MONASH University

# Pursuing a novel adjuvant therapy for preeclampsia

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Department of Obstetrics and Gynaecology

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# Dedication

This thesis is dedicated to my family who have always supported me, to Euan who has shaped the person that I have become and to the brave women who participated in my studies. Most of all, this thesis is dedicated to the countless women diagnosed with preeclampsia every day.

# Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

# Acknowledgments

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Completing this PhD was the most challenging and most rewarding experience I have undertaken in my life to date. I have developed invaluable skills in problem solving, overcoming challenges and in dealing with failure. Undertaking this PhD was worthwhile alone for the manner in which I have grown as a person, a clinician and a researcher. I am eternally grateful for the extraordinary group of people I have met along the way, and how each has shaped the person I am continuing to become.

# Table of Contents

# Abbreviations

| | |
|---|---|
| ACh | Acetylcholine |
| ARE | Anti-oxidant Response Element |
| ARII | Activin Receptor II |
| ALK4 | Activin Like Tyrosine Kinase 4 |
| BK | Bradykinin |
| cAMP | Cyclic Adenosine Monophosphate |
| cGMP | Cyclic Guanosine Monophosphate |
| cGTP | Cyclic Guanosine Triphosphate |
| CO | Carbon Monoxide |
| $pEC_{50}$ | Half Maximal Effective Concentration |
| ELISA | Enzyme Linked Immuno Solubility Assay |
| eNOS | Endothelial Nitric Oxide Synthase |
| Eng | Endoglin |
| EDH | Endothelium Dependent Hyperpolarisation |
| Flt- 1 | Fms-like Tyrosine Kinase 1 |
| GCA | Guanylate Cyclase |
| Gclc | Glutamate-cysteine ligase catalytic subunit |
| GST | Glutathione S-transferase |
| HBSS | Hank's Balanced Salt Solution |
| HIF-1α | Hypoxia Inducible Factor 1 Alpha |
| HO-1 | Heme Oxygenase 1 |
| HUVEC | Human Umbilical Vein Endothelial Cells |
| ICAM-1 | Intracellular Adhesion Molecule 1 |
| $IK_{Ca}$ | Intermediate-Voltage Potassium-Mediated Calcium-Channels |

| | |
|---|---|
| IL-β | Interleukin Beta |
| ISSHP | International Society for the Study of Hypertensive Disorders of Pregnancy |
| KEAP-1 | Kelch-like-ECH-associated Protein-1 |
| mRNA | Messenger Ribonucleic Acid |
| MAPK | Mitrogen-activated protein kinase |
| NADPH | Nicotinamide Adenine Dinucleotide Phosphate |
| Nfκβ | Nuclear Factor Kappa Beta |
| NO | Nitric Oxide |
| Nox2 | NADPH Oxidase 2 |
| NQO1 | NADPH Dehydrogenase (Quinone) 1 |
| NFE2L2 | Nuclear Related ECH-like Related Factor 2 |
| NFE2L3 | Nuclear Related ECH-like Related Factor 3 |
| $O_2$ | Oxygen |
| PE | Phenylephrine |
| PIGF | Placental Growth Factor |
| $PGI_2$ | Prostaglandin I2 |
| ROS | Reactive Oxygen Species |
| $R_{max}$ | Maximum Relaxation |
| $SK_{Ca}$ | Small-Voltage Potassium-Mediated Calcium-Channels |
| SNP | Sodium Nitroprusside |
| SOMANZ | Society of Obstretric Medicine of Australia and New Zealand |
| sFlt-1 | Soluble Fms-like Tyrosine Kinase 1 |
| SFN | Sulforaphane |
| TGFβ-1 | Transforming Growth Factor Beta 1 |
| TGFβ-3 | Transforming Growth Factor Beta 3 |
| TNF-α | Tumour Necrosis Factor alpha |
| VCAM-1 | Vascular Cell Adhesion Molecule 1 |
| VEGF | Vascular Endothelial Growth Factor |

# Preamble

Preeclampsia is defined by the Society of Obstetric Medicine of Australia and New Zealand (SOMANZ) as new onset hypertension after the 20th week of pregnancy with associated maternal organ dysfunction and/or fetal growth restriction[1]. This syndrome complicates 2-8% of pregnancies globally. Every year 15,000 Australian women are diagnosed with preeclampsia; 60,000 women die worldwide and more than 2 million neonates are delivered prematurely. Preeclampsia remains a leading cause of maternal and fetal morbidity and mortality worldwide, and a priority for obstetric research. The burden of disease is particularly heavy in low resource settings where preeclampsia represents the single greatest cause of maternal mortality[1,2]. The only "cure" for preeclampsia is delivery of the placenta, and therefore the fetus[1,2]. The challenge facing treating clinicians is balancing the complications of early delivery for the baby with delayed delivery for the mother. In severe disease, delivery may be necessary at very early gestations to save the mother's life. As such, the perinatal disease burden attributable to preeclampsia is high[2]. For women, preeclampsia carries the risk of acute complications including seizure, stroke, renal failure, liver failure and haematological dysfunction as well as long-term cardiovascular disease[3,4].

Over recent years, an increased understanding of disease pathogenesis has led to improvements in prediction[5], prevention[6,7] and diagnosis[8] of the condition. However, few advancements have been made in the management of those women with early-onset established disease[2,9]. Once a woman is diagnosed with preeclampsia, beyond blood pressure control[10], little can be done to delay disease progression. The improvements in perinatal outcomes from pregnancies complicated by preeclampsia have largely resulted from advances in neonatal care, allowing earlier delivery and resolution of the condition[2]. Future advances in outcomes are likely to now come from widespread uptake of screening and prevention[5,7,8], or from more effective treatment, beyond simply managing the hypertension[11,12]. Much scope remains to explore

novel therapies for the management of established preeclampsia, informed by recent insights into the mechanisms that underlie the hypertension and end-organ injury of the maternal syndrome.

