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Sulfasalazine reduces placental secretion of antiangiogenic factors, up-regulates the secretion of placental growth factor and rescues endothelial dysfunction

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ABSTRACT

Background: Preeclampsia is a major complication of pregnancy with no medical treatment. It is associated with placental oxidative stress, hypoxia and inflammation leading to soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sENG) secretion and reduced placental growth factor (PlGF). This results in widespread endothelial dysfunction causing hypertension and multisystem organ injury. Sulfasalazine is an anti-inflammatory and antioxidant medication used to treat autoimmune disease. Importantly, it is safe in pregnancy. We examined the potential of sulfasalazine to quench antiangiogenic factors and endothelial dysfunction and increase angiogenic factor secretion.

Methods: We performed functional experiments using primary human pregnancy tissues to examine the effects of sulfasalazine on sFlt-1, sENG and PlGF secretion. Sulfasalazine is known to inhibit nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and upregulate heme-oxygenase 1 (HO-1) thus we explored the effect of these transcription factors on sFlt-1 secretion from human cytotrophoblasts. We examined the ability of sulfasalazine to reduce key markers of endothelial dysfunction and dilate whole blood vessels.

Findings: We demonstrate sulfasalazine administration reduces sFlt-1 and sENG and upregulates PlGF secretion from human placental tissues. Furthermore sulfasalazine mitigates endothelial dysfunction in several *in vitro/ex vivo* assays. It enhanced endothelial cell migration and proliferation, promoted blood vessel dilation (vessels obtained from women at caesarean section) and angiogenic sprouting from whole blood vessel rings. The effect of sulfasalazine on the secretion of sFlt-1 was not mediated through either the NFκB or HO-1 pathways.

Interpretation: We conclude that sulfasalazine reduces sFlt-1 and sENG secretion and endothelial dysfunction and upregulates PlGF. Sulfasalazine has potential to treat or prevent preeclampsia and warrants investigation in clinical trials.

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1. Introduction

Preeclampsia affects 5–8% of pregnancies and is annually responsible for 60,000 maternal deaths and over 500,000 stillbirths or neonatal deaths [1,2]. Key aspects in the pathophysiology of preeclampsia are the presence of placental oxidative stress, inflammation and hypoxia. This is accompanied by an excess secretion of the anti-angiogenic factors soluble fms-like tyrosine kinase 1 (sFlt-1) [3] and soluble endoglin

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Research in context

Evidence before this study

Preeclampsia is a serious complication of pregnancy with no medical treatment. There has been recent interest in repurposing medications that upregulate innate placental anti-oxidants and reduce hypoxic regulated transcription factors to treat preeclampsia. We have studied statins and identified esomeprazole and metformin as possible therapeutics for preeclampsia as they reduce placental sFlt-1 secretion and reverse features of endothelial cell dysfunction. Here in we identify sulfasalazine, a medication safe in pregnancy, as a possible therapeutic. Importantly its potent anti-inflammatory properties accompanied by anti-oxidant effects identify it as an exciting and novel potential therapeutic. In this study we examined its effect on quenching key features of preeclampsia.

Added value of this study

This work identifies sulfasalazine, a medication safe in pregnancy, with anti-inflammatory and anti-oxidant properties as a possible treatment for preeclampsia. Using primary placental cells we demonstrate sulfasalazine reduces the secretion of sFlt-1 and sENG and increases PlGF. Furthermore using primary endothelial cells and omental vessels we show sulfasalazine mitigates vascular dysfunction and dilates whole vessels.

Implications of all the available evidence

Sulfasalazine is a potential treatment for preeclampsia and investigation in clinical trials is warranted.

(sENG) [4] into the maternal circulation and is accompanied by a reduction in the angiogenic factor placental growth factor (PlGF). This imbalance in antiangiogenic factors accompanied by increased inflammatory cytokines in the presence of widespread endothelial dysfunction leads to hypertension, multisystem organ injury or failure [5–7]. Currently, there is no medical treatment and delivery is the only way to stop disease progression [1]. A medical therapeutic that can stabilise the disease and reduce its severity would be a major advance, and could save many lives globally.

There has been recent interest in repurposing medications that upregulate innate placental anti-oxidant defences and reduce hypoxia. The medications that have been repurposed so far target placental hypoxia and upregulating anti-oxidant defences. An important part of the pathophysiology of preeclampsia is increased placental and systemic inflammation [14–17]. Increased circulating proinflammatory cytokines are likely to further exacerbate endothelial dysfunction. Therefore another approach in developing a treatment for preeclampsia would be to quench inflammation. In this study, we considered the potential of anti-inflammatory medication, sulfasalazine as a prevention or treatment for preeclampsia. Sulfasalazine has anti-inflammatory and immune modulating properties and potentially induces anti-oxidants by upregulating the transcription factor HO-1 [18]. It is widely used to treat inflammatory arthritis and bowel disease and is considered safe to administer in pregnancy (FDA pregnancy category B). Sulfasalazine reduces cytokine secretion by inhibiting both transcription and the nuclear translocation of the inflammatory transcription factor NFκB [19]. It has been reported to reduce vascular neointimal hyperplasia likely through up-regulating endogenous anti-oxidant defences [18]. Therefore we reasoned that sulfasalazine might offer a prevention or treatment for preeclampsia with the added advantage of being an anti-inflammatory drug.

We examined whether sulfasalazine reduces sFlt-1 and sENG secretion, upregulate PlGF secretion and might rescue endothelial dysfunction in preeclampsia. If so, it may have the potential to treat or prevent preeclampsia. This study had three main objectives: 1) to assess the effects of sulfasalazine on sFlt-1, sENG and PlGF secretion from primary human placental and endothelial cells/tissues; 2) to explore whether the effects of sulfasalazine on sFlt-1 and sENG secretion are mediated through its ability to induce HO-1 or inhibit NFκB and 3) to assess whether sulfasalazine can reduce endothelial dysfunction, enhance dilation of human vessels and reverse sFlt-1 inhibition of angiogenesis in mouse aortic ring explants.

2. Materials and methods

Using primary human cells we performed functional experiments by administering sulfasalazine to examine its effect on key aspects of preeclampsia. Specifically we assessed the effect of sulfasalazine on placental secretion of sFlt-1, sENG and PlGF and on reducing markers of endothelial dysfunction on cells and in whole omental vessels. We also explored whether nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and heme-oxygenase 1 (HO-1) affected sFlt-1 secretion using gene overexpression or silencing. To perform these experiments we firstly isolated primary cells.

2.1. Isolating and treating primary human umbilical vein endothelial cells (HUVECs)

We obtained umbilical cords from normal term placenta and the cord vein was infused with 10 ml (1 mg/ml) of collagenase (Worthington, Lakewood, New Jersey) and cells were isolated as previously described [20]. We used cells between passage 2 to 4 and we plated them at 24,000 cells/cm² and treated at 80% confluency with 0, 200, 500, 1000 μM sulfasalazine (sigma) n = 3 for 24 h at 37 °C in 20% O₂ and 5% CO₂. We collected conditioned media for assessment of sFlt-1, sENG and PlGF secretion and cell lysates for RNA and protein extraction.

2.2. Isolating and treating primary human cytotrophoblast cells

We collected term placentas from women having elective caesarean sections and isolated human cytotrophoblasts as previously described [20,21]. We plated cells at different cell densities and treated them for different periods of time as described below (n = 3).

We assessed the effect of sulfasalazine on cytotrophoblasts by plating cells at 24,000 cells/cm² and treating them with increasing doses of sulfasalazine at 0, 50, 100, 200, 300, 500, 750 and 1000 μM for 24 h. We assessed the effect of gene silencing or gene overexpression by plating cells at 175,000 cells/cm² and 125,000 cells/cm² respectively. For silencing experiments, we transfected cytotrophoblasts with Lipofectamine RNAiMAX (life Technologies) and 1) 10 nM siRNA targeting NFκB (Qiagen) or 10 nM siRNA targeting HO-1 (Qiagen) or 2) 16 μg/ml of IKBKB or NFκB p65 over expression plasmid and compared the output to a scrambled siRNA control (Qiagen) or an empty vector plasmid (Origene) respectively. We washed cells 24 h after treating them and changed the media every 48 h for a total treatment time of 96 h. For the experiment assessing HO-1 siRNA we treated the transfected cells with sulfasalazine at 750 μM for the last 24 h of the experiment. All experiments were cultured at 37 °C in 8% O₂ and 5% CO₂.

We collected conditioned media from sulfasalazine, NFκB siRNA and overexpression plasmid (NFκB, p65 and IKBKB) experiments and assessed sFlt-1 and sENG secretion and from the HO-1 siRNA experiment we assessed sFlt-1 secretion. We collected cell lysates for RNA and protein extraction.

