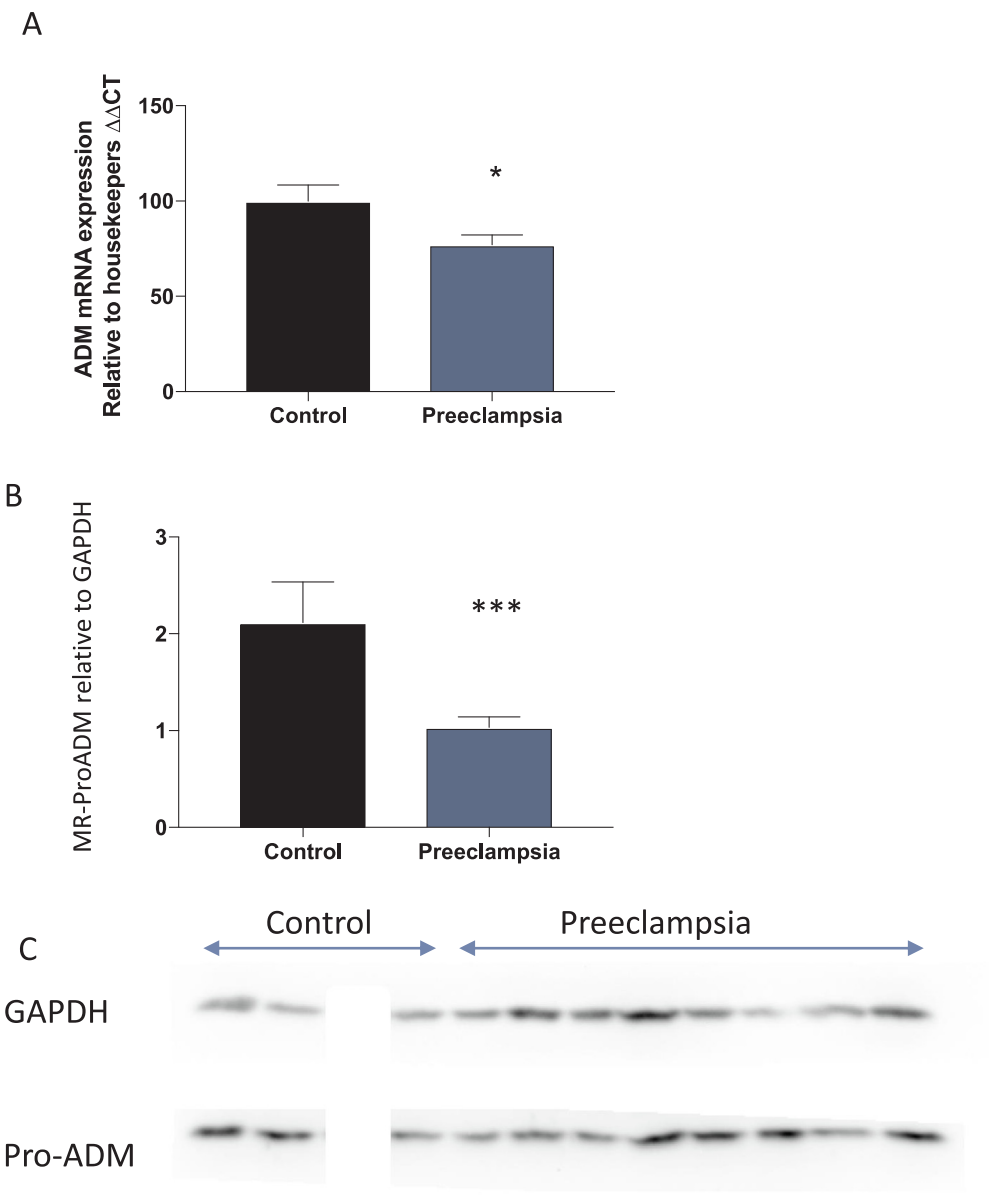

Fig. 3. ADM mRNA and Pro-ADM protein are significantly reduced in preeclamptic placentas. Expression of both *ADM* mRNA and protein were assessed in placentas collected from patients with severe early onset (< 34 weeks gestation) preeclampsia compared to pre-term control placentas. A, *ADM* mRNA expression was significantly decreased ($p = 0.02$) in placentas from patients with severe early onset preeclampsia ($n = 34$) compared with controls ($n = 12$). B, Similarly, pro-ADM protein expression ($p = 0.0002$) was significantly decreased in preeclamptic placentas ($n = 53$) compared to controls ($n = 17$) as measured by Western Blot and densitometric analysis (C). A representative western blot is shown. Data expressed as mean ± SEM.

that altered endothelial expression of *ADM* does not contribute to the high levels of circulating sFlt-1 and sEng found in the circulation of preeclamptic patients.

