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Evidence-Based Revised View of the Pathophysiology of Preeclampsia

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Abstract

Preeclampsia is a life-threatening vascular disorder of pregnancy due to a failing stressed placenta. Millions of women risk death to give birth each year and globally each year, almost 300,000 lose their life in this process and over 500,000 babies die as a consequence of preeclampsia. Despite decades of research, we lack pharmacological agents to treat it. Maternal endothelial oxidative stress is a central phenomenon responsible for the preeclampsia phenotype of high maternal blood pressure and proteinuria. In 1997, it was proposed that preeclampsia arises due to the loss of VEGF activity, possibly due to elevation in anti-angiogenic factor, soluble Flt-1 (sFlt-1). Researchers showed that high sFlt-1 and soluble endoglin (sEng) elicit the severe preeclampsia phenotype in pregnant rodents. We demonstrated that heme oxygenase-1 (HO-1)/carbon monoxide (CO) pathway prevents placental stress and suppresses sFlt-1 and sEng release. Likewise, hydrogen sulphide (H_2S)/cystathionine-γ-lyase (Cth) systems limit sFlt-1 and sEng and protect against the preeclampsia phenotype in mice. Importantly, H_2S restores placental vasculature, and in doing so improves lagging fetal growth. These molecules act as the inhibitor systems in pregnancy and when they fail, preeclampsia is triggered. In this review, we discuss what are the hypotheses and models for the pathophysiology of preeclampsia on the basis of Bradford Hill causation criteria for disease causation and how further *in vivo* experimentation is needed to establish 'proof of principle'. Hypotheses that fail to meet the Bradford Hill causation criteria include abnormal spiral artery remodelling and inflammation and should be considered associated or consequential to the disorder. In contrast, the protection against cellular stress hypothesis that states that the protective pathways mitigate cellular stress by limiting elevation of anti-angiogenic factors or oxidative stress

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and the subsequent clinical signs of preeclampsia appear to fulfil most of Bradford Hill causation criteria. Identifying the candidates on the roadmap to this pathway is essential in developing diagnostics and therapeutics to target the pathogenesis of preeclampsia.

Keywords
Preeclampsia • sFlt-1 • HO-1 • Inflammation • Hypoxia • Activin A • Gasotransmitter • microRNA • Oxidative stress • Angiogenic factors

1 Introduction

It is believed that in 400 B. C, Hippocrates was the first to state that convulsion during pregnancy was a sign of bad pregnancy, which resulted in imbalance in the 'humours' (Bell 2010). Today preeclampsia is still being debated as the "disease of theories" reported as a 'two-stage model'. The first stage being asymptomatic, characterized by an abnormal formation of the placenta and the release of placental factors into the maternal circulation. The second stage is symptomatic resulting in hypertension and proteinuria that can eventually culminate in angiospasm in the brain to cause eclampsia (Hladunewich et al. 2007). Preeclampsia is characterised as *de novo* hypertension (bp $\geq$140/90) and proteinuria ($\geq$300 mg/24 h) occurring after 20 weeks of pregnancy, however, neither are specific to the pathophysiology of preeclampsia (Brown et al. 2001). The prevalence of preeclampsia ranges from 5 to 8 % of pregnancies depending on the geographical location and affects 8.5 million pregnancies globally, accounting for over 70 000 maternal deaths and 500 000 infant deaths per year (Ramma and Ahmed 2014; Lowe et al. 2009).

The causes of preeclampsia remain largely unknown. Recent studies, however, have shed new light on factors originating in the placenta likely to cause the condition due to an imbalance in 'autacoids' factors (Ahmed and Ramma 2015). Preeclampsia still lacks a reliable means of diagnosis and prediction with no effective therapy or pharmacological agents available to treat the disease. The only solution is the early delivery of the pregnancy. If left untreated, preeclampsia can be life threatening and may progress to eclampsia with complications of HELLP syndrome (Elevated liver enzymes, haemolysis, and low platelets), placental abruption, acute renal failure and pulmonary oedema (Arulkumaran and Lightstone 2013). Although maternal symptoms appear to be largely resolved with the delivery of the baby, data are accumulating that preeclampsia is associated with long-term maternal cardiovascular and other complications such as renal diseases (Smith et al. 2001; Saxena et al. 2010; Garovic and Hayman 2007; Veerbeek et al. 2016).

As Steve Jobs once said "You can only connect the dots looking backwards". To gain a deeper insight into the pathophysiology of preeclampsia, we need to identify the "dots" critical in the pathogenesis of preeclampsia in order to discover the roadmap. What are the intersections that connect the dots to make up the roadmap of the preeclampsia phenotype? To discover the 'roadmaps' that connect the dots of preeclampsia, proof-of-principle experimentation that goes beyond association-type approaches need to be undertaken. Studies focused on *in vitro* experimentations to make bold claims of links to preeclampsia are misrepresenting the facts and provide limited insight into the mechanisms. Therefore, *in vitro* studies *per se,* should not be considered as representative vehicles for 'proof of principle'. Animal models which mimic the "preeclampsia phenotype", are better vehicles to connect and test the 'dots'. Even though animals rarely get preeclampsia, many phenotypes of the disorder can be modelled in rodents. In such a system, it is possible to test whether a potential cause can be reversed, ultimately leading to the rectification of symptoms and establishment of

Table 1 Bradford-Hill causation criteria

Criterion	Explanaion
Temporality	The cause always precedes the effect
Dose response	An increase in exposure results in an increase in the risk
Strength	The stronger the association, the more likely it is that there is a causal relationship between the associated factors
Consistency	The results can be replicated by different people or in different studies
Specificity	A single cause will result in a specific effect
Coherence	There must be coherence between the epidemiology and the experimental findings.
Plausibility	The cause can be linked to the effect through a plausible mechanism

'proof of principle'. Any hypotheses put forward for investigation should be critically analysed using the Bradford Hill causation criteria (Hill 1965), rather than claiming the 'cause and effect' status without it (Ramma and Ahmed 2011).

In this review, we discuss what are the hypotheses and models for the pathophysiology of preeclampsia. A revised view is presented to test the evidence for the role of circulating factors such as soluble fms-like tyrosine kinase-1 (sFlt-1) and inhibin, inflammation, oxidative stress and gasotransmitters on the basis of the Bradford Hill causation criteria for disease causation (see Table 1).