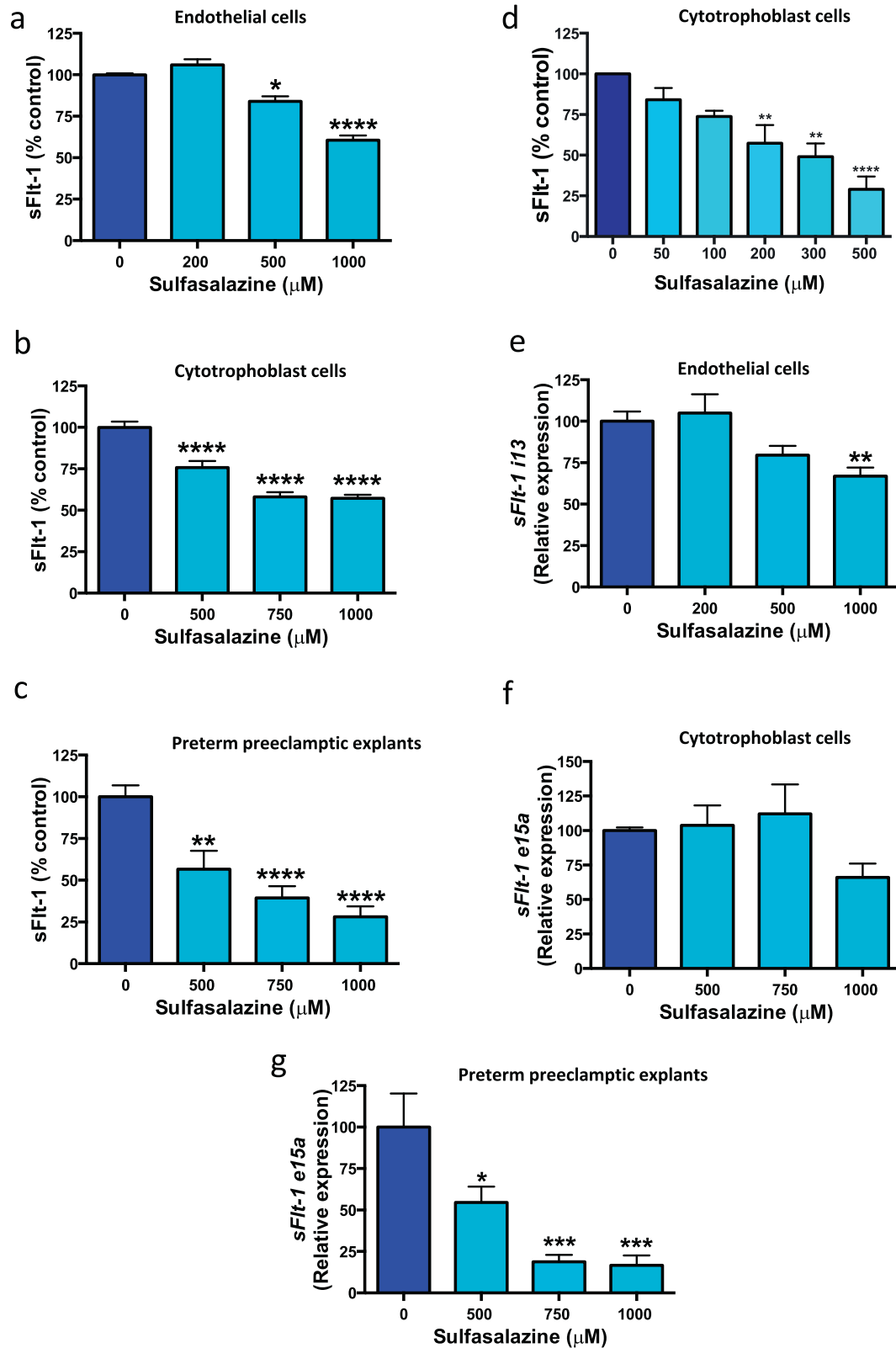


Fig. 1. Effect of sulfasalazine on sFlt-1 secretion and transcription in endothelial and cytotrophoblast cells and preeclamptic placental explants. Sulfasalazine dose dependently reduced sFlt-1 secretion from a) endothelial cells, b) cytotrophoblast cells and c) preterm preeclamptic placental explants. At low doses sulfasalazine reduced sFlt-1 secretion from d) cytotrophoblast cells. Sulfasalazine reduced endothelial cell expression of e) *sFlt-1 i13* isoform, did not change transcription of f) *sFlt-1 e15a* isoform in cytotrophoblasts however g) reduced *sFlt-1 e15a* expression in preterm preeclamptic explants. Data are means \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Kruskal-Wallis).

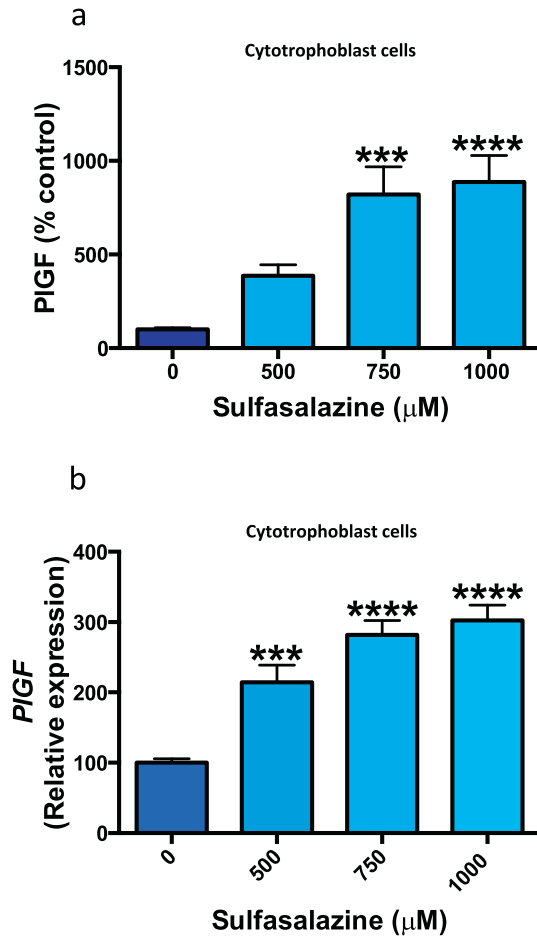


Fig. 2. Effect of sulfasalazine on PIGF secretion from cytotrophoblasts cells. Sulfasalazine dose dependently increased PIGF protein secretion a) and mRNA expression b) from cytotrophoblast cells. Data are means \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Kruskal-Wallis).

2.3. Culture of placental explants

We collected human placental tissue from 4 women with early onset, proteinuric preeclampsia (delivered at ≤ 34 weeks gestation). Preeclampsia was defined using the 2013 American College of Obstetricians and Gynecologists (ACOG) guidelines: the presence of hypertension $>140/90$ on two occasions 4 h apart and any of the following: proteinuria >300 mg/day, renal insufficiency, impaired liver function, thrombocytopenia or visual disturbance [22].

Preterm preeclamptic explants were prepared as previously described [20]. After we cultured the explants in media for 24 h, we treated them with 500, 750, 1000 μ M sulfasalazine (sigma) for 24 h then re-treated and cultured them for a total of 72 h under 8% O_2 and 5% CO_2 at 37 $^\circ C$. We collected the conditioned media and assessed sFlt-1 and sENG levels. We normalized protein secretion against placental explant weights and collected the tissue for RNA extraction.

2.4. Cell viability assay (MTS assay and calcein stain)

We assessed cell viability using CellTiter 96-Aqueous One solution (Promega, Madison WI) assay according to the manufacturer's instructions or we stained the cells with calcein-AM (Merck Millipore, Darmstadt, Germany) and fluorescent signal was detected using Fluostar omega fluorescent plate reader (BMG labtech, Victoria, Australia).

2.5. Endothelial dysfunction in primary HUVECs

We induced endothelial dysfunction by treating primary HUVECs ($n = 4$) with a constant dose of 1) 10 ng/ml TNF α (Sigma) or 2) by administering a pool of 10% serum obtained from three patients with preterm preeclampsia (comparison against 10% serum from a pooled sample from 3 gestationally matched normotensive controls). We administered increasing doses of sulfasalazine at 0, 200, 500, 750 μ M for 24 h at 20% O_2 , 5% CO_2 and 37 $^\circ C$. We collected lysates for RNA or protein extraction.