3.7. Silencing ADM causes increased ET-1 mRNA and protein expression in HUVECs

We next investigated the downstream intracellular effects of reduced endothelial *ADM* expression. *ADM* was silenced in primary endothelial cells using siRNA and measured its effect on markers of endothelial dysfunction. We first confirmed that there was efficient silencing of *ADM* in primary HUVECs (Fig. 5A, $p < 0.0001$). Interestingly, silencing endothelial ADM resulted in a significant decrease in *VCAM-1* expression (Fig. 5B, $p = 0.002$) but significantly increased *ET-1* mRNA expression (Fig. 5C, $p = 0.0002$). *ET-1* is a potent vasoconstrictor that is highly expressed in endothelial cells. Consistent with this finding, we found silencing ADM increased the concentrations of *ET-1* protein secreted into the media (Fig. 5D, $p = 0.033$). Thus, whilst inducing endothelial dysfunction with cytotrophoblast conditioned media or TNFα increases *VCAM-1* expression, our findings suggests *ADM* does not play a role in increased *VCAM-1* expression seen in preeclampsia. In contrast, we have generated functional evidence to suggest *ADM* may regulate *ET-1* expression, which is in keeping with previously published evidence which demonstrated a regulatory role in vascular smooth muscle cells [19].

4. Discussion

Preeclampsia is a leading cause of pregnancy related death for both mothers and babies [20]. Predicting patients who will go onto develop preeclampsia is a major research imperative in obstetrics. Poon and Nicolaides [21] demonstrated that combining maternal risk factors, uterine artery Doppler, mean arterial pressure, maternal serum pregnancy associated plasma protein-A and placental growth factor in a test done at the end of the first trimester has a high predictive value for early onset preeclampsia (but not later onset preeclampsia). Tan et al. [22] demonstrated that this algorithm can predict 75% of preterm preeclampsia, however only 41% of patients who will subsequently develop term preeclampsia were identified. Therefore, there remains a need to identify altered biomarkers before the clinical signs and symptoms of late onset preeclampsia in order to produce an algorithm to identify these 'at risk' patients.