Preeclampsia is, by definition, a syndrome; a manifestation of underlying pathological processes with specific characteristics, as defined by SOMANZ.[1] However, while the features of women fit within the diagnostic criteria, the exact underlaying pathophysiology may vary significantly. The maternal endothelial dysfunction characteristic of preeclampsia is thought to arise from placental production of antiangiogenic compounds to a degree that overwhelms the maternal vasculature leading to blood vessel irritation, vasoconstriction and endothelial cell activation triggering systemic inflammatory cascades[13]. In women with placentally driven disease this is likely due to overwhelming placental production of anti-angiogenic factors in a stress response to repeated hypoxic-reperfusion injury[13–15], far in excess of what an even previously health vasculature can tolerate. Alternatively, the syndrome of preeclampsia may manifest in women with healthy placentae but whose vasculature is already irritated and cannot tolerate normal pregnancy production of antiangiogenic compounds. In even healthy pregnancies, women will have increasing levels of antiangiogenic factors as the placenta ages[16]. However, for women with a previously healthy vasculature, this does not manifest as a disease state. Women who have pre-existing or underlaying vascular dysfunction will develop endothelial irritation and present with preeclampsia despite levels of antiangiogenic compounds that would not cause issue in women with baseline healthy vasculature.

In reality, these pathological processes of vascular and placental disease are likely not discrete with varying degrees of contribution of each. It is likely however, that the contribution of each process relates to the timing of presentation. In general women in whom disease is driven by placental insufficiency likely present earlier than those whose disease is largely of vascular

origin. As such, the term “early onset preeclampsia” is often used to explain women with placental insufficiency, while “late onset preeclampsia” is used to describe women with vascular disease. It must be noted however this is only an association and this stratification of women is not based on pathology. Because clinical outcomes are generally worse with earlier presentations, given the progressive nature of the disease, and because, though both currently termed “preeclampsia” these pathologies are in essence different diseases, this thesis will focus on disease of placental insufficiency, or “early onset” preeclampsia[2].

In a normal pregnancy, spiral arteries, the vessels responsible for placental vascularisation, are remodelled to become wide diameter, low calibre vessels, to facilitate placental oxygenation across pregnancy in accordance with increasing fetal demand[17,18]. In women who subsequently develop early onset, or placentally driven preeclampsia, shallow remodelling and persistently vasoactive spiral arteries means the placenta is exposed to repeated hypoxic-reperfusion injury[19,20]. Hypoxic-reperfusion injury impairs mitochondrial electron transport and results in reactive oxygen species (ROS) build up[21–23], inducing oxidative stress as endogenous protective antioxidant mechanisms are progressively overwhelmed. This cellular oxidative stress is evidenced by superoxide formation, lipid peroxidation and cellular destruction as well as activation of immune cells[24,25]. Supercharged oxygen species damage DNA and alter gene transcription. In turn, these induce cellular dysfunction including alternate splicing of mRNA, abnormal protein production and excessive release of various anti-angiogenic factors, such as the soluble forms of membrane receptors; soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng), and activin, with a corresponding reduction in proangiogenic factor placental growth factor (PlGF) [26–28]. sFlt-1 competitively binds vascular endothelial growth factor (VEGF), reducing nitric oxide (NO) production and impairing endothelial health[29,30]. Without adequate PlGF to support endothelial function, widespread microvascular endothelial dysfunction follows. Similarly, activin A is a proinflammatory cytokine that triggers a body

wide inflammatory response. In addition, recurrent and progressive trophoblast injury disrupts syncytialisation leading to increased shedding of trophoblast debris into the maternal circulation[31,32]. These trophoblast micro emboli activate the maternal inflammatory scavenger system[24] and further add to endothelial dysfunction. Endothelial dysfunction together with systemic inflammation promotes vascular smooth muscle contraction, which manifests as hypertension and maternal organ dysfunction; the maternal syndrome of preeclampsia[13,19,33,34]. The role for oxidative stress in the mitochondria, in the placenta and on the maternal vasculature suggests that antioxidants, particularly those targeting key pathways, offer an appealing therapeutic option to delay disease progression.

The overarching aim of this PhD was to investigate sulforaphane as a potential adjuvant therapy for preeclampsia, to be used alongside antihypertensives in women with established disease. At the outset of these studies, a series of integrated *in vitro*, *ex vivo* and clinical studies were designed to first assess whether sulforaphane offered any protective effects in the placenta and/or vasculature, to explore mechanisms by which sulforaphane may mediate any such protection, and to determine the dose of sulforaphane likely required as a therapy. All in aid of informing the design of a clinical trial to determine the efficacy of a broccoli extract rich in sulforaphane as an adjuvant therapy for preeclampsia.