2.6. Endothelial cell migration and proliferation

We used the xCELLigence system (Roche diagnostic, New South Wales, Australia) to assess changes in endothelial cell migration and invasion. We used vascular endothelial growth factor (VEGF) (Bio-scientific, New South Wales, Australia) at 12.5 ng/ml as the chemoattractant in the lower well. We plated HUVECs between passage 2–4 at 160,000 cells/cm 2 in the upper well and treated them with 0 μ M or 200 μ M sulfasalazine to assess migration. We plated HUVECs between passage 2–4 were plated at 20,000 cells/cm 2 and treated with 0 μ M or 200 μ M sulfasalazine to assess proliferation. We measured electrical impedance for migration and proliferation in each well every minute for 2 h then every hour for 24 h.

2.7. Whole omental artery pressure myography

We obtained omental arteries ($n = 5$) from patients undergoing elective caesarean section at term and prepared experiments as previously described [12]. Briefly, omental arteries were mounted on glass cannulae in a pressure myograph organ bath (Living Systems Instrumentation, Burlington, VT, USA) and pre-constricted with 0.1 μ M U46619 (to reach 70% of maximum constriction). We treated omental arteries with sulfasalazine or vehicle (DMSO, dimethyl sulfoxide) at increasing increments (0.1–3000 μ M with 2.5 min exposure at each dose) and the outer diameter of the vessel was assessed. The maximum final concentration of DMSO in the organ bath was 0.1%.

2.8. Mouse aortic ring explants

We assessed the effects of sulfasalazine on angiogenesis *ex vivo* using mouse aortas that we scavenged and dissected as previously described [23]. Briefly, we cut the vessel into 0.5 mm rings and serum starved it in Optimem at 20% O_2 , 5% CO_2 at 37 $^\circ C$ overnight. We embedded it in a collagen matrix (1 mg/ml in DMEM, pH adjusted so slightly basic using NaOH) in a 96 well plate with one mouse aortic explant per well. We treated the rings in media containing Optimem with 2.5% fetal calf serum (FCS) (Sigma, St Louis, United States) and antibiotic antimycotic 1% (Life Technologies) \pm 250 ng/ml sFlt-1 and 0 or 25 μ M sulfasalazine (Sigma). We obtained $n = 5$ rings for each condition per mouse sample $n = 4$. We changed the treatment every 48 h and continued the experiment for a total of 144 h. We added calcein-AM (Merk Millipore) to the wells for 45 min at 37 $^\circ C$ and images were obtained at the same magnification ($\times 40$) using the EVOS FL microscope (Life Technologies). We determined vessel outgrowth by calculating area of growth using the Image J software (<http://imagej.nih.gov/ij/>) using a blinded observer.

2.9. ELISA analysis

We measured concentrations of sFlt-1, sENG and PIGF in conditioned cell/tissue culture media using the DuoSet VEGF R1/Flt-1 kit (R&D systems by Bioscience, Waterloo, Australia), a DuoSet Human Endoglin CD/105 ELISA kit (R&D systems) or DuoSet Human PIGF kit (R&D systems) according to manufacturer's instructions.

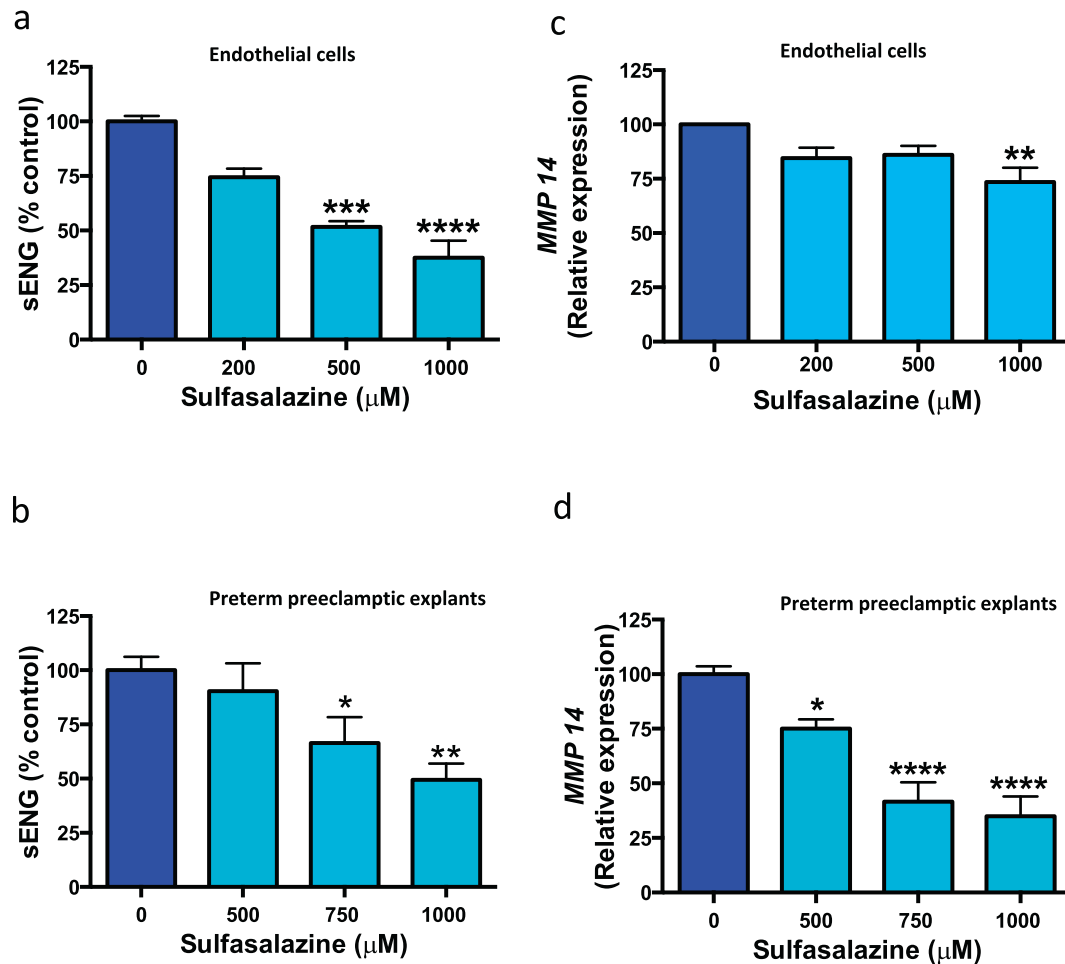


Fig. 3. Effect of sulfasalazine on sENG secretion and MMP 14 expression from endothelial cells and placental tissue. Sulfasalazine reduced sENG secretion from a) endothelial cells and b) preterm preeclamptic placental preterm preeclamptic explants. Sulfasalazine also reduced transcription of MMP 14 (cleavage protease of membrane bound endoglin) in c) endothelial cells and d) preterm preeclamptic placental preterm preeclamptic explants. Data are means \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (Kruskal-Wallis).

2.10. RT-PCR

We extracted RNA from placental explants and HUVECs using an RNeasy mini kit (Qiagen, Valencia, CA) and quantified this using the Nanodrop ND 1000 spectrophotometer (NanoDrop technologies Inc., Wilmington, DE). We converted 0.2 μ g of RNA to cDNA using the Applied Biosystems high capacity cDNA reverse transcriptase kit (Life Technologies) as per manufacturer guidelines.

We assessed gene expressions of *VCAM-1* (Life Technologies), *MMP 14* (Life Technologies), *PIGF* (Life Technologies), *HO-1* (Life Technologies), *NF κ B p65* (Life Technologies), *IKKB* (Life Technologies) and *NF κ B* (Life Technologies), *Rel A* (Life Technologies) and *IKKB* (Life Technologies) by real time PCR (RT-PCR) on the CFX 384 (Bio-Rad, Hercules, CA) using FAM-labeled Taqman universal PCR mastermix and its specific primer/probe set (Life Technologies) with the following run conditions: 50 $^{\circ}$ C for 2 min; 95 $^{\circ}$ C for 10 min, 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 1 min (40 cycles). SYBR RT-PCR was carried out to assess gene expressions of *sFlt-1 e15a* and *sFlt-1 i13* and *GAPDH*. We designed primers as previously described (Geneworks, South Australia, Australia) [24]. We performed quantitative RT-PCR using the run conditions: 95 $^{\circ}$ C for 20 min; 95 $^{\circ}$ C for 0.01 min, 60 $^{\circ}$ C for 20 min, 95 $^{\circ}$ C for 1 min (39 cycles), melt curve 65 $^{\circ}$ C to 95 $^{\circ}$ C at 0.05 $^{\circ}$ C increments at 0.05 s.

We normalized data to GAPDH, Topo or Cyt-c as a housekeeper and calibrated against the average C_t of the control samples. Results were expressed as fold change from control.