2 Evaluating the Current Hypothesis of Preeclampsia

Decades of research into placental histopathology of preeclampsia established certain dogmas. These now need to be challenged if we are to find therapies apart from prematurely delivering the baby. These dogmas include (i) abnormal spiral artery remodelling (Khong et al. 1986; Feinberg et al. 1991; Brosens et al. 2002; Burton et al. 2009) and (ii) failed trophoblast invasion (Zhou et al. 1997); accepted as the early primary defect causing preeclampsia. It is further claimed that the failure of trophoblast remodelling of spiral arteries precedes abnormal placentation to cause the secretion of placental factors into the maternal circulation that lead to (iii) aberrant maternal systemic inflammation, (iv) oxidative stress and (v) angiogenic imbalance (Redman and Sargent 2009; Maynard et al. 2008). These ultimately lead to the manifestation of clinical signs and symptoms of preeclampsia. A closer scrutiny based on the Bradford Hill criteria has brought many of the established dogmas into question.

3 Role of Inflammation in Abnormal Spiral Artery Remodelling

Evidence to support any mechanistic understanding of the processes of abnormal spiral artery remodelling or trophoblast invasion is lacking apart from association and descriptive studies. The two separate processes are highly interrelated and it is accepted that spiral artery remodelling involves the maternal immune system and the placenta. Recent studies established that spiral artery remodelling may be a common underlying contributing factor of abnormal placentation, but it is not specific to preeclampsia (Lyall et al. 2013).

Uterine natural killer cells (uNK), part of the innate immune response, differ from the classical natural killer cells in the peripheral blood. One possible role of the uNK cells is to maintain immunological tolerance for the semi-allogeneic fetus by antagonising the T_H17 subset of T cells through the secretion of Interferon-gamma (IFN-γ) (Fu et al. 2013). Loss of this regulation is linked to spontaneous abortion with a rise in T_H17 cells and local inflammation (Fu et al. 2013). However, the uNK also accumulate around uterine spiral arteries and are abundant in early pregnancy (Robson et al. 2012). Robson and co-workers showed that uNK-conditioned media containing secreted factors, IFN-γ, VEGF-C (Vascular Endothelial Growth Factor C), angiopoietin-1 and angiopoietin-2, impact upon vascular smooth muscle cell separation and extra cellular matrix remodelling *In vitro* studies (Robson et al. 2012), speculating that

similar changes occur during spiral artery remodelling.

There is contrasting data on the levels of uNK in preeclampsia; immunohistochemistry and flow cytometry showed an increase in uNK levels (Stallmach et al. 1999; Bachmayer et al. 2006) whereas a reduced population was detected in placental bed biopsies from pre-eclampsia (Williams et al. 2009). These associations need to be supported by mechanistic *in vivo* experimentation. Therefore, more studies are necessary to establish the role of uNK in spiral artery remodelling and trophoblast invasion during preeclampsia. Furthermore, it is not clear whether the immunological alteration that occurs early in pregnancy contributes to the onset of preeclampsia or whether activation and elevation of pro-inflammatory mediators are a consequence of the disease (Ramma and Ahmed 2011; Cheng and Sharma 2016). Indeed immune deficient $Rag2^{-/-}/\gamma_c{}^{-/-}$double-knockout mice that lack spiral artery remodelling remain normotensive and do not develop proteinuria, fetal growth restriction or other preeclampsia-like phenotype (Burke et al. 2010).

Interestingly, a temporal relationship between excessive inflammation and the onset of pre-eclampsia does not exist, evident from the lack of changes seen in the levels of pro-inflammatory cytokines before the onset of the disorder (Djurovic et al. 2002; Kronborg et al. 2011; Carty et al. 2012). Moreover, the level of inflammation does not correlate with the severity of the disorder, supported by the absence of significant quantitative differences in serum TNF-α and IL-6 (Ozler et al. 2012). Pregnant women with increased levels of IL-6 have normal angiogenic status with no symptoms of hypertension or proteinuria (Ramma et al. 2012). Finally, corticosteroid treatment in women with severe preeclampsia did not resolve the condition and only transiently reduced the levels of IL-6 and C-reactive protein along with improving the clinical manifestations for approximately 48 h (Nayeri et al. 2014). Thus, it can be said that treatment of inflammation does not remove the stimulus of the disorder.

4 Hypoxia and Spiral Artery Remodelling

Extravillous trophoblast cells become invasive, allowing the transformation of vessels to accommodate the increase in maternal blood flow (Carter et al. 2015). Oxygen levels were reported to be a regulating factor for this differentiation as Genbacev and colleagues demonstrated that at 2 % oxygen tension, trophoblasts maintained their proliferative state *in vitro*, whereas in 20 % oxygen they acquire an invasive phenotype (Genbacev et al. 1996). Therefore, hypoxia must be prolonged for the failure of trophoblast invasion to occur and must precede abnormal spiral artery remodelling (Huppertz et al. 2014). This is in mark contrast to the argument proposed by the Two Stage model where failure of trophoblast invasion leads to failed spiral artery remodelling, which leads to placental hypoxia (Roberts and Hubel 2009; Redman and Sargent 2005).

During the hypoxic conditions of the placenta, HIF-1α expression is high and is found to positively regulate trophoblast invasion though Transforming Growth Factor-β3 (TGFβ3) signalling *in vitro*. Inhibition of HIF-1α in hypoxic explants inhibited TGFβ3 expression leading to markers of an invasive trophoblast phenotype (Caniggia et al. 2000). In normal pregnancy, HIF-1a levels decrease in line with the increase in the placental oxygen tension throughout gestation (Rajakumar and Conrad 2000a; b). Interestingly these same authors showed that when placental villous explants obtained from women with preeclampsia were exposed to hypoxia (2 % oxygen) HIF-1a was not up-regulated as was the case in normal placental tissue indicating that something other than hypoxia affects the relative high levels of HIF-1a in preeclamptic placenta (Rajakumar et al. 2003a; b). These are at best loss association studies and collectively question the significance of hypoxia as a trigger for the pathogenesis of preeclampsia.