By way of introduction to my experimental chapters this thesis includes two reviews. In the first of these, *Chapter one*, the literature surrounding the current and future therapeutics available for the management of preeclampsia is summarised, to better understand the need for novel and targeted therapies. *Chapter two* looks at the *in vitro*, *ex vivo* and *in vivo* literature supporting a role for the antioxidant melatonin as a therapeutic for preeclampsia. From here, this thesis outlines investigations of the ability of sulforaphane to protect endothelial and placental cells from preeclamptic-like injury, as shown in *Chapter three*. This thesis then

describes further mechanistic investigations, exploring whether sulforaphane can prevent the damaging effect of hypoxia and superoxides on placental mitochondrial function. These studies form *Chapter four*. The experiments of this thesis used *in vitro* techniques, outlined in *Chapter five*, to assess the effect of sulforaphane on the systemic vasculature. *Chapter six* marked the beginnings of the clinical translation of this experimental work, through development of a high throughput method to quantify plasma levels of sulforaphane and active metabolites – a technique needed for clinical trials of sulforaphane. In the experiments in *Chapter seven* these methods are used to evaluate the bioavailability of sulforaphane and metabolites after administration of two broccoli extract preparations in non-pregnant women and for a limited dose escalation study in women with hypertensive disorders of pregnancy. *Chapter eight* presents a protocol for a phase III randomised control trial administering broccoli extract or placebo to women with early onset preeclampsia.

*Chapter nine* of this thesis reflects on the strengths and limitations of the experiments, sharing insights that are not possible within the word limits of published manuscripts. Finally, this thesis provides considerations of likely future directions of this and related research.

# Chapter one

## *Current and emerging pharmacotherapy for emergency management of preeclampsia*

Before outlining experimental studies for an adjuvant therapy for preeclampsia, this thesis summarises the literature on the current management of preeclampsia, particularly acute management. The closing paragraphs of this review foreshadow future therapies, including some of the work this thesis would subsequently include. This review did not detail efforts made to prevent preeclampsia.

From this review it became apparent that, beyond optimising blood pressure, treatment options for established preeclampsia remain limited[1,2]. Managing hypertension is important to reduce stroke risk. However, it largely neglects the underlying pathological processes of the disease and carries fetal risks by reducing blood flow to an already under-perfused placenta[1,2,35]. Indeed, there remains much debate about which antihypertensive is most suitable for the management of preeclampsia[12]. Of the four most commonly used antihypertensives, labetalol, methyldopa, nifidepine and hydralazine, labetalol perhaps generates the most controversy and debate about possible adverse fetal outcomes associated with use[36–39]. Methyldopa, though safe, has lower efficacy, and hydralazine is generally accepted as only used in emergency situations with risks of significant fetal effects associated with dramatic reductions in blood pressure[12,40,41]. While optimising blood pressure will always play an essential role in the management of preeclampsia, and has served the field well in greatly reducing maternal and perinatal mortality and morbidity, there is a need for disease targeted treatments to address the underlying placental and vascular oxidative stress of the condition[11].

This review covers the current management options used for the emergency control of preeclampsia and cast light on those therapies emerging as potential novel treatments.

The following information was not included in the review however warrants mention in this thesis. The serotonin 2 receptor antagonist ketanserin may offer benefit in the preeclamptic population with evidence of a better safety profile, lower rates of HELLP and lower rates of abruption[42] compared to hydralazine. Contention in the evidence surrounding the efficacy of ketanserin as an antihypertensive may relate to discrepancies in dosing[43–45]. It might be that studies disputing antihypertensive efficacy of ketanserin use inadequate doses, highlighting the importance of dose-finding before clinical trial[43–45]. Ketanserin may offer benefits that extend beyond diagnosed preeclampsia with reduced rates of preeclampsia in at risk women on oral ketanserin, compared to placebo in studies of prevention[46]. Similarly, no benefits in preventing preeclampsia have been identified from metformin, also attributed to insufficient doses[47–49]. The gastrointestinal side effects of higher doses of metformin limit clinical utility and capacity for further dose escalation. Further dose finding may elucidate clinical importance of both ketanserin and metformin in the prevention and management of preeclampsia.

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REVIEW



# Current and emerging pharmacotherapy for emergency management of preeclampsia

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**ABSTRACT**

**Introduction**: Preeclampsia is a disease specific to pregnancy characterised by new onset hypertension with maternal organ dysfunction and/or fetal growth restriction. It remains a major cause of maternal and perinatal morbidity and mortality. For fifty years, antihypertensives have been the mainstay of treating preeclampsia, reducing maternal morbidity and mortality. With increased knowledge of the mechanisms underlying the disease has come opportunities for novel therapies that complement antihypertensives by protecting the maternal vasculature.

**Areas covered**: In this review, the authors consider, in detail, the antihypertensives commonly used today in the emergency care of women with severe preeclampsia. They also review less common antihypertensive agents and discuss the role of magnesium sulphate in the management of preeclampsia and the prevention of eclampsia. Finally, they explore novel therapeutics for the acute management of preeclampsia.

**Expert opinion**: The rapid control of maternal hypertension will, and must, remain the mainstay of emergency treatment for women with severe preeclampsia. The role of magnesium sulphate as a primary prevention for eclampsia is context dependant and should not displace a focus on correcting blood pressure safely. The exploration of novel adjuvant therapies will likely allow us to prolong pregnancy longer and improve perinatal outcomes safely for the mother.

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## 1. Introduction

The term 'eclampsia' is derived from the Greek word 'lightening'. It is thought to have been first used by the 2nd-century Greek philosopher Celsus to describe a seizure disorder unique to pregnant women that had a strikingly sudden onset and was associated with disastrous maternal and fetal consequences [1]. It wasn't until the mid 19th century that oedema and proteinuria were identified as preceding the development of seizures, giving rise to the concept of a prodromal condition and the introduction of the term 'pre-eclampsia', literally, 'before the eclampsia' [2]. Another fifty years passed before hypertension was recognised as part of preeclampsia. To this day, it is the hypertension that remains the key treatment target [2,3], particularly in the emergency setting. Indeed, until only very recently preeclampsia was understood to be a disease defined by hypertension and proteinuria, accompanied by maternal organ dysfunction in severe disease. However, the recognition that preeclampsia arises from a dysfunctional placenta and so was frequently associated with fetal growth restriction, particularly in early-onset disease, recently led to the inclusion of fetal disease into the definition. Today, preeclampsia is defined by new onset hypertension occurring after 20 weeks gestation with associated maternal organ dysfunction and/or fetal growth restriction [4].