2.11. Western blot analysis

We separated HUVEC or cytotrophoblast protein lysates (20 μ g) on 10% polyacrylamide gels with wet transfer to PVDF membranes (Millipore, Billerica, MA). We blocked membranes prior to incubation overnight with the primary antibody (Either anti-HO-1 at 1:500 dilution (ADI-SPA-896 (ENZO Life Sciences, Farmingdale, New York) or anti-VCAM 1 at 1:200 dilution (SC-1504), Santa Cruz, Biotechnology, Dallas, Texas)). We assessed heme-oxygenase 1 (HO-1) by blocking blots with 5% skim milk followed by incubation with primary rabbit anti-HO-1 antibody (1:500) overnight at 4 $^{\circ}$ C, and incubated them with secondary anti-rabbit-HRP antibody for 1 h at room temperature. We examined vascular cell adhesion molecule 1 (VCAM 1) by blocking protein blots with 5% skim milk, followed by incubation with primary goat anti-VCAM 1 antibody (1:200) in Canget 1 overnight at 4 $^{\circ}$ C then incubated with secondary anti-goat-HRP antibody for 1 h at room temperature. We applied ECL prime reagent (GE healthcare Life Sciences, Pittsburgh, PA) and immune-reactive bands were visualized using a Chemidoc XRS (BioRad, Hercules, CA). We stripped blots with Restore

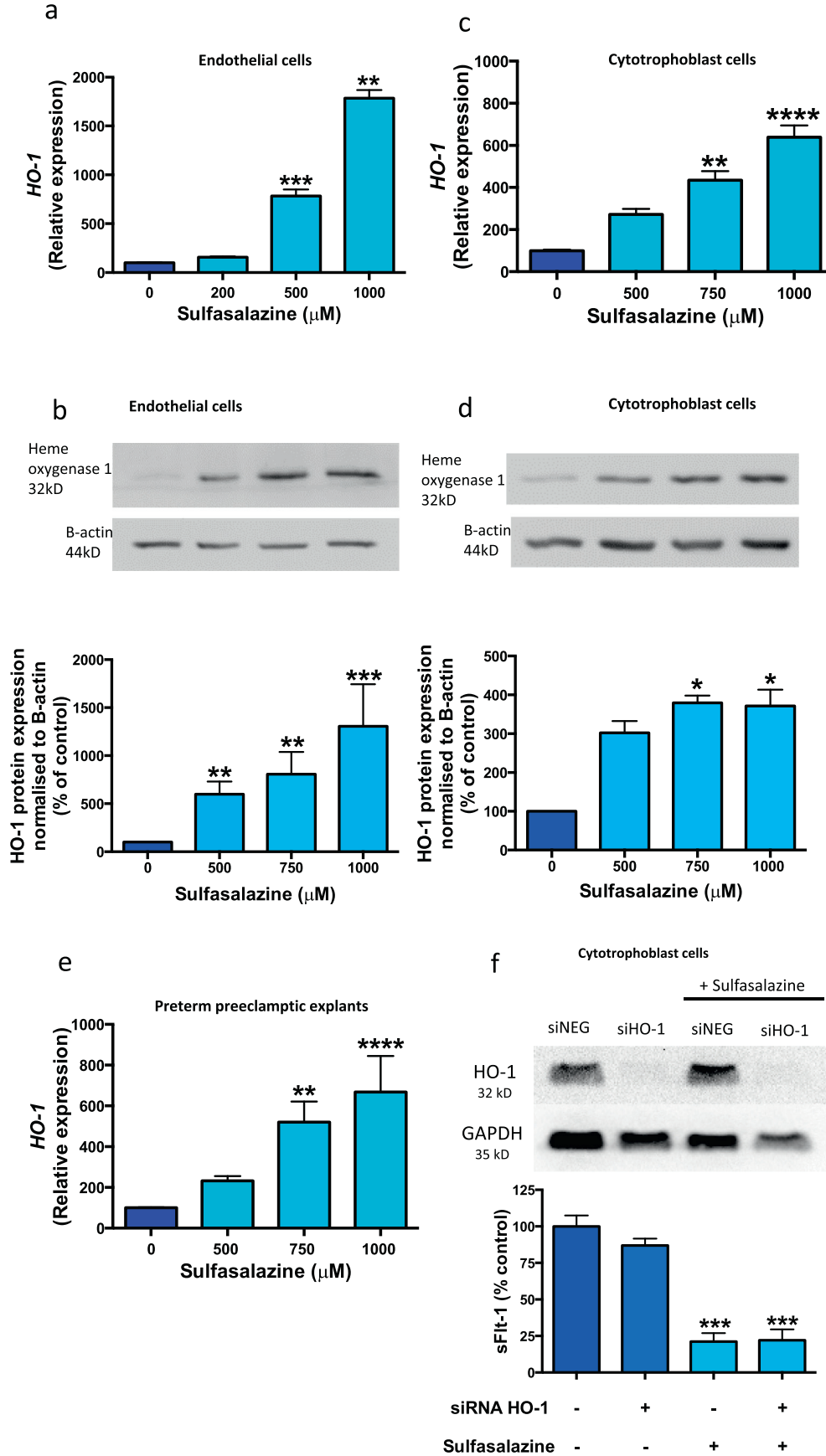


Fig. 4. Effect of sulfasalazine on heme-oxygenase 1 (HO-1) expression and the effect of silencing HO-1 on sFlt-1 secretion \pm sulfasalazine. Sulfasalazine upregulates heme-oxygenase 1 (HO-1) a) mRNA expression and b) protein production in endothelial cells. Furthermore it upregulates HO-1 c) mRNA expression and d) protein production in cytotrophoblasts cells. There is also an increase in HO-1 e) protein expression in preterm preeclamptic placental explants treated with sulfasalazine. Silencing HO-1 gene in cytotrophoblasts cells did not affect f) sFlt-1 secretion and sulfasalazine continued to reduce sFlt-1 secretion from cytotrophoblasts in the presence of HO-1 silencing. Data are means \pm SEM of three independent experiments. $p < 0.05$, $** p < 0.01$, $*** p < 0.001$, $**** p < 0.0001$. (Kruskal-Wallis).