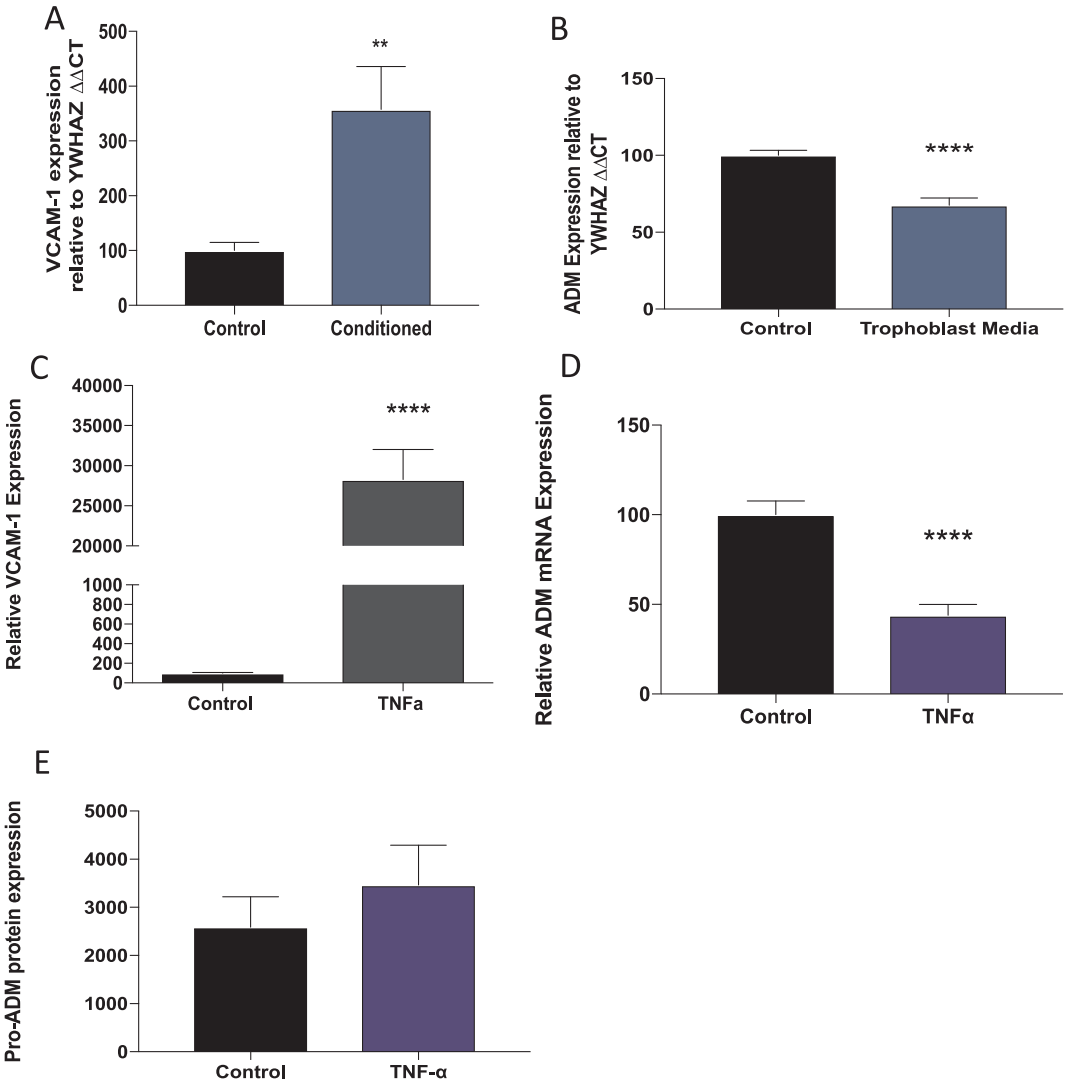

Fig. 4. Endothelial *ADM* mRNA is significantly decreased by placental factors and TNFα. To induce endothelial dysfunction, primary HUVECs were treated with either cytotrophoblast conditioned media or TNFα. After exposure to cytotrophoblast conditioned media there was significantly A, increased *VCAM-1* ($p = 0.005$) and B, decreased *ADM* ($p < 0.0001$). When HUVECS were treated with TNFα we found C, significantly increased *VCAM-1* ($p < 0.0001$) along with D, significantly decreased *ADM* ($p < 0.0001$). E, There was no change in ADM protein secretion by cells treated with TNFα ($p = 0.41$). Experiments were repeated a minimum of $n = 3$ times, with each 'n' representing a separate primary HUVEC isolation and including experimental triplicates. Data expressed as mean ± SEM.

Our investigations have identified three distinct preeclamptic cohorts in which *ADM* mRNA is altered: those with established late onset preeclampsia, established early onset preeclampsia, and before the onset of clinical preeclampsia in those who are destined to develop term disease. In this last group, we have demonstrated significantly down regulated *ADM* mRNA at two time points: 28 and 36 weeks gestation, in a case control cohort. Furthermore, when comparing *ADM* mRNA in those destined to develop preeclampsia in the entire first 1004 FLAG participants (a group which is comparative to the incidence of preeclampsia in the population ~4% [1]) where other placental pathologies, such as SGA without preeclampsia are present are considered in the control group, we still found *ADM* mRNA to be downregulated. This demonstrates that reduced *ADM* mRNA in the maternal circulation could be used as part of an algorithm to distinguish those at risk of developing term preeclampsia from those at risk of other placental pathologies.

One of the main strengths of our study is the size of the cohorts available to us in the FLAG study. We have significant numbers in our established early onset preeclamptic versus control groups, as well as in our placental investigations. Although there are far fewer numbers in the late onset versus control cohort, the fact that we have proven significance in such a small cohort leads us to believe this significance would only increase if larger numbers of patient samples were available.

Our functional studies were carried out in primary tissues, giving us strong evidence that the origin of the circulating *ADM* mRNA may be either placental or endothelial. It is thought that the pathophysiology of late-onset preeclampsia is likely to be vascular, compared to the poor placental implantation characteristic of pre-term disease [11]. Certainly, our functional studies in primary endothelial cells demonstrate that there is downregulation of *ADM* mRNA in states of endothelial cell dysfunction. It has been suggested that ADM has a regulatory role in the production of the potent vasoconstrictor ET-1 [19]. Our functional studies in which ADM is silenced supports this theory, demonstrating a significant increase both *ET-1* mRNA and protein production. We propose that this may be a mechanism by which reduced *ADM* mRNA contributes to the pathophysiology of the disease, vasoconstriction and high blood pressure.