Preeclampsia complicates 2–8% of pregnancies. While rates vary slightly across nations this is mostly due to variable detection and reporting rather than actual variance in incidence. In all countries, preeclampsia remains a leading cause of perinatal morbidity and mortality, largely due to iatrogenic prematurity [5]. While management of hypertension is essential in reducing maternal morbidity and mortality, ultimately, delivery of the placenta is the only cure for preeclampsia. The timing of delivery requires a balance between prolonging pregnancy to gain sufficient fetal maturity and delivering in time to prevent serious maternal complications [5]. Despite this, targeted management options for preeclampsia, to safely prolong pregnancy, are largely lacking. In essence, treatment is restricted to the optimisation of maternal blood pressure [3,6]. This focus on hypertension neglects the underlying pathological processes of the disease and may increase fetal risks by reducing blood flow to an already under-perfused placenta [5]. As such, the stringency of blood pressure control in preeclampsia has been under debate [7]. Reassuringly, as will be discussed in detail later, for women with mild-moderate preeclampsia tight blood pressure control has been shown to reduce risks of severe disease without increasing the risks of fetal compromise [7,8].

However, while an essential component of management, whether blood pressure should be the *sole* target for treatment remains debatable. Hypertension, like renal

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**Article highlights**

- Tight and rapid blood pressure control remains the mainstay of emergency management of severe preeclampsia.
- Oral nifedipine and intravenous labetalol are the first line agents of choice to control blood pressure.
- Magnesium sulphate is the sole agent of choice for the prevention of eclampsia but blood pressure control is more important.
- Predictive modelling can accurately identify which women require escalated care.
- Future improvements in care will come from therapies directly targeting endothelial damage

This box summarizes key points contained in the article.

disease or neurological excitability, is merely a reflection of the underlying organ damage. Specifically, it arises from maternal endothelial dysfunction secondary to systemic oxidative stress and excessive release of antiangiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1), activin, and soluble endoglin (sEng), from a chronically and progressively ischaemic placenta [2]. New insights gleaned over the past decade have identified the fundamental mechanisms that underlie preeclampsia, opening a variety of avenues for novel treatments [3]. It is now accepted that addressing the pathophysiology behind the disease, rather than simply controlling blood pressure alone, is integral to improving maternal and perinatal outcomes [5,9].

In this review we will examine the current use of antihypertensives in the emergency management of preeclampsia and detail emerging pharmacological interventions that promise to further reduce the disease burden of the condition. While there have been important advances in the prevention of preeclampsia, for example through aspirin [10,11] and calcium supplementation [12], in this review we focus on the pharmacotherapy available for managing women with established disease.

## 2. Antihypertensive medications

It is principally the need to reduce the risk of stroke, and the related morbidity and mortality, that necessitates the urgent use of antihypertensives to treat preeclampsia, particularly in the setting of severe, uncontrolled hypertension [13–15]. However, blood pressure targets must be balanced with the risk of acute fetal compromise, typically arising in the emergency setting of sudden reduction in extreme hypertension and fetal growth restriction, that may accompany over-zealous control of maternal blood pressure. Managing this balance has sparked debate regarding how aggressively blood pressure should be controlled and which antihypertensive agents should be preferred. At present, the majority of expert clinical practice guidelines support the use of nifedipine, labetalol, methyldopa, or hydralazine as first and second line antihypertensives [4,5,16]. Mechanisms of action, and the benefits and risk of each of these therapeutics will be explored in this review [17].

### 2.1. Methyldopa

Discovered in 1960, methyldopa exerts antihypertensive effects by two central mechanisms that both decrease sympathetic nervous system drive. First, methyldopa is metabolised to alpha-methylnoradrenaline, an agonist for centrally (brainstem) located inhibitory alpha-adrenergic receptors. Activation of these inhibitory receptors reduces sympathetic drive and lowers blood pressure. Second, methyldopa is also a competitive inhibitor of aromatic L-amino acid decarboxylase, the enzyme necessary to convert L-DOPA to dopamine. By reducing the production of dopamine, the precursor of noradrenaline and thence adrenaline, methyldopa further reduces sympathetic drive and lowers blood pressure [17]. Though effective in reducing blood pressure, the central action of methyldopa has a slight delay in the onset of antihypertensive effects, typically becoming apparent only some 24 hours after starting treatment [17]. For this reason, while commonly used in mild to moderate disease, methyldopa is not an ideal agent for acute or emergency management of hypertensive crises.

At high doses the mood altering side effects of methyldopa also limit acceptability, particularly in women with a history of mood disorders [18]. These limitations can be mitigated by combining it with another antihypertensive to attain the desired blood pressure targets at lower doses [17]. Reassuringly, there is no evidence that methyldopa increases the likelihood of postnatal depression [19].

The fetal safety profile of methyldopa is well documented. Indeed, methyldopa was introduced as an antihypertensive suitable for pregnancy when Chris Redman, working with John Bonnar, first reported that its use in 242 hypertensive women was associated with improved pregnancy outcomes [20]. This safety profile was subsequently confirmed by a randomised controlled trial of methyldopa in pregnant women with chronic hypertension, reducing the risks of superimposed preeclampsia with no apparent maternal or fetal adverse effects [21], and by longer term follow up of children [22]. So, while not particularly suitable as an emergency antihypertensive agent, methyldopa has rightly earned its place as a mainstay therapy for more chronic treatment.