Recent studies challenge the view that abnormal spiral artery remodelling leads to the hypoxia during preeclampsia. Immunohistochemical analysis using Hypoxyprobe™-1 to detect

cellular hypoxia showed that oxygen delivery to the placenta is not impaired in $Rag2^{-/-}Il2rg^{-/-}$ double knock-out pregnant mice, which fail to undergo normal physiological spiral arterial remodelling (Leno-Duran et al. 2010). In fact, it is known that 'hypoxia' is key to normal placentation during the first trimester of pregnancy, and increase in oxygen tension above 20 mmHg, through the dissolution of trophoblast plugs in the spiral arteries, can result in spontaneous abortion (Huppertz et al. 2014). Based on the above evidences, hypoxia as an initial stimulus for the causation of preeclampsia can be disputed. Levels of sFlt-1 are elevated in preeclamptic placental explants compared to normal placental explants, when cultured under atmospheric conditions (Ahmad and Ahmed 2004), demonstrating that sFlt-1 elevated before the onset of preeclampsia is not increased due to placental hypoxia. Thus, the Two Stage model of failed remodelling of spiral arteries leading to hypoxia and subsequent alteration in the downstream factors in preeclampsia is a misconception (Ahmed and Ramma 2015). The availability of materials limits the study of human spiral artery remodelling with no standard practice to follow for placenta collection. Animal models such as the murine 'reduced uterine placenta perfusion' model can generate the symptoms of preeclampsia through placental perfusion (Burke and Karumanchi 2013), however, this does not prove ischemia and hypoxia are key elements on the roadmap to preeclampsia phenotype.

5 Role of sFLT-1 in Preeclampsia

The most prominent factor linked to the pathogenesis of preeclampsia is the 'loss of VEGF activity' as proposed originally in 1997 (Ahmed 1997). Several groups showed elevated levels of naturally occurring anti-angiogenic molecule, sFlt-1, in women with preeclampsia, which is strongly linked to the clinical sign of hypertension through antagonising VEGF and PlGF (He et al. 1999; Ahmad and Ahmed 2001; Maynard et al. 2003; Levine et al. 2004).

Soluble Flt-1 is a splice variant of *flt-1* gene and belongs to the vascular endothelial growth receptor (VEGFR) family. Several isoforms of sFlt-1 have been identified (Thomas et al. 2009) with sFlt-1e15a thought to make up at least 80 % of placental sFlt-1 while sFlt-1i13 makes up ~15 % (Jebbink et al. 2011). The amount of sFlt-1e15a, coupled with its placental specificity, is considered as the main isoform responsible for the preeclampsia phenotype (Palmer et al. 2016). Recent *in vivo* studies reveal that mice injected with adenovirus to full length human sFlt-e15a, have increased levels of creatinine/albumin in the urine and elevated mean arterial pressure (Szalai et al. 2014).

The administration of sFlt-1 into mouse models induces hypertension and proteinuria as well as other clinical manifestations of preeclampsia (Maynard et al. 2003). Furthermore, reduction in sFlt-1 levels in preeclamptic women using 'dextran sulfate' apheresis, reduced proteinuria and stabilised blood pressure (Thadhani et al. 2011; 2016). Loss of VEGF activity causing preeclampsia-like syndrome comes from human cancer chemotherapy patients receiving anti-VEGF treatment (Cross et al. 2012). Two patients treated with bevacizumab, (VEGF neutralising antibody), developed a preeclampsia-like syndrome characterised by hypertension, proteinuria, liver enzyme elevation and central nervous system irritability (Cross et al. 2012). Interestingly, VEGF increased sFlt-1 mRNA expression and release in cultured endothelial cells. Furthermore, adenovirus overexpression of VEGF-A in mice resulted in an eight-fold increase in circulating sFlt-1 levels (Ahmad et al. 2011). Following these observations *in vivo*, it is reasonable to state that the 'loss of VEGF activity' may play a central role in the pathogenesis of preeclampsia.

Hypoxic conditions induce VEGF expression (Shweiki et al. 1992) and the same relationship exists for sFlt-1 (Ahmad and Ahmed 2004; Palmer et al. 2016). Further contradicting the 'hypoxia hypothesis' as contributing to preeclampsia pathogenesis. However, hypoxia may affect cell types differently *in vitro*; an increase in sFlt-1 secretion and mRNA expression in

cytotrophoblasts exposed to 8 % or 2 % oxygen concentration is not seen in endothelial cells and villous fibroblasts (Nagamatsu et al. 2004). Interestingly, endothelial cells incubated with VEGF-A revealed an increase in sFlt-1 mRNA expression and secretion, which was then replicated *in vivo*, using adenovirus overexpression of VEGF-A in mice, resulting in an 8-fold increase in circulating sFlt-1 levels (Ahmad et al. 2011).

An important growth factor in placental development appears to be placenta growth factor (PlGF) that is also antagonised by sFlt-1. It is dramatically decreased before the onset of pre-eclampsia and remains suppressed during the disorder in relation to the severity (Levine et al. 2004, 2006). The temporal relationship between PlGF levels and the onset of preeclampsia signifying a clear role of PlGF in preeclampsia, however, this remains unknown and unproven.

6 Role of Soluble Endoglin in Preeclampsia

Membrane glycoprotein, endoglin is a co-receptor for TGFβ signalling. Its cleaved isoform, soluble endoglin (sEng), is increased along with sFlt-1 before the onset of clinical symptoms of preeclampsia (Levine et al. 2006). Transgenic mice that express sEng (*Sol.eng*$^{+}$) exhibit symptoms of hypertension, small pup size, proteinuria and renal damage, which mimic the signs of preeclampsia (Valbuena-Diez et al. 2012).

An increase in sEng, blocking the TGFβ downstream signalling, results in a reduction in endothelial nitric oxide synthase (NOS3) activity (Venkatesha et al. 2006). This ultimately leads to a decrease in the vasodilator, NO. As TGFβ1 and TGFβ3 are also anti-inflammatory cytokines, sEng levels may influence the polarisation of different T cell subsets, mainly Tregs and T_H17 cells, both of which are found to be decreased and increased respectively in pre-eclamptic patients when compared to normotensive pregnancy controls (Darmochwal-Kolarz et al. 2012). This dysregulation of signaling may lead to the exacerbation of the inflammatory response seen in preeclampsia and would suggest that a rise in sEng precedes the immune response. However, *in vivo* studies regarding the relationship between sEng and T cell populations are needed to support any associative link.