ADM is a hypoxic-regulated gene and its transcription factor is HIF1α [23]. We confirmed that placental explant *ADM* mRNA and primary trophoblast ADM protein is increased by hypoxia, however found that in early onset preeclamptic placentas *ADM* mRNA was

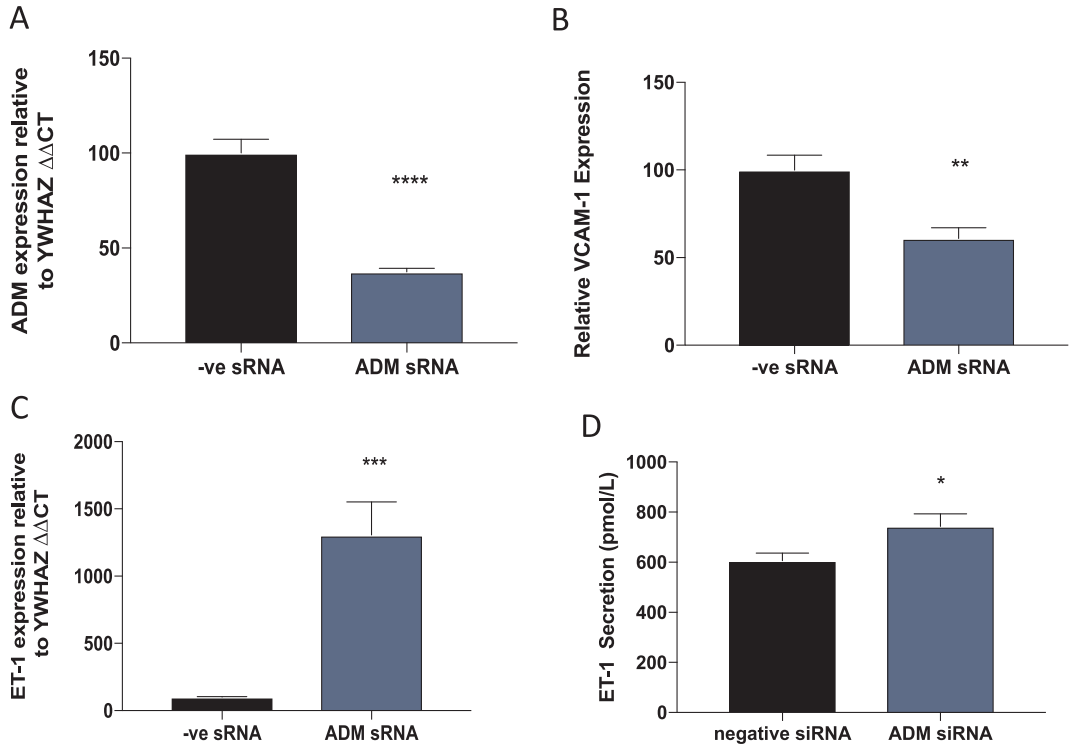

Fig. 5. Silencing *ADM* causes increased ET-1 mRNA and protein expression in HUVECsTo reduce ADM expression, siRNA targeting ADM was added to primary HUVECs. A, As expected, *ADM* mRNA was significantly with ADM siRNA relative to negative or control siRNA ($p < 0.0001$). B Silencing *ADM* in primary HUVECs resulted in significantly decreased *VCAM-1* mRNA expression ($p = 0.002$). C, *ET-1* mRNA ($p = 0.0002$) and D, ET-1 protein ($p = 0.03$) were significantly increased when ADM was silenced. Experiments were repeated a minimum of $n = 3$ times, with each 'n' representing a separate primary HUVEC isolation and including experimental triplicates. Data expressed as mean ± SEM.

significantly reduced. This indicates that there may be other factors regulating ADM in preeclampsia placentas. We provide evidence here the reduced free circulating *ADM* mRNA preceding term preeclampsia diagnosis may in fact be produced by either or both the placenta and maternal endothelium.

There are several limitations to our studies. Of note, there is a significantly higher number of patients with gestational diabetes (GDM) in the 28 week cohort. GDM is a risk factor for preeclampsia and this could represent a confounding factor. However, there is no significant difference in GDM in the 36 week cohort nor in the first 1004, and so we expect that the difference in ADM is due to the difference in preeclampsia rather than GDM.

Furthermore, in those with established preeclampsia, we found consistently reduced *ADM* mRNA. In the established early onset cohort we found increased levels of both ADM protein and pro-ADM protein. We suggest that the inverse correlation between mRNA and protein is due to post transcriptional modification.

5. Conclusion

In summary, *ADM* mRNA is significantly decreased in the circulation of patients destined to develop preeclampsia, up to 10–12 weeks before the onset of clinical signs and symptoms of the disease. In discovering biomarkers that are altered in the circulation of those patients who are destined to develop late onset preeclampsia, we hope to formulate an algorithm using both endothelial and placentally derived biomarkers to triage the intensity of antenatal surveillance, therefore reducing the risks of preeclampsia to both mum and baby.

6. Athors contribution

CW carried out experiments and wrote the manuscript.

TM and SW were principle investigators of the FLAG study and responsible for sample collection.

NP characterized and collated patient demographic data.

NH provided intellectual input and scientific direction.

PC, VN, RH, NDA assisted with scientific experiments.

ST provided intellectual input and scientific direction.

TKL conceived the experiments and provided intellectual input and scientific direction.

All authors reviewed the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2019.02.003.

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