### 2.2. Labetalol

Labetalol is an α,β1,β2-adrenergic receptor antagonist. It was specifically created to combine both α- and β-adrenergic receptor blocking properties and was the first drug to do so. Reflecting that dual purpose, labetalol exerts antihypertensive effects both peripherally, through α -adrenergic blocking, and centrally at the heart via β1, β2-adrenergic receptor blocking [17]. The relative balance of these two mechanisms is largely dependant on treatment duration and mode of administration, either oral or intravenous (IV). Oral labetalol principally drives alpha blockade, having a more profound effect on peripheral vascular resistance [17]. In contrast, IV labetalol equally effects alpha and beta-receptors influencing peripheral resistance as well as heart rate and stroke volume [17]. Acute, short term use induces mainly alpha blockade while chronic exposure is more likely to impact cardiac contractility and stroke volume via β-blockade [17]. An understanding of these differential effects of route of administration and duration of therapy are important for the optimum management of a haemodynamically unstable preeclamptic woman and for an awareness of her risks of cardiac compromise with sustained high dose treatment.

However, both oral and IV labetalol are commonly used for blood pressure control in preeclampsia [23]. The availability of oral and intravenous preparations allows for a two-tiered approach to the use of labetalol in preeclampsia. Acute hypertensive crises demand IV labetalol for rapid blood pressure control while oral formulations are ideal for use in chronic management and milder cases.

In acute hypertensive crises labetalol has been shown to act significantly faster and be more effective than oral methyldopa in reducing blood pressure, and was associated with fewer caesarean sections [24]. Not surprisingly, intravenous labetalol is ranked highly by clinicians for the management of hypertension in pregnancy, likely due the perception that intravenous administration affords faster attainment of the blood pressure target [23–25]. However, while intravenous labetalol certainly has a more rapid onset of action that methyldopa, oral nifedpine may be just as rapid and easier to deliver [26]. Intravenous labetalol is also much less likely to impair uterine perfusion and cause acute fetal compromise that the other commonly used intravenous antihypertensive, hydralazine [27,28]. That is not to say that intravenous labetalol is without fetal-neonatal effects. Neonatal hypotension and bradycardia, secondary to fetal cardiac beta blockade, can occur even with acute maternal treatment with labetalol [29], albeit these are less common that acute hypotension caused by hydralazine.

Regarding longer term maintenance of blood pressure control following management of an acute crisis, oral labetalol is broadly equivalent to methyldopa [24,30]. However, at least one group has reported better perinatal outcomes in women treated with methyldopa [31] compared to beta blockers, including a lower risk of stillbirth [31]. As with all antihypertensives, labetalol should be used cautiously, particularly in the setting of fetal compromise [32]. Perhaps the most important consideration regarding labetalol, particularly in the maintenance setting, is the potential effect on fetal growth. Impaired fetal growth has been associated with both oral and intravenous labetalol. Undoubtedly, some of the fetal growth impairment is due to the placental dysfunction that underlies the preeclampsia but, when compared to methyldopa, the majority, but not all [33,34], of studies suggest labetalol increases the risk of fetal growth restriction [31,32,35,36].

In summary, labetalol has been a favoured antihypertensive in the management of preeclampsia for over thirty years [33,34,37,38]. Availability in both intravenous and oral preparations allows its use in both the emergency and maintenance management of preeclampsia with lower risks of acute fetal compromise than hydralazine. An awareness of the need to monitor fetal growth and wellbeing following acute management will ensure good perinatal outcomes.

### 2.3. Nifedipine

Nifedipine is a dihydropyradine calcium channel blocker that acts on L-type calcium channels. The first clinical trial reporting its use as a therapy in preeclampsia was in 1993 [39]. Unlike other calcium channel blockers, nifedipine is relatively selective for peripheral smooth muscle and therefore does not greatly affect cardiac muscle contractility [40]. In this regard it is also quite different to the beta-blockade actions of labetalol. Extensive clinical experience with nifedipine over the past 20 years has shown it to be a both safe and effective antihypertensive, particularly in the acute crisis setting [26,41]. Indeed, compared to intravenous labetalol, oral nifedipine is at least as effective at controlling blood pressure in a hypertensive crisis [26,42], requiring fewer doses to achieve desired pressures [26]. While labetalol is associated with negative ionotropic effects, this is not observed with nifedipine [26]. Given that severe preeclampsia is associated with impaired myocardial and endomyocardial function [43–45] this cardio-protectant property of nifedipine may be beneficial. Specifically, a lack of a negative ionotropic effect on an already compromised myocardium may reduce the risk of pulmonary oedema [46], an important cause of maternal death [13]. Regarding acute fetal effects, oral nifedipine appears at least as effective [47] and perhaps better [25] than intravenous labetalol in reducing blood pressure. While hypotension and excessively rapid reductions in blood pressure have been associated with capsule formulations of nifedipine, these are no longer commercially available [48]. Nifedipine has also proven safe and effective in maintaining normal blood pressure longer term without the higher rates of fetal growth restriction associated with labetalol [49]. Not surprisingly, nifedipine has emerged as preferred agent for both acute and chronic management of preeclampsia.