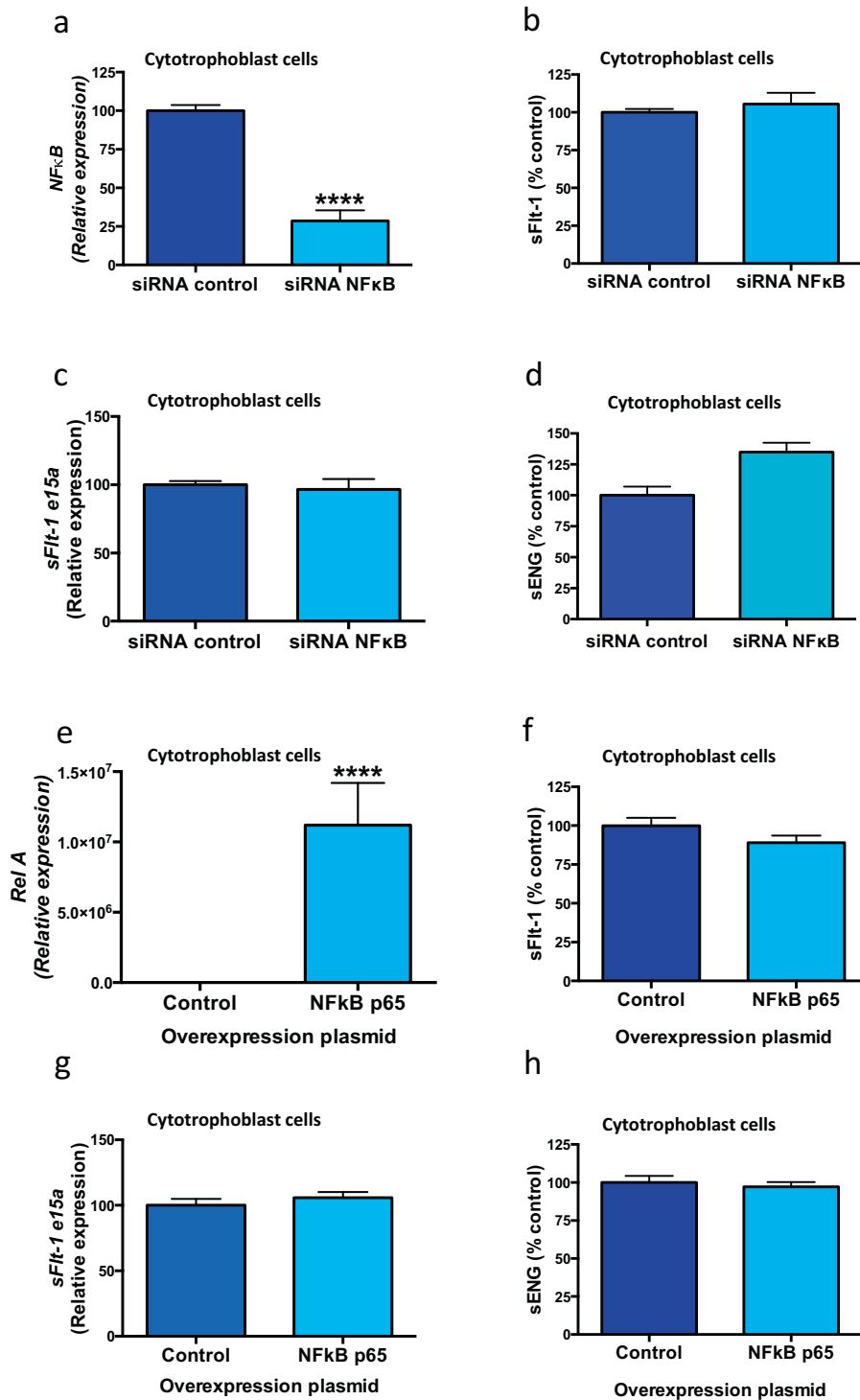


Fig. 5. Silencing and overexpressing genes in the $\text{NF}\kappa\text{B}$ pathway and effect on sFlt-1 and sENG secretion. Silencing $\text{NF}\kappa\text{B}$ in cytotrophoblast cells significantly reduced a) *NFκB* expression however this did not change b) sFlt-1 secretion or c) *sFlt-1 e15a* mRNA expression or d) sENG secretion. Overexpression of $\text{NF}\kappa\text{B}$ p65 resulted in an increase in e) *Rel A* expression however no change in f) sFlt-1 secretion, g) *sFlt-1 e15a* expression or h) sENG secretion. Furthermore overexpression of *IKKβ* in cytotrophoblast cells resulted in an increase in i) *IKKβ* expression however there was no change in j) sFlt-1 secretion, k) *sFlt-1 e15a* expression or l) sENG secretion. Data are means \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (*t*-test).

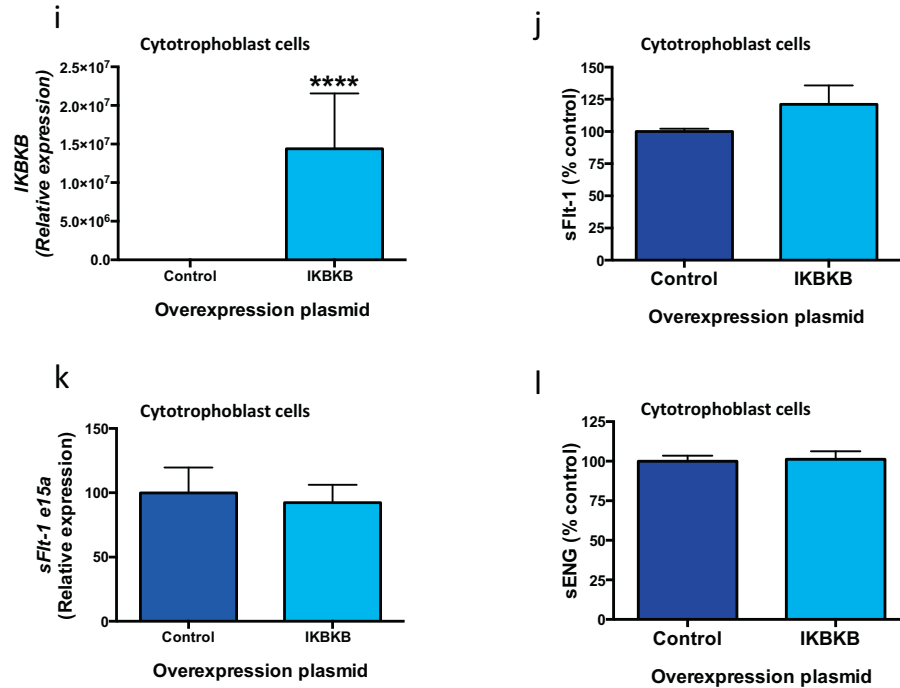


Fig. 5 (continued).

Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA) and re-probed it with mouse anti-B-actin (1:10,000) antibody overnight at 4 °C for the protein loading control.

2.12. Ethics approval

Our study was approved by The Mercy Health Human Research Ethics Committee (Institutional review board number R11/34 and R14/11) and all women gave written informed consent. We sourced mouse aortas from animals after sacrifice in ethically approved experiments conducted by other research groups within the Institute.

2.13. Statistical analysis

We performed a minimum of three technical triplicates for each biological replicate, with a minimum of three biological replicates (each from different patients) performed for each *in vitro* study. We assessed two groups using a *t*-test (parametric) or a Mann-Whitney test (non-parametric) and a 1-way ANOVA (parametric) or a Kruskal-Wallis test (non-parametric) when three or more groups were compared. We used the GraphPad Prism 6 (GraphPad Software, La Jolla, CA) for statistical analysis. 'n' represents the number of different patients used during the whole omental vessel experiments. We fitted the concentration response curves from omental arteries to a sigmoidal curve with nonlinear regression to calculate the sensitivity for sulfasalazine (pEC₅₀) or maximum relaxation (E_{max}) as previously described [12]. We analysed the response curves using two-way repeated measures ANOVA with Bonferroni post-hoc analysis (treatment *versus* concentration). All data were expressed as mean ± SEM; P values <0.05 were considered significant.

3. Results

3.1. Effect of sulfasalazine on sFlt-1 secretion from primary HUVECs and placental tissues

To examine the effect of sulfasalazine on sFlt-1 secretion we obtained primary human placental tissues. We isolated human umbilical vein endothelial cells (HUVECs) and cytotrophoblasts from the placenta

(as endothelial and trophoblast cells are the main source of sFlt-1) using a protocol previously described [25]. Placental explants were also examined from patients with preterm preeclampsia. Sulfasalazine dose dependently reduced sFlt-1 secretion from primary HUVECs (Fig. 1a). Sulfasalazine also dose dependently reduced sFlt-1 secretion from cytotrophoblasts (Fig. 1b) and placental explants obtained from women with preterm preeclampsia (Fig. 1c). We explored the effect of low doses of sulfasalazine on cytotrophoblasts and there was a dose dependant reduction in sFlt-1 secretion (Fig. 1d).

We investigated the effect of sulfasalazine on the transcription of sFlt-1 splice variants (e15a and i13) [26,27] in HUVECs, cytotrophoblast and preterm preeclamptic placental explants. sFlt-1 i13 is the most abundantly expressed isoform in endothelial cells [26]. Addition of sulfasalazine to HUVECs significantly reduced sFlt-1 i13 transcription (Fig. 1e). sFlt-1 e15a is the dominant sFlt-1 isoform expressed in placenta [26]. Whilst sulfasalazine did not change sFlt-1 e15a transcription in cytotrophoblasts (Fig. 1f), there was a dose dependent reduction in transcription in preeclamptic placental explants (Fig. 1g). Therefore, we conclude sulfasalazine reduces sFlt-1 secretion from HUVECs and placental cells/tissues, including placental explants obtained from patients with preterm preeclampsia.

3.2. Effect of sulfasalazine on placental growth factor (PlGF) secretion from primary human tissues

Placental growth factor is a circulating pro-angiogenic protein secreted from the placenta that is neutralised by sFlt-1 and reduced in preeclampsia. We explored the effect of sulfasalazine on PlGF secretion and expression from primary cytotrophoblasts. With increasing doses of sulfasalazine there was a dose dependant increase in PlGF protein secretion (Fig. 2a) and this corresponded to a significant increase in PlGF mRNA expression (Fig. 2b).

3.3. Effect of sulfasalazine on sENG secretion in primary endothelial cells and placental tissues

Soluble endoglin is an anti-angiogenic factor produced from cleavage of membrane bound endoglin and is increased in preeclampsia.