7 The Role of Anti-angiogenic Factors in Endothelial Dysfunction

Maternal endothelial dysfunction is central to the hypertensive phenotype and other clinical manifestations of preeclampsia. Endothelial cell surface adhesion molecules are markers of endothelial activation (Farzadnia et al. 2013). These markers are elevated in preeclampsia, soluble E-selectin was significantly higher at 12–16 weeks' gestation in women who subsequently developed preeclampsia (Carty et al. 2012). A recent study also revealed soluble VCAM-1 and ICAM-1 levels to be elevated in severe preeclampsia compared to normotensive pregnancy controls (Farzadnia et al. 2013). Both of these adhesion molecules are key to identifying endothelial activation (Farzadnia et al. 2013). However, no significant differences were seen in both adhesion molecules between normal and mild preeclamptic pregnancy.

Soluble Flt-1 and sEng are factors thought to induce endothelial dysfunction in preeclampsia. It was reported that *in vitro*, sFlt-1 alone does not cause endothelial dysfunction but works in concert with pro-inflammatory cytokines (TNF-α) to sensitise and amplify endothelial dysfunction (Cindrova-Davies et al. 2011). However, sFlt-1 alone does induces endothelial dysfunction *in vivo* (Bergmann et al. 2010). This contrast in results, demonstrates potential false positive nature of *in vitro* studies *per se*. In vitro studies should refrain from over claims unless further support for the argument comes from *in vivo* studies used to complete 'proof of principle'.

Inhibition of TGFβ signaling using an adenovirus to overexpress sEng in mice, increased leukocyte rolling, demonstrating a chemokinetic response (Walshe et al. 2009). Soluble VCAM-1 and E-selectin expression also increased correlating with a decrease in leukocyte velocity.

Thus, this study shows sEng to be involved in the activation of endothelial cells, ready for an immunological response (Walshe et al. 2009). Overexpression of sFlt-1 and sEng together in mice demonstrates a synergistic approach to induce endothelial dysfunction, ultimately resulting in severe vascular damage, hypertension, proteinuria, fetal growth restriction and HELPP syndrome (Venkatesha et al. 2006). In a separate rodent study, it was also found to cause focal vasospasm, and increased vascular permeability leading to brain odema, which is associated with eclampsia (Maharaj et al. 2008). Therefore, given the evidence for their temporal relationship with preeclampsia, sFlt-1 and sEng elevation may lie close to the root cause of endothelial dysfunction in preeclampsia, further supports to the possibility of these two anti-angiogenic factors being important 'dots' on the roadmap of preeclampsia development.

8 Oxidative Stress

Oxidative stress reflects on the imbalance of oxidative substances, such as reactive oxygen species (ROS) over the innate anti-oxidants that make up the endogenous defence system. Oxidative stress acts through several mechanisms such as DNA damage, inhibition of protein synthesis, protein nitration and mitochondrial modification (Sanchez-Aranguren et al. 2014). An increase in oxidative stress in the placenta and maternal circulation is found in preeclampsia at which point, multiple factors converge leading to endothelial dysfunction, systemic inflammation and more oxidative stress (Alpoim et al. 2016; Tjoa et al. 2006). Measurement of mitochondrial dysfunction using oxygen consumption rate revealed dysfunction in trophoblast mitochondrion isolated from preeclamptic patients (Maloyan et al. 2012; Muralimanoharan et al. 2012).

The placental syncytiotrophoblast is sensitive to exposure to high oxygen and low anti-oxidant levels from the mother, and so oxidative stress is present in normal pregnancy (Myatt and Webster 2009). It is argued that the inadequate trophoblast invasion resulting in placental hypoxia, causes the formation of free radicals and a reduction in anti-oxidant molecules and thus increases oxidative stress in preeclampsia. However, this description of events is not supported by temporal evidence as discussed earlier in this review. Similar to hypoxia, failed trophoblast invasion and abnormal spiral artery remodelling are dispelled as causational factors, so the process by which oxidative stress produces free radicals needs to be identified. Do the answers lie with mitochondrial dysfunction, identified in the trophoblast cells of the preeclamptic placenta (Maloyan et al. 2012; Muralimanoharan et al. 2012)? What cannot be ruled out is that oxidative stress is part of the final common pathway to a preeclampsia phenotype. Superoxide can react with NO to produce peroxynitrite, leading to a reduction in the bioavailability of NO, thereby reducing vasodilation while promoting the production of vasoconstrictors (Sankaralingam et al. 2006). This is plausible and needs to be tested in vivo using mouse models, which produce the preeclampsia phenotype. Furthermore, many proteins are nitrated in the presence of NO and superoxide which can lead to either loss or gain of protein function (Peluffo and Radi 2007). Indeed, placental peroxynitrite expression is increased in preeclampsia (Myatt et al. 1996) and tyrosine nitration is increased in cardiovascular disease (Peluffo and Radi 2007).

The natural oxidative stress already present during pregnancy may be intensified by the decreased anti-oxidant levels (enzymatic and non-enzymatic) observed in the circulation of women with preeclampsia. However, there was no correlation found between the level of enzymatic anti-oxidant and the severity of the disease (Llurba et al. 2004). A systematic review showed that supplementation of anti-oxidant does not reduce the risk of preeclampsia (Salles et al. 2012), ruling oxidative stress as a secondary phenomenon to the pathogenesis of preeclampsia.

9 Protective and Stress Model

Pregnancy can be viewed as a 'journey in a car' with the accelerator and brake system controlling the movement and progression of the vehicle

towards a successful birth. Getting in a car and getting to your destination is equivalent to a successful pregnancy outcome. If the car breaks down, it can be viewed as pregnancy complication but if the brakes fail altogether, the system crashes that's preeclampsia. What are the brakes in pregnancy that hold back the fuel for the accelerator? The fuel for the accelerator includes inflammation, oxidative stress and anti-angiogenic factors, while the brakes work to maintain control of the car through regulating the amount of fuel in the accelerator pathway. In this review we demonstrate that it is the failure in the braking system, representing the protective pathways, that causes the car to loose control until the system crashes, manifesting itself as preeclampsia. Identifying the braking systems (the protective pathways) and discovering how to enhance their effects, may restore balance leading to a possible prevention or cure of preeclampsia.