That is not to say that nifedipine treatment is wholly without risk. Case reports outline hypocalcaemia [50] and an admission to intensive care in the context of acute hypoxia – likely related to respiratory depression – when nifedipine was used for tocolysis in threatened preterm delivery [51]. Perhaps most concerning are reports of neuromuscular blockade [52,53] and severe hypotension when nifedipine was used concurrently with magnesium sulphate ($MgSO_4$) [40]. However, these risks are generally associated with underlying pathology and, with the risk at <1%, are not considered a significant concern at a population level [54]. The use of nifedipine is certainly not contraindicated in the setting of $MgSO_4$, or vice versa [54].

### 2.4. Hydralazine

Hydralazine is a hydrazinophthalazine. It acts to directly dilate resistance vessels by inducing smooth muscle relaxation, thereby reducing peripheral resistance [17]. Hydralazine is particularly effective in rapidly lowering blood pressure and has long been a favoured therapy for the acute management of hypertensive crises, including severe preeclampsia. However, this sudden reduction in peripheral vascular resistance does carry risks of profound, acute hypotension that can be difficult to correct [55]. A meta-analysis by Magee and colleagues showed consistently higher rates of maternal hypotension with hydralazine than with either nifedipine or labetalol [55]. In an antenatal setting this is important because such sudden maternal hypotension is associated with acute changes in uterine perfusion and so in fetal oxygenation and wellbeing. A not uncommon effect of over-zealous use of hydralazine is acute fetal compromise, as evidenced by sudden changes in the cardiotocograph (CTG), requiring urgent delivery; not what is usually intended [55]. Much of the risk of maternal hypotension

can be offset by a rapid fluid preload, to fill the vascular space prior to vasodilatation [56]. Typically, 500mL acute crystalloid can be given safely to preload ahead of hydralazine administration [56]. The acute volume delivery itself may also induce transient vasodilatation allowing for a reduced dose of hydralazine [57]. However, as discussed previously, both labetalol and nifedipine are equally effective as hydralazine in the acute setting and are much less likely to cause acute profound hypotension [58]. Indeed, nifedipine has a shorter time to target blood pressure and requires less dose escalation than intravenous hydralazine [59–61]. Some of the side effects of hydralazine also mimic worsening preeclampsia. This can make it more difficult for the attending clinician to discern deterioration. For these reasons, hydralazine has lost favour as the first-line agent for managing acute hypertension in many centres. However, hydralazine is an effective therapy for resistant hypertension, poorly responsive to either nifedipine or labetalol.

It also appears that maternal and perinatal outcomes are better following treatment with nifedipine than with hydralazine [60]. Women given nifedipine required fewer doses, had greater urinary output before and after delivery, and had a significantly longer interval between treatment and a consecutive hypertensive crisis [60]. Sublingual nifedipine is no longer used clinically, but when it was it was associated with higher Apgar scores than hydralazine with similar time to target blood pressure [61].

### 2.5. Other antihypertensive agents

While labetalol, nifedipine and hydralazine are the most commonly used first and second line agents in the management of acute severe preeclampsia, a number of other antihypertensives have been assessed for the treatment of preeclampsia. However, a combination of safety concerns, limited evidence and impracticalities in modality of treatment mean they are not widely incorporated into current treatment guidelines. Ketanserin initially appeared effective reducing blood pressure [62–64] but was shown to be inferior to methyldopa, more likely to be associated with persistent hypertension and was unsafe in pregnancy [65]. Diazoxide appeared to have comparable efficacy to hydralazine [66] but was less safe than labetalol due to larger and less predictable reductions in blood pressure [67], a dose-response effect that precluded use at therapeutic doses [67]. Prazosin, more commonly employed as a second lime agent, proved much less effective than nifedipine and so has not been widely adopted [68]. While a small study comparing urapidil and niccardipine found unacceptable levels of side effects in women taking the nicardapine [69], it was shown to be more effective in lowering blood pressure than metoprolol [70], lending growing support to the use of calcium channel blockers *per se* in women with preeclampsia in preference to beta blockers. In that regard, small studies have found isradipine to be similarly effective in hypertensive crises to methyldopa [71] and hydralazine [72]. However, the evidence for these newer calcium channel blockers remains limited and the successful use of nifedipine makes it unnecessary to assess them further.

Aerosol isosorbide mononitrate, given as a sublingual spray, was an attractive prospect as an emergency therapy for acute severe preeclampsia. However, it was unable to adequately control blood pressure [73] and its delivery was associated with significant reductions in both maternal and fetal heart rates [73]. Nitroglycerine infusion, while not been extensively studied, appeared to have a similar safety profile to the no longer available sublingual nifedipine [74]. Angiotensin converting enzyme (ACE) and angiotensin II inhibitors, though highly effective in reducing blood pressure, have not been adequately assessed in the setting of hypertensive crises of preeclampsia. This is because ACEi and ATIIi have been associated with an increased risk of fetal malformation when taken early in pregnancy [75–78]. As such they have been deemed unsafe in pregnancy and not recommended for the treatment of preeclampsia [4,5,16]. Given the success of labetalol, it is not surprising that other beta-blockers have been assessed. Small studies of oxprenolol [79,80], propranolol [81] and metoprolol [82,83] suggest that they are safe to use in pregnancy but their relative effectiveness, that is are they better than labetalol? has not been adequately explored. While an early report of atenolol suggested that it was both safe and effective in reducing maternal blood pressure [83], it has since been associated with adverse fetal growth [84], not dissimilar to labetalol. Similarly, pindolol is an effective antihypertensive [85], but at the cost of significant fetal growth restriction [86].

### 2.6. Antihypertensive selection by disease severity

As will be apparent, some agents are more suitable than others for the management of severe preeclampsia and others for either maintenance therapy or in a non-severe setting.