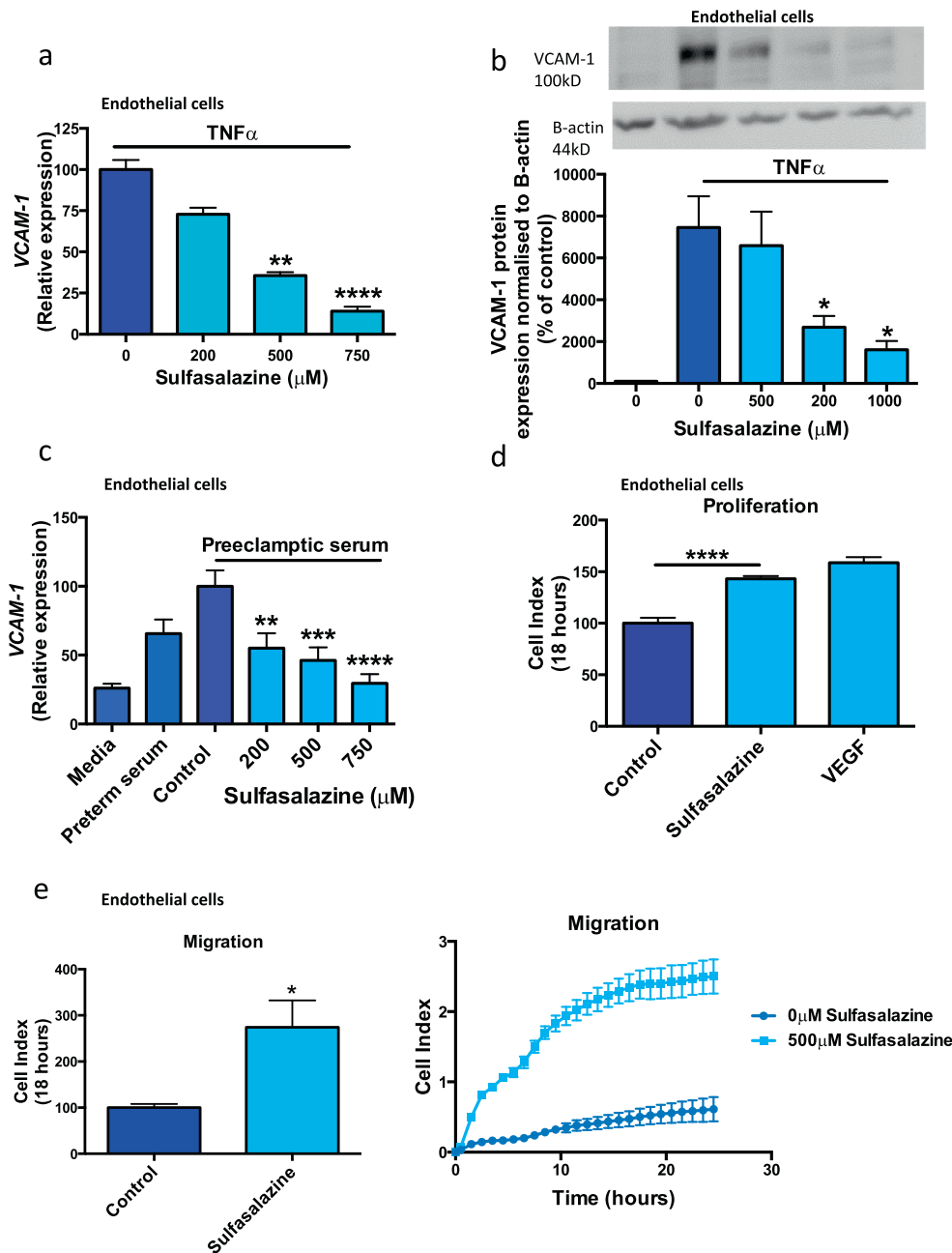


Fig. 6. Effect of sulfasalazine on endothelial cell *vascular cell adhesion molecule 1* (VCAM 1) transcription and endothelial cell proliferation and migration. Sulfasalazine dose dependently reduced TNF α induced a) VCAM-1 mRNA expression and b) VCAM-1 protein in HUVECs. Furthermore serum obtained from patients with preterm preeclampsia increased endothelial cell c) VCAM 1 expression, and this was significantly reduced with increasing doses of sulfasalazine. Sulfasalazine increased endothelial cell d) proliferation and e) migration towards chemoattractant vascular endothelial growth factor. Data are means \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (Kruskal-Wallis).

Next we investigated the effects of sulfasalazine on sENG secretion. Sulfasalazine dose dependently reduced sENG secretion from HUVECs (Fig. 3a) and from placental explants obtained from patients with preterm preeclampsia (Fig. 3b).

MMP 14 is a membrane bound protease, responsible for the release of sENG into the circulation [28]. We examined the effect of sulfasalazine on the expression of MMP 14 mRNA in the same cells/tissues where sENG was reduced. Sulfasalazine reduced MMP 14 mRNA expression in both HUVECs (Fig. 3c) and preterm preeclamptic placental explants (Fig. 3d).

3.4. Effect of sulfasalazine on heme-oxygenase 1 in primary human tissues

Given sulfasalazine has been shown to induce HO-1 (a molecule with anti-oxidant properties) in vascular smooth muscle cells [18], we explored whether sulfasalazine might also up-regulate HO-1 in placenta. Indeed, we found sulfasalazine dose dependently increased HO-1 expression (Fig. 4a) and protein (Fig. 4b) in HUVECs. Additionally sulfasalazine increased HO-1 mRNA (Fig. 4c) and protein (Fig. 4d) in cytotrophoblast, and in preterm preeclamptic placental explants (Fig. 4e).

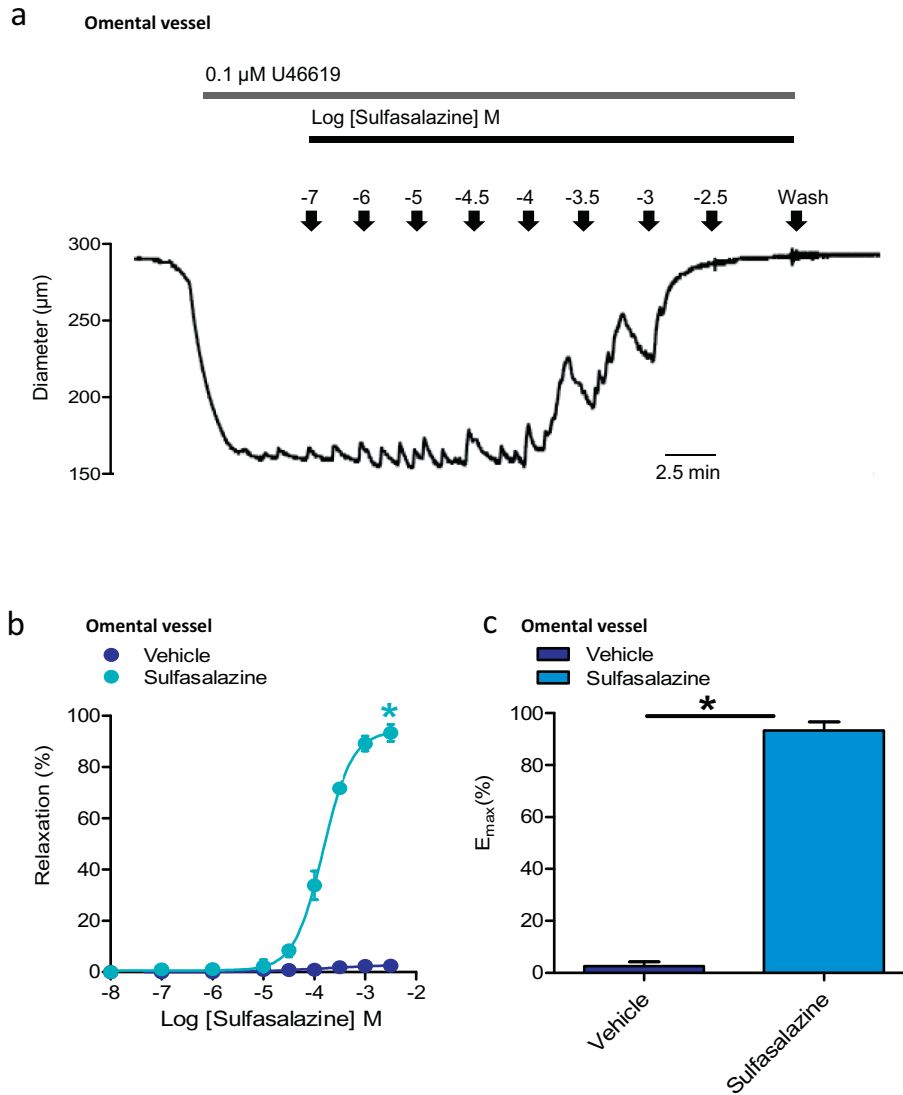


Fig. 7. Effect of sulfasalazine on whole blood vessel function. Sulfasalazine dose-dependently dilated whole omental blood vessels that were pre-constricted with the thromboxane A2 receptor agonist, U46619. Omental vessels were first incubated in U46619, which caused a reduction in vessel diameter. With the cumulative addition of increasing doses of sulfasalazine, the blood vessel diameter increased as demonstrated in this representative trace a). Sulfasalazine caused a significant increase in vessel relaxation b) and maximum relaxation (E_{max}) c) compared with vehicle (DMSO) controls. Data are mean \pm SEM of three independent experiments. * $p < 0.05$ (t-test).