10 Gasotransmitters

Gaseous signalling molecules, NO, CO and H_2S, have potential to be part of the 'braking' system based on their roles in angiogenesis, cytoprotection and regulation of vascular tone. Numerous studies show NO, CO and H_2S as well as their related enzymes promote placental blood vasodilation *in vitro* and *in vivo* (Gude et al. 1990; Myatt et al. 1991; Learmont and Poston 1996; Ahmed et al. 2000; Bainbridge et al. 2002; Zhao et al. 2008; Cindrova-Davies et al. 2013; Wang et al. 2013) suggesting a role in the pathophysiology of preeclampsia (Fig. 1).

11 HO/CO System

Heme oxygenase (HO) is the rate-limiting enzyme responsible for the degradation of heme in the endoplasmic reticulum to generate equimolar amount of biliverdin, free iron and carbon monoxide (CO) (Tenhunen et al. 1969). Biliverdin is rapidly reduced to bilirubin, a potent antioxidant, by the cytosolic enzyme biliverdin reductase. Atmospheric CO is lethal; cellular CO is a potent vasodilator with anti-apoptotic properties (Dulak et al. 2008).

HO exists in two main isoforms; HO-2 (36 kDa) is constitutively expressed in several tissues around the body with high concentrations in the brain and vascular endothelium. Reduced HO-2 immunostaining at the maternal-placental interface reported to be associated with reduction in trophoblasts' invasion in preeclampsia and linked to abnormal placentation (Lyall et al. 2000). In mammalian tissues, the inducible

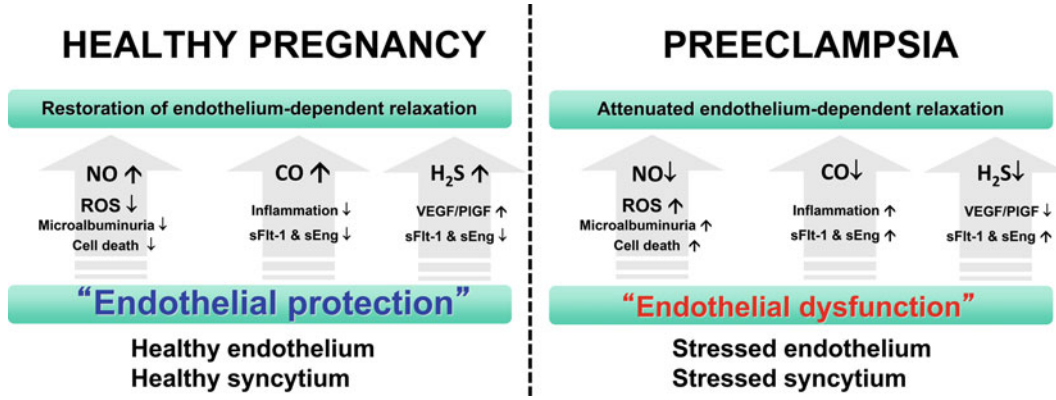

Fig. 1 Schematic diagram illustrating the molecules involved in the pathogenesis of preeclampsia. The upstream events consist of decrease in nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S) leads to increase in microalbuminuria, cell death, inflammation, elevation in soluble Flt-1 (sFlt-1) and soluble Endoglin (sEng) and decrease in placenta growth factor (PlGF). These biochemical changes lead to generation of reactive oxygen species (ROS) and endothelial dysfunction that contributes to the pathogenesis of preeclampsia

isoform, HO-1, is found in the spleen and liver in high concentration but can be identified throughout the body. HO-1 expression is thought to be induced mainly by its substrate, heme, but can also be regulated by other stimuli such as peroxynitrite, cytokines, hypoxia, hypothermia, endotoxins, and metalloprphyrins (Sikorski et al. 2004). HO-1, via its products, inhibits inflammation, oxidative stress and is anti-apoptotic (Dulak et al. 2008). Indeed, deficiency in HO-1 results in severe endothelial damage marked by an increase in thrombomodulin and von Willebrand factor (Yachie et al. 1999).

12 HO/CO Pathway in Pregnancy

The HO-1/CO axis plays a crucial role in the maintenance of uterine quiescence during normal pregnancy (Acevedo and Ahmed 1998) and acts on the utero-placental circulation (Ahmed et al. 2000; Lyall et al. 2000). Placental HO protects human placenta from cellular damage and reduced HO-1 expression is associated with preeclampsia (Ahmed et al. 2000). HO-1 deficient mice that develop hypertension at the beginning of pregnancy (Linzke et al. 2014) further support the role of HO-1 in preeclampsia. Preeclamptic pregnant women also have decreased concentrations of CO compared to healthy pregnant women in their exhaled breath signifying a decrease in HO-1 enzymatic activity (Baum et al. 2000). Deletion of the *HO-1* gene leads to pregnancy complications including intrauterine growth restriction and fetal lethality as well as hypertension (Zenclussen et al. 2014). Furthermore, a recent murine study showed that treating HO-1-deficient animals with CO normalised the number of uNK and angiogenic factor expression as well as restoring spiral artery remodelling (Venditti et al. 2014) that was reported as deficient spiral artery remodelling in HO-1-deficient animals (Zenclussen et al. 2014). However, defect of spiral artery remodelling may be a generalised phenomenon rather than specific to preeclampsia, pointed out earlier in this review as it fails to meet most of the Bradford Hill criteria. More importantly, human studies using fetal placenta cells (chorionic villous sampling, CVS) showed HO-1 mRNA expression to be reduced in women before the onset of preeclampsia (Alpoim et al. 2016; Farina et al. 2008) and so meets one of the key Bradford Hill causation criterion for disease causation.