#### 2.6.1. Severe preeclampsia

Intravenous labetalol and oral nifedipine form the mainstay of treatment for severe hypertension, with hydralazine reserved as second line treatment for resistant hypertension. Though IV routes of administration are often considered more rapid, oral nifedipine appears to have equal or better time to target blood pressure than IV labetalol [26,42,47] without the negative inotropic risks [26]. In turn, IV labetalol is much less likely than hydralazine to cause acute, profound hypertension, a side effect important to avoid if the fetus is still *in utero* [27,28,55].

#### 2.6.2. Non-severe preeclampsia and maintenance therapy

The impressive safety profile of methyldopa [20,21,22] explains why it remains a popular agent for maintenance and non-severe preeclampsia. High doses of methyldopa may carry risks of mood derangement [18] but these can be avoided by safely combining with either oral labetalol or oral nifedipine; agents commonly used in maintenance and therapy of non-severe disease [17]. The slower onset of methyoldopa complements action of the more rapid oral nifedipine [41] to act in concert for maintenance control. Maintenance therapy and chronic treatment with oral labetalol may affect fetal growth [31,35,36], though these findings remain controversial [33,34].

## 3. Magnesium sulphate

While blood pressure control is the first priority in emergency management of severe preeclampsia, the care of the woman with severe preeclampsia extends beyond blood pressure. In particular, acute management requires the assessment and prevention of seizures – eclampsia. The challenge facing the clinician caring for the woman with severe preeclampsia is not which agent to give to prevent eclampsia – unlike the choice of different antihypertensive agents, the only agent suitable for the prevention and treatment of eclampsia is magnesium sulphate ($MgSO_4$) [87] – it is whether to give magnesium sulphate at all. With a prevalence of eclampsia of only 1 in 10,000 pregnancies in a high-income setting, and with the majority of eclamptic seizures taking place prior to admission to hospital, on average a clinician will need to treat over 300 women to prevent one eclamptic event [6,88,89]. The question is whether this is merited or not. Useful guidance can be found in expert clinical guidelines [90,91]. These suggest that $MgSO_4$ should certainly not be administered universally but should be reserved for women with severe hypertension, headaches/visual disturbance, right upper quadrant pain, platelet count <100,000 $10^9$/L, progressive renal insufficiency and/or elevated liver enzymes. It can be safely given antenatally, intrapartum or postpartum. Unfortunately, there are no sensitive tools to predict which women with severe preeclampsia will progress to eclampsia and which will not [6,88,89]. There also appears to be no significant improvements in maternal outcome afforded by $MgSO_4$ seizure prophylaxis in high-income countries – quite different from low and middle-income settings. The use of $MgSO_4$ prophylactically certainly halves the risk of eclampsia [92]. However, timing the drug delivery and selecting women who will progress to eclampsia, limits the value of this intervention [6,88,89]. In fact, the majority of eclamptic fits are self-limiting and occur in isolation. Further, these convulsions largely have no lasting consequences on perinatal or maternal wellbeing. There are no significant differences in 2-year death or morbidity between women given $MgSO_4$ prophylactically and those not [93]. While there are no adverse outcomes from $MgSO_4$ administration in infants at 18 months and women at 2 years [94,95], there are also no benefits. If the purpose of giving $MgSO_4$ prophylactically is to decrease important morbidity and/or mortality, whether maternal or perinatal, then, at least in a high-income setting, it does not do that. Indeed, maternal mortality from preeclampsia in a high income setting typically relates to complications of preeclampsia, not eclampsia [11,93]. The key to preventing maternal death is better and more rapid blood pressure control.

Nonetheless, it is important that clinicians are comfortable with $MgSO_4$ administration and are aware that it is not without some risk. $MgSO_4$ significantly increases the risk of side effects including severe hypotension (4-fold increase) and respiratory depression (doubled) [92]. For every five women treated with $MgSO_4$, one will experience an adverse reaction. While relatively inexpensive, there are also financial costs incurred from $MgSO_4$ use. With a number needed to treat of 324, the estimated cost of $MgSO_4$ to prevent one case of eclampsia is about $21,000 [87]. However, while given primarily to prevent eclampsia, $MgSO_4$ may have neuroprotective effects for the very preterm fetus [97] and reduce the risk of cerebral palsy for babies born under 30 weeks [98]. In the management of the woman with severe preeclampsia at such early gestations the longer term benefits, and associated health economics, are likely to be significantly greater.

## 4. Beyond lowering blood pressure: newer approaches to management

The impressive decline in maternal morbidity associated with antenatal monitoring of blood pressure has been attributed largely to antihypertensives and the acute reduction in life threatening blood pressure [13]. However aggressively managing blood pressure must be balanced with potential effects on the fetus with a view to prolonging pregnancy for as long as is maternally safe [7,97–101].