We explored whether the effects of sulfasalazine on sFlt-1 secretion were mediated by its ability to induce HO-1 expression. To do this, we concurrently silenced HO-1 expression in cytotrophoblasts and added sulfasalazine. Despite efficient HO-1 silencing, the addition of sulfasalazine consistently reduced sFlt-1 secretion (Fig. 4f). Notably, efficient silencing of HO-1 in the absence of sulfasalazine did not affect sFlt-1 secretion (Fig. 4f), which is consistent with the findings of our previous report which showed, HO-1 does not directly regulate sFlt-1 production [29]. Thus, it appears that the reduction in sFlt-1 secretion from cytotrophoblasts induced by sulfasalazine is not mediated through HO-1.

3.5. Effect of inhibiting and overexpressing molecules in the NF κ B pathway on sFlt-1 and sENG secretion

Sulfasalazine is known to inhibit the inflammatory transcription factor NF κ B [6], likely through stabilising proteins that stop its translocation into the nucleus [19]. Given that sulfasalazine has such effects on NF κ B activity, and that it also reduces sFlt-1 and sENG secretion, we examined whether the NF κ B pathway regulates sFlt-1 and sENG secretion. We silenced NF κ B in cytotrophoblasts using siRNA (siNF κ B), which

reduced its mRNA expression by 71% (Fig. 5a). siNF κ B did not alter the secretion of sFlt-1 (Fig. 5b), expression of sFlt-1 e15a (Fig. 5c) or sENG (Fig. 5d). Similarly, overexpressing NF κ B p65 in cytotrophoblasts upregulated the p65 subunit Rel A (Fig. 5E), did not change sFlt-1 secretion (Fig. 5f), sFlt-1 e15a expression (Fig. 5g) or sENG secretion (Fig. 5h). Furthermore, overexpressing I κ BKB increased I κ BKB (Fig. 5i), however did not alter sFlt-1 secretion (Fig. 5j), sFlt-1 e15a expression (Fig. 5k) or sENG secretion (Fig. 5l). Taken together, our data suggests the NF κ B pathway does not regulate sFlt-1 or sENG secretion from placental cytotrophoblasts.

3.6. Effect of sulfasalazine on VCAM-1 transcription and protein and the proliferation and migration of HUVECs

Endothelial dysfunction is associated with increased expression of vascular cell adhesion molecule-1 (VCAM-1) [30,31]. VCAM-1 is up-regulated by pro-inflammatory stimuli and is expressed on the luminal surface of blood vessels where it can bind and sequester leukocytes, erythrocytes and platelets. VCAM-1 expression is induced by pro-inflammatory stimuli. Preeclampsia is associated with increased circulating TNF α [15], a pro-inflammatory cytokine that up-regulates

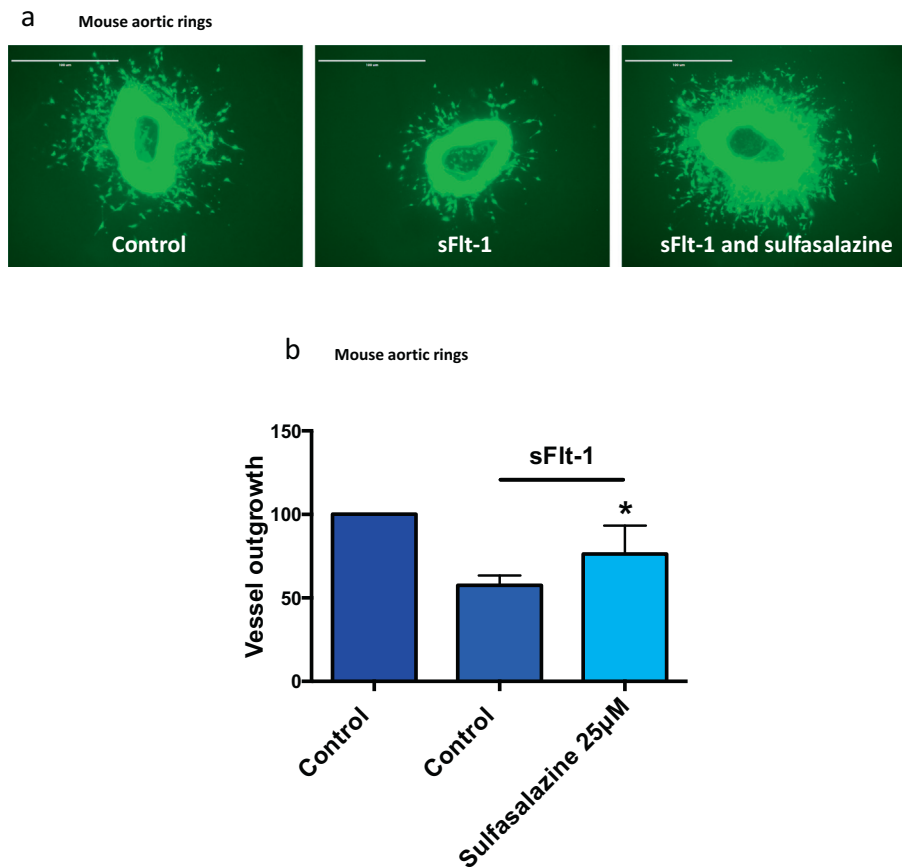


Fig. 8. Effect of sulfasalazine on angiogenesis. Mouse aortic rings cultured with sFlt-1 have reduced vessel outgrowth, which is restored with the addition of sulfasalazine (a) and b). White arrows point at vessel outgrowth. Representative micrographs are shown. Data are means \pm SEM of three independent experiments. * $p < 0.05$ (t-test).

VCAM-1 [30,31]. We therefore performed an *in vitro* endothelial dysfunction assay where HUVECs were treated with TNF α to upregulate VCAM-1 and then co-administered sulfasalazine. Sulfasalazine potentially reduced the TNF α induced VCAM-1 mRNA expression (Fig. 6a) and VCAM-1 protein (Fig. 6b). Next, we administered pooled serum from three patients with preterm preeclampsia to primary HUVECs, which induced a significant increase in VCAM-1 expression (Fig. 6c) that was quenched with the addition of sulfasalazine (Fig. 6c).

We then examined whether sulfasalazine affected HUVEC proliferation and migration by monitoring cells using the xCELLigence system (measures experiments in real time). We demonstrated sulfasalazine increased HUVEC proliferation (Fig. 6d) and enhanced HUVEC migration towards the chemoattractant VEGF (Fig. 6e and f).

Thus, sulfasalazine reduced VCAM-1 in HUVECs in two *in vitro* endothelial dysfunction models and enhanced endothelial proliferation and migration. These data suggest sulfasalazine rescues endothelial dysfunction.

3.7. Effect of sulfasalazine on whole maternal vessel dilation isolated from the omentum

In order to determine if sulfasalazine affected vessel diameter, we undertook whole artery functional experiments. Arteries were isolated from omental tissue obtained from women having a caesarean section. Arteries were then subjected to pressure myography to examine vascular function. As expected, the administration of the thromboxane A2 receptor agonist U46619 caused constriction of the vessel (reduced arteriole diameter). However, in the presence of sulfasalazine, this was significantly augmented with an increase in relaxation (pEC₅₀ 150 \pm 8 μ M, E_{max} 93.4 \pm 3.3%) compared to the vehicle controls, an effect that

was dose dependent (Fig. 7 a, b and c). This suggests that sulfasalazine can induce dilation in a pre-constricted artery.

3.8. Sulfasalazine effect on angiogenic sprouting from mouse aorta

Preeclampsia is believed to be associated with decreased angiogenesis [5,32]. We explored whether sulfasalazine could rescue sFlt-1 induced inhibition of angiogenesis by performing a mouse aortic angiogenesis assay [23]. Mouse aortic explants were cultured in the presence of recombinant human sFlt-1 \pm sulfasalazine (Fig. 8 a and b). Administering sFlt-1 alone reduced *de novo* angiogenic sprouting from the aortic rings. Sulfasalazine rescued the inhibition of angiogenic sprouting seen with the addition of sFlt-1, suggesting that it may have pro-angiogenic actions (Fig. 8 a and b).

4. Discussion

Preeclampsia is one of the most serious complications of pregnancy and can place the life of mother and fetus at risk. Currently, there is no medical treatment and delivery is the only way to stop disease progression. At early gestations, the need to deliver to save the mother can inflict complications of prematurity on the infant including death, cerebral palsy, chronic lung disease and intracerebral bleeding [33]. The risks for mothers and babies continue throughout life as they are at increased risk of cardiovascular morbidity and mortality from ischaemic heart disease and stroke [34–36].