When the first link between sFlt-1 and HO-1 was established (Cudmore et al. 2007), the significance of HO-1 in preeclampsia gained traction and other researchers entered the field. Adenoviral HO-1 expression or COP-releasing donors inhibited VEGF-mediated sFlt-1 release and IFN-γ and TNF-α-induced sEng release in cultured endothelial cells (Cudmore et al. 2007). Likewise, knockdown of HO-1 increased sFlt-1 release. In addition, a clinical study showed HO-1 mRNA levels to increase in samples of villous trophoblast, obtained from women between 6 and 11 weeks of gestation undergoing elective abortion, while the mRNA expression of sFlt-1 was decreased with gestational age (Miyagami et al. 2013). It is no surprise therefore that human trophoblast cells from patients with preeclampsia cultured with CO had reduced ability to secret sFlt-1 (Zenclussen et al. 2014). Indeed, women who smoke have reduced circulating sFlt-1 levels (Levine et al. 2006), we would argue this is due to an increase in CO levels. Despite a recent paper challenging the ability of Hmox1 to inhibit sFlt-1 production (Tong et al. 2015) an overwhelming number of studies show that a number of drugs that inhibit sFlt-1 do so via up-regulation of HO-1 (Onda et al. 2015; McCarthy et al. 2011); sildenafil suppresses sFlt-1 from trophoblast via HO-1 (Jeong et al. 2014). In addition, HO-1 induction attenuates ischemia-induced hypertension in pregnant rats (George et al. 2011) and proteinase-activated receptor-2 mediated sFlt-1 release (Al-Ani et al. 2010). Moreover, diastolic blood pressures and plasma sFlt-1 levels were significantly elevated in HO-$1^{+/-}$ pregnant mice (Zhao et al. 2009). HO-1 mRNA expression is also decreased in women destined to develop

preeclampsia (Farina et al. 2008) and an Hmox1 promoter polymorphism is associated with preeclampsia (Kaartokallio et al. 2014) indicating HO activity is reduced in preeclampsia. Collectively, these studies show that HO-1 acts as a negative regulator of sFlt-1 and sEng (Cudmore et al. 2011) and support the argument that partial loss of HO-1 activity early in gestation maybe the cause of preeclampsia. In vivo proof-of-principle experiments are needed to validate this theory.

13 The Cth/H_2S System

Hydrogen sulfide (H_2S) is a gaseous signaling molecule promotes vasodilatation (Zhao et al. 2001), exhibits cytoprotective anti-inflammatory properties (Zanardo et al. 2006), protects against reperfusion injury induced cellular damage (Elrod et al. 2007) or lethal hypoxia (Blackstone and Roth 2007) and stimulates angiogenesis (Papapetropoulos et al. 2009). H_2S production is regulated by three enzymes; cystathionine γ-lyase (Cth, also known as CSE), cystathionine β synthase (CBS), and 3-mercaptopyruvate sulfurtransferase (MST) and is generated from the substrates cystathionine, homocysteine, cysteine, and mercaptopyruvate respectively (Wang et al. 2015). CBS is most abundant in the brain whereas Cth is primarily responsible for endogenous H_2S production in vasculature (Wang et al. 2015). Administration of the Cth selective inhibitor (DL-propargylglycine, PAG) leads to elevated blood pressure and vascular remodelling in rats (Yan et al. 2004), and mice lacking Cth develop age-dependent hypertension, severe hyperhomocysteinaemia, and endothelial dysfunction (Wang et al. 2015).

14 Cth/H_2S Pathway in Pregnancy

H_2S is produced by the placenta and other uterine tissues (Patel et al. 2009) and Cth and CBS both localise to the endothelium in chorionic and stem villi of the placenta (Holwerda et al. 2012). In normal vasculature, H_2S is vasodilatory (Leffler et al. 2006) and as expected perfusion of normal placenta with a H_2S donor causes vasorelaxation of pre-constricted vasculature *in vitro* (Cindrova-Davies et al. 2013). A dysregulation in H_2S levels in preeclampsia was reported due to reduced plasma H_2S levels in the maternal circulation and reduced expression of Cth in the placenta of these patients (Wang et al. 2013).

Recent studies regarding Cth and CBS expression levels in preeclampsia are inconsistent. Holwerda and colleagues observed in placental villous tissue from early onset preeclampsia, no changes in Cth expression, but a decrease in CBS expression using immunohistochemistry, mRNA and protein expression (Holwerda et al. 2012). In contrast, Cth immunoreactivity was reduced in placenta from pregnancies with severe early-onset growth-restriction and preeclampsia (Cindrova-Davies et al. 2013; Wang et al. 2013). Real-time PCR confirmed reduced Cth mRNA in preeclamptic women and was associated with decreased levels of plasma H_2S (Wang et al. 2013). These are observational studies and provide little insight into the contribution this enzyme system makes in preeclampsia.

Interestingly, the Cth pathway shows similar capabilities to the HO-1 system. Endothelial Cth knockdown by siRNA increased the release of sFlt-1 and sEng, while adenoviral-mediated Cth overexpression inhibited their release from endothelial cells (Wang et al. 2013). Furthermore, inhibition of Cth activity by administration of PAG to pregnant mice induced hypertension, liver damage, elevated sFlt-1 and sEng and promoted abnormal labyrinth vascularisation in the placenta as well as decreased fetal growth (Wang et al. 2013). These symptoms were reversed when the inhibitor was supplemented with GYY4137, a slow releasing H_2S-generating compound, demonstrating that the effect of PAG was due to inhibition of H_2S production (Wang et al. 2013). These findings strongly

support a link between H_2S and the anti-angiogenic factors and implicate H2S/Cth as a player on the roadmap of preeclampsia phenotype.

Treatment with H_2S donor, SG-1002, offers cardio protection via upregulation of the VEGF–Akt–NOS3–NO pathway (Kondo et al. 2013). This is further supported by the uncoupling of eNOS in Cth KO mice, reducing the bioavailability of NO (Polhemus et al. 2014). This phenotype can be rescued with the restoration of H_2S levels. Heart failure patients with decreased levels of NO and H_2S, treated with SG-1002, showed an increase in NO bioavailability and plasma H_2S levels (Polhemus et al. 2014). This was also replicated in healthy individuals, illustrating the potential for exogenous H_2S to reverse some of the damaging changes in preeclampsia (Ahmed and Wang 2014, patent WO2014132083 A2). Interestingly, H_2S is known to stimulate VEGF and exposure of vascular smooth muscle cells to H_2S, up-regulates HIF-1α and VEGF protein levels and increased HIF-1α binding activity under hypoxic condition (Liu et al. 2010). Recently, VEGF receptor-2 was reported as the direct target of H_2S and VEGF receptor inhibitor suppressed angiogenesis induced by H_2S (Tao et al. 2013), suggesting that H_2S promotes angiogenesis via VEGF receptor activation. Studies regarding the impact of H_2S/Cth in preeclampsia indirectly support the original idea that loss of VEGF activity (Ahmed 1997) may be the major initiator of preeclampsia.