While there has long been debate about whether blood pressure should be aggressively controlled or not, it is now clear that maintaining tight blood pressure targets (diastolic blood pressure 85 mmHg) significantly reduces the likelihood of severe preeclampsia compared to accepting less tight blood pressure control (diastolic blood pressure 100 mmHg), without any adverse effects on fetal wellbeing [97]. However, while episodes of severe hypertension certainly place women at risk of adverse outcomes [8], high blood pressure itself is not particularly predictive of all serious adverse outcomes [7]. Even cerebrovascular disease, a phenomenon generally attributed to hypertensive crises, does not always coincide with hypertension [14]. Eclampsia in particular, and the corresponding cerebral oedema, is more closely linked with markers of endothelial dysfunction than hypertension [102]. Given that preeclampsia is a complex syndrome where hypertension is only one of many involved organ systems, it is not surprising that blood pressure is not an all encompassing prognostic marker of disease progression. Indeed, accurately identifying which women merit earlier delivery and additional care, such as magnesium sulphate or antenatal corticosteroids, has historically been very challenging. The recent development and validation of predictive tools, such as the fullPIERS predictive model [103,104] has begun to change this. The fullPIERS uses key maternal variables (gestational age, chest pain or dyspnoea, oxygen saturation, platelet count, creatinine, and aspartate transaminase) – note not blood pressure – to predict those women who will develop deteriorating preeclampsia requiring early delivery [103,104]. The level of predictive accuracy is good with 75% of women needing early intervention correctly identified. It is interesting to reflect that the predictive variables in the model reflect outcome related to endothelial dysfunction – pulmonary vascular leakage, platelet activation, hepatic and renal injury. This highlights that advances in the management of preeclampsia are likely to come from targeting the endothelium rather than just blood pressure [8].

Indeed, with an improved understanding of the underlying pathophysiology of preeclampsia over the past decade or more, new opportunities for more effective therapies beyond simple blood pressure control are becoming realistic prospects. These therapeutic developments are important because they may be able to both prevent and mitigate severe, early-onset preeclampsia, avoiding the need for 'crisis'

management in the future. In particular, in the setting of women presenting with severe preeclampsia, those with a high fullPIERS score, there is growing evidence that endothelial targeted adjuvant treatments can prolong pregnancy safely for both mother and baby [9,105], improving the outcomes for the baby. In the future, the emergency management of severe preeclampsia will not simply be about controlling blood pressure and preventing eclamptic seizures. Improving blood vessel function to allow safe prolongation of pregnancy and better perinatal outcomes will become a major focus. The benefits of such targeted therapy are also likely to extend well beyond the acute emergency that is severe preeclampsia. While the acute syndrome of preeclampsia is considered 'cured' after delivery of the placenta, maternal cardiovascular and renal consequences of this disease continue to manifest over time [106]. About one in five women who have preeclampsia will develop chronic hypertension in less than a decade [107]. Indeed, preeclampsia is recognised as a key lifetime risk factor for future cardiovascular [108] and cerebrovascular disease [109], even in otherwise previously healthy women [109–111].

Accordingly, while in this review we are principally focused on the emergency management of severe preeclampsia, the near future importance of these emerging therapies is such that some mention here of what that future is likely to involve is worthwhile. In essence, the adjuvant therapies under current evaluation are predicated on the understanding that preeclampsia is a disease arising from maternal endothelial dysfunction. This dysfunction is secondary to excessive oxidative stress and inflammation driven by the placental production of anti-angiogenic and inflammatory compounds [112]. As such, the novel therapeutics have two main targets: direct vasodilators to mitigate vessel constriction and antioxidant compounds to reduce placental and endothelial oxidative stress. Here, we will only discuss those agents that have already progressed to clinical trial.

### 4.1. Targeting the vasculature

Sildenafil citrate acts directly on vessel endothelium, inhibiting the action of phosphodiesterase type 5 (PDE5) thereby preventing degradation of cyclic guanosine monophosphate (cGMP). The resulting increased levels of cGMP induce localised vessel relaxation. Several case reports suggested that sildenafil increases uterine artery pulsation [113,114], leading to the hypothesis that it may increase placental flow and improve fetal wellbeing in growth restricted infants. It has since been investigated in a preeclamptic cohort, for both maternal and perinatal outcomes. In a randomised trial of 100 women with severe preeclampsia, presenting at 24 to 33 weeks, 50 mg of sildenafil was given every 8 hours [113]. Women taking sildenafil had a longer interval between diagnosis and delivery, of about 4 days, than the women taking the placebo. The women taking sildenafil also had better acute blood pressure control, with less need for escalation of antihypertensive treatment. While the lower resistance in uterine and umbilical arteries in the women taking sildenafil group hinted at improved uterine perfusion no difference in neonatal outcomes were observed. The dose of sildenafil is likely to be important because in a dose escalation trial, starting at 20mg three times a day (TDS) rising to 80mg TDS, while blood pressure was better controlled there was no significant prolongation of pregnancy [115]. The women in the latter study were also treated at a more advanced gestation and so the lack of pregnancy prolongation may have related to a lower threshold for delivery.

The biggest concern with sildenafil as an adjuvant therapy is its effect on the fetus. In addition to being assessed as a therapy for preeclampsia it has been recently evaluated as a therapy for fetal growth restriction. A series of randomised trials, specifically planned and designed to be run separately but to share outcome data collectively, called STRIDER have been reporting [116]. Neither the UK nor the Australian and New Zealand STRIDER trials found that sildenafil was at all beneficial in improving perinatal outcomes. Nor did they observe any reduction in rate of preeclampsia in women with fetal growth restriction randomised to sildenafil. However, most recently the Dutch STRIDER was stopped early because an interim safety analysis revealed a higher rate of fetal deaths in the treatment arm. The Canadian trial was stopped in response to the Dutch findings [117]. Interestingly, a large animal study of sildenafil in fetal growth restriction had raised concerns of fetal harm [118]. Despite the apparent safety in the UK and New Zealand trial, albeit with no beneficial effects, it is now most unlikely that clinicians will have the appetite to continue exploring sildenafil as an adjuvant therapy, whether for preeclampsia or fetal growth restriction.

L-arginine, is a direct vasodilator that acts as an early substrate in the formation of endothelial NO, thereby inducing vessel relaxation. While it has been shown to reduce blood pressure in women with early-onset preeclampsia it did not appear to prolong pregnancy or improve either maternal or fetal outcomes