Our group and others have demonstrated statins reduce sFlt-1 secretion from cell lines [8] and from primary human placental tissues and pravastatin also reverses key features of endothelial dysfunction [9,10]. We identified esomeprazole [11] and metformin [12] as

medications that reduce sFlt-1 and sENG secretion from primary placental cells and tissues and mitigate key aspects of endothelial dysfunction in primary endothelial cells and whole vessels. Given these promising results these medications have progressed to randomised clinical trial. The StAmP trial (A Proof of Principle, Double-Blind, Randomised, Placebo-Controlled, Multi Centre Trial of pravaStatin to Ameliorate Early Onset Pre-eclampsia) has finished recruitment in the United Kingdom and we await the results of its primary outcome assessing the levels of sFlt-1 48 h post randomisation. In collaboration with colleagues in South Africa we have completed the PIE (Preeclampsia Intervention with Esomeprazole) trial randomising 120 women with preterm preeclampsia to esomeprazole or placebo. There was no difference in our primary outcome of gestation prolongation [13]. Following on from this trial we have recently commenced the PI2 (Preeclampsia Intervention 2) trial examining metformin as a treatment for preeclampsia and we will recruit 150 women with preterm preeclampsia to see whether metformin might prolong gestation.

We present a body of work suggesting sulfasalazine may be a promising prevention or treatment for preeclampsia. We have performed multiple functional assays using primary human tissues to demonstrate sulfasalazine may mitigate key features involved in the pathophysiology of preeclampsia. Firstly, we demonstrate sulfasalazine reduced sFlt-1 and sENG secretion from primary endothelial cells, cytotrophoblast cells and placental explants obtained from cases of preterm preeclampsia. This reduction in sFlt-1 secretion does not appear to be mediated through the HO-1 or NF κ B pathways. We also demonstrate sulfasalazine can increase PlGF expression and secretion from cytotrophoblasts. Furthermore sulfasalazine reduces endothelial dysfunction, vasodilate whole vessels and enhance vessel angiogenesis.

There are no previous reports demonstrating effects of sulfasalazine on pro and anti-angiogenic molecules. We show that sulfasalazine not only reduces sFlt-1 and sENG secretion, but importantly increases PlGF secretion. Sulfasalazine likely reduces sFlt-1 secretion by decreasing transcription. sFlt-1 arises from alternative splicing of the Flt-1 pre-mRNA gene. Two sFlt-1 mRNA variants predominate in preeclampsia; sFlt-1 i13 which is widely expressed in many tissues including the endothelium, and the primate specific variant sFlt-1 e15a highly expressed in placenta. Sulfasalazine reduced mRNA expression of sFlt-1 i13 in endothelial cells and e15a in placental explants. Sulfasalazine also reduced sENG secretion, possibly by inhibiting expression of MMP14. MMP14 is thought to cleave membrane bound Endoglin to produce circulating sENG [28]. Our finding of increased PlGF secretion and transcription is exciting because it strengthens the potential of sulfasalazine as a medication for preeclampsia – not only for its capacity to reduce anti-angiogenic molecules, but also for its ability to enhance vasoprotective molecules such as PlGF.

We also set out to try to identify the upstream pathways that sulfasalazine may be acting on to reduce sFlt-1 and sENG secretion. We were limited by the fact that the molecular regulation of these factors is still not well understood. Sulfasalazine is known to inhibit NF κ B and also upregulate the antioxidant enzyme HO-1. Despite silencing or overexpressing genes involved in the NF κ B pathway in cytotrophoblasts, there was no change in sFlt-1 or sENG secretion suggesting the NF κ B pathway may not be involved in their regulation. This is consistent with reports demonstrating the administration of corticosteroids, known to inhibit NF κ B [37], do not change serum sFlt-1 and sENG levels in patients with preeclampsia [38]. Furthermore, silencing HO-1 did not affect the ability of sulfasalazine to reduce sFlt-1 secretion from cytotrophoblasts. This is consistent with our previously published findings showing that silencing or upregulating HO-1 in cytotrophoblasts does not affect sFlt-1 or sENG secretion [29]. Therefore, it appears sulfasalazine is not exerting its effects on sFlt-1 and sENG through inhibiting NF κ B or upregulating HO-1 pathways and another upstream mechanism, yet to be determined, must be present.

An important feature of a drug that may be useful to treat or prevent preeclampsia would be the ability to reduce endothelial dysfunction.

We have obtained evidence from multiple functional assays using primary cells and tissues to show that sulfasalazine can mitigate endothelial dysfunction. Sulfasalazine is known to reduce markers of inflammation by inhibiting NF κ B [18,39]. We demonstrated sulfasalazine quenches VCAM-1 expression in HUVECs in the presence of TNF α , which is increased in preeclampsia. Furthermore, sulfasalazine enhanced endothelial cell proliferation and migration. Sulfasalazine also induced vasodilation of whole human vessels. It rescued sFlt-1 inhibition of vascular outgrowths from mouse aortic rings. Given these apparent vasoprotective effects of sulfasalazine, further exploration of sulfasalazine as a treatment for cardiovascular conditions may be warranted.

Whether the doses of sulfasalazine used *in vitro* correspond to the levels achieved *in vivo* at the level of the placenta is unknown. The *in vitro* doses of sulfasalazine we used were similar to other studies examining the effect of sulfasalazine on the vasculature [18]. The pharmacokinetics of sulfasalazine in normal healthy individuals would suggest steady state serum concentrations reach around 45 μ g/ml [40] when administered at 4 g per day which corresponds to around 113 μ M in serum. Therefore we examined the effect of sulfasalazine *in vitro* at doses straddling this threshold and found sulfasalazine reduced sFlt-1 secretion from cytotrophoblasts. Studies focusing on levels of sulfasalazine in tissues show it concentrates in connective tissues in animal studies [40,41]. Unfortunately studies examining its distribution in pregnancy focus on its concentration in the cord blood and breast milk however have not examined the concentrations at the level of the placenta [40]. Therefore we are embarking on a pharmacokinetics study examining the concentration of sulfasalazine at the level of the placenta in patients with preterm preeclampsia (Australia and New Zealand Clinical Trials Registry 12,617,000,226,303).

Sulfasalazine treatment has been linked to renal and liver impairment in case reports. So far there are 5 case reports detailing 6 cases of sulfasalazine induced nephritis. In each case sulfasalazine was administered at 1000 mg daily to 1000 mg three times daily generally for >12 months to treat inflammatory bowel disease. In 5 cases the nephritis completely resolved once sulfasalazine was ceased and the patient was commenced on steroids [1–4]. In one case the patient proceeded directly to renal transplant [5]. Reassuringly a review of the side effects of sulfasalazine was performed and despite 4.7 million scripts of sulfasalazine filled in the UK from 1991 to 1998 there were no reports of interstitial nephritis [6]. Furthermore a study examining the effect of long term sulfasalazine use (mean of 10 years) found it to be safe and free of nephrotoxic side effects [7]. Animal studies examining the effect of sulfasalazine on the kidneys revealed that at supra-physiological doses, ten times the dose administered to humans, that interstitial inflammation and oxidative stress occurred [8]. Given the rarity of renal complications coupled with the long-term treatment administered before a complication arose we believe the risk of renal injury is minimal in our population. Given patients with preeclampsia often have renal impairment it is possible that sulfasalazine may in fact improve this if it targets and treats aspects of the preeclampsia disease process. Therefore evaluating renal function will be an important outcome to examine in any trial evaluating the effect of sulfasalazine on preeclampsia treatment or prevention. Sulfasalazine use has also been linked to hepatic dysfunction. In the reported cases all patients experiencing hepatic dysfunction were on concomitant hepatotoxic medications. In the majority of case reports, once sulfasalazine is ceased, the impairment completely resolves. There was one case series involving 2 patients on sulfasalazine that required a liver transplant and one of these patients died a few weeks later [15]. As pregnant patients are not on hepatotoxic medications we believe this complication would be exceedingly rare in preeclamptic patients.

As sulfasalazine mitigates angiogenic factor production and endothelial dysfunction, it holds promise as a potential prevention or treatment for preeclampsia. In contrast to other medications studied that are safe in pregnancy, including proton pump inhibitors [11] and

metformin [12], sulfasalazine has an added benefit of potentially exerting systemic anti-inflammatory effects. Therefore we believe clinical trials examining its potential as a treatment or prevention may be warranted.

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Declarations of interests

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Author contributions

FCB designed and performed experiments, analysed the data and wrote the manuscript. NJH designed experiments and corrected the manuscript. PC, VN and RH performed experiments. LJP, SS designed and performed experiments. TJKL and ST designed experiments, analysed data and assisted with drafting the manuscript.

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