In preeclampsia, the maternal circulating level of PlGF is decreased well before the onset of the symptoms (Levine et al. 2004; 2006), and Cth dysregulation offers an explanation. Inhibition of Cth activity in early (first trimester) human placental explants obtained from termination of pregnancy results in a marked reduction in PlGF production (Wang et al. 2013). Administration of PlGF in lentiviral sFlt-1-infected non-pregnant mice depresses the level of sFlt-1 and ameliorates hypertension, glomerular endotheliosis and proteinuria (Kumasawa et al. 2011). Furthermore, mice deficient in PlGF ($PlGF^{-/-}$) and treated with an adenovirus containing sFlt-1 developed severe hypertension and proteinuria and H_2S releasing agent. GYY4137, reduced these symptoms (personal communication, Dr Shakil Ahmed). Therefore, Cth/H_2S activity may be upstream to PlGF and could be an earlier biomarker as well as a key regulator that keep the level of PlGF and VEGF activity sufficiently high to counteract antagonism by sFlt-1.

15 The NOS3/NO System

Nitric oxide (NO) is synthesized from the non-essential amino acid L-arginine by one of the three isoforms of NO synthase (NOS); NOS1 (neuronal), NOS2 (inducible), and NOS3 (endothelial). NO produced by NOS3 is important for the relaxation of smooth muscle cells and subsequently the promotion of vasodilation. It is critical for neovascularisation (Bussolati et al. 2001; Ahmad et al. 2006). Loss of NOS3 activity is an established contributor to endothelial dysfunction (Heitzer et al. 2001), a key sign associated with preeclampsia. In endothelial cells, NOS3 exists in a homodimeric complex that is stabilized by the cofactor tetrahydrobiopterin (BH4). Decreased availability of BH4 results in "uncoupling" of NOS3 activity and an increase in superoxide (d'Uscio et al. 2001; Bendall et al. 2005).

16 NOS3/NO Pathway in Pregnancy

Blocking the production of NO by administering a NOS-inhibiting agent produces virtually all the symptoms of preeclampsia in pregnant mice and rats, suggesting that the NOS pathway is a key player in this disorder (Lowe 2000). A meta-analysis showed that genetic variations in the *NOS3* gene contribute to an increased risk for preeclampsia (Dai et al. 2013). In non-pregnancy mice lacking NOS3, the sFlt-

induced preeclampsia phenotype is aggravated (Li et al. 2012). Indeed, BH4 doubled NOS3 activity in a concentration dependent manner in homogenates of first trimester and term placenta (Kukor et al. 2000) and uncoupled NOS3 and oxidative stress in a rat model of pregnancy-induced hypertension (Mitchell et al. 2007). However, BH4 concentrations in preeclamptic placenta were reported to be comparable with those of normal placenta (Kukor et al. 2000). A nested case-control study of screening for pre-eclampsia revealed that asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO formation, normally increases during pregnancy but the concentrations in the second trimester were significantly elevated in pregnancies that later developed preeclampsia (Rizos 2012, #5348).

Reduced availability of the NOS3 substrate L-arginine is also linked to impaired endothelial NO production. Twenty years earlier, it was reported that blood pressure in men could be reduced through intravenous administration of L-arginine as well as improving renal plasma flow and decreased renal vascular resistance (Higashi 1995, #5535). In small clinical trials, L-arginine supplementation used in complicated pregnancy was without conclusive outcomes. L-arginine supplementation reduced the risk of preeclampsia in high-risk women (Vadillo-Ortega et al. 2011), while showed little or no beneficial effects (Staff et al. 2004). As L-arginine supplementation increases the levels of asymmetric dimethylarginine and arginase, direct and indirect competitive inhibitors of endothelial NOS (Jabecka et al. 2012), high expression of these enzymes can induce the uncoupling of NOS as a source of superoxide in the vasculature. A number of studies suggested that L-arginine supplementation could lead to a further increase in peroxynitrite (Xia et al. 1996; Sankaralingam et al. 2010). Indeed, long-term L-arginine supplementation is harmful both in animal models (Chen et al. 2003) and in patients with cardiovascular diseases (Schulman et al. 2006). Finally, there is still an absence of a solid mechanistic model for impact of L-arginine in preeclampsia.

17 Activin A and Inhibin A in Preeclampsia

Activin A and inhibin A are members of TGFβ superfamily. The main function of activin A is in trophoblast proliferation while both molecules have opposing effects on follicle stimulating hormone (FSH) production from the pituitary (Muttukrishna et al. 1997; Caniggia et al. 1997). They are elevated before the onset of the pre-eclamptic phenotype. An eight-fold and ten-fold increased serum concentration in activin A and inhibin A respectively was reported in women with preeclampsia compared to normal pregnancy (Muttukrishna et al. 2000). The more severe the preeclampsia, the higher the levels of inhinin A (Kang et al. 2008) supporting a dose-response correlation. However, the mechanism by which the interaction between activin A and inhibin A is not clear. Perhaps it contributes to the pathogenesis of preeclampsia potentially through decreasing protective factors or increasing ROS activity and is an area worthy of further investigation.

18 Role of microRNA in Preeclampsia

Micro RNA's (miRNA) are small, non-coding, single stranded RNA molecules that target specific mRNA. By annealing to their mRNA partner, they can manipulate the expression pattern of the mRNA (Lagos-Quintana et al. 2002). A large number of miRNAs are now being discovered in pregnancy. Pro-angiogenic miRNA-126 correlated with VEGF expression and was decreased in preeclampsia pregnancies (Hong et al. 2014). Another miRNA found to be reduced in endothelial cells from preeclampsia patients is miRNA-155, believed to down-regulate angiogenic factors, possibly contributing to the angiogenic imbalance seen in preeclampsia (Zhang et al. 2010). MiRNA-155 also linked to endothelial NOS3 modulation (Li et al. 2014) and trophoblast function (Dai et al. 2011). Exact role of these miRNA in

pregnancy and preeclampsia remains to be defined in mechanistic and in vivo models as well as in clinical studies. MicroRNAs are not only found in the placenta but also in the maternal circulation and may be potentially important as diagnostic targets for preeclampsia (Mouillet et al. 2011).

19 Biochemical Markers to Predict Preeclampsia

Identified risk factors are used to screen pregnant women at high risk of developing preeclampsia. These include age, obesity, diabetes mellitus, renal disease, multiple pregnancy and history of preeclampsia in earlier pregnancies. Indeed, none of these alone can predict preeclampsia sufficiently, but combining these pre-dispositions with possible biomarkers may lead to effective diagnosis and potential predictions of disease onset (Bartsch et al. 2016). Both sFlt-1 and PlGF have been proposed as highly selective biomarkers for diagnosis of preeclampsia (Levine et al. 2006; Chappell et al. 2013). Placental protein 13 is another biomarker, which when combined with Doppler ultrasound pulsatility index showed a prediction rate of 90 % in first trimester of pregnancy (Nicolaides et al. 2005). However, ideal biomarkers needs to detected in plasma without the need of a sophisticated ultrasound or an obstetrician input. Biomarkers used to predict preeclampsia must precede the onset of preeclampsia, correlate with the severity of the disease and must show a high (>90 %) sensitivity and specificity and be overall low cost to existing management systems and ideally replace them.

20 Future Perspective and Conclusion

Despite substantial advances in the scientific and technological field, we have failed to provide effective treatment and diagnostic means for patients with preeclampsia. There is a vast amount of information but we are no closer to solving the enigma of preeclampsia. In order to identify suitable diagnosis, treatment and prevention strategies for preeclampsia, the roadmap of preeclampsia phenotype needs to be discovered and manipulated to illustrate a causation link. This review can only stress that any hypothesis proposed should be analyzed based on the Bradford Hill causation criteria in order to make claims for a given set of molecules to be implicated as a contributor in the causation of preeclampsia.

Both sFlt-1 and sEng are increased before the onset of symptoms. They both impact on VEGF and NO signaling pathways ultimately resulting in hypertension and other clinical symptoms. It is also evident that by removing these molecules, symptoms can improve. This cannot be said to be true for the "excessive inflammation" hypothesis seen during preeclampsia as corticosteroid treatment only transiently reduced symptoms. Both sFlt-1 and HO-1 pathways fit the disorder criteria to a greater extent, as there is a correlation between the levels of expression within the disorder progression as well as their regulatory relationship. Recent studies involving activin A and inhibin A expression also show a relationship between expression and severity. However, more in vivo research is needed to pinpoint the roles these molecules in roadmap of preeclampsia phenotype.

Limitations within the research field, as well as the dogma's compiled for the disorder, have blocked or created false intersections between the 'dots' of preeclampsia. *In vitro* studies should not be considered as the only means to demolish these roadblocks. There are now many mouse models, which rely on a number of different techniques to produce a preeclamptic phenotype. These *in vivo* models are and should be used to gather 'proper' evidence with regards to 'connecting the dots looking backwards' in order to answer; what 'dots' are inside the black box of causation? (Fig. 2) and how do the intersections between them fit together? Only by taking this approach can we hope to control and abolish this major disorder of pregnancy.

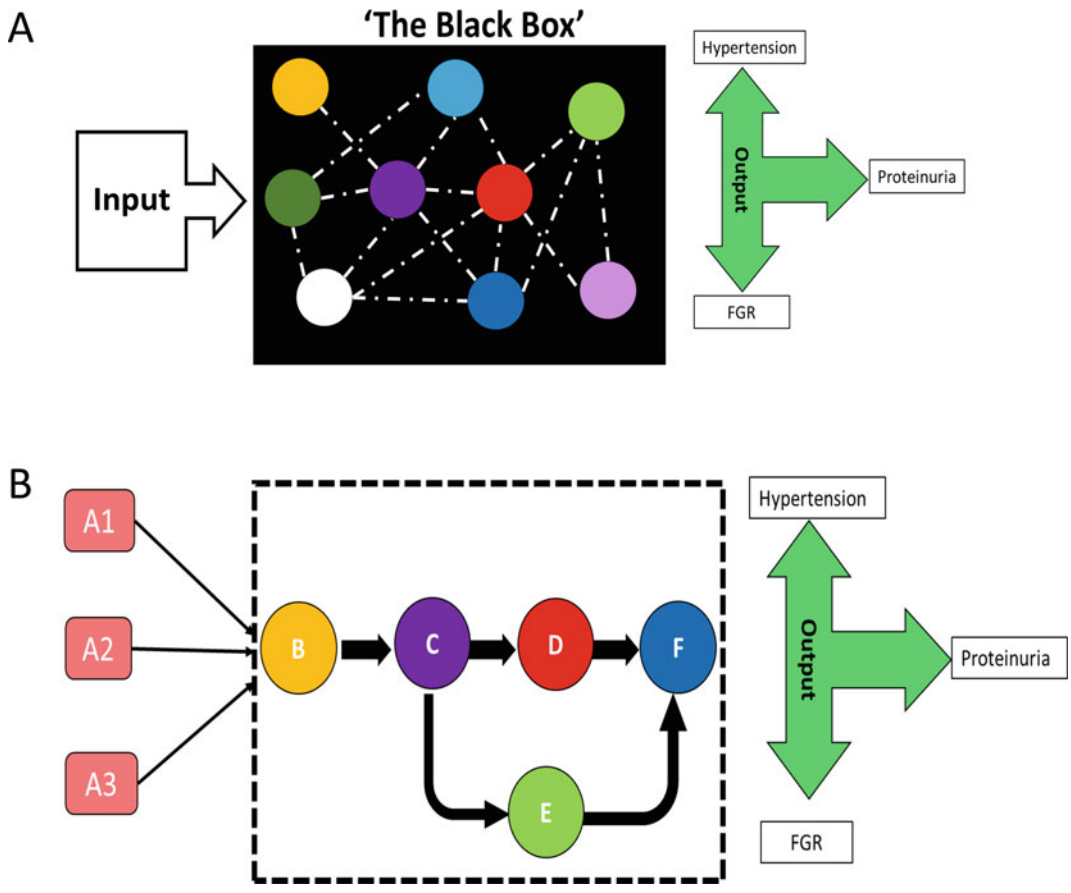

Fig. 2 The black box model. (**a**) Schematic representation for the roadmap of preeclampsia. An input equates to agents challenging pregnancy. These trigger series of factors denoted as *dots* in "the black box" and interconnected temporarily by "association studies" until the intersections are confirmed and established as solid connections using in vivo 'proof of principle' experimentations. (**b**) The schematic representation of preeclampsia roadmap pathway established by connecting the 'dots looking back' based on in vivo experimentations by gain or loss of the preeclampsia phenotype by manipulation of these *dots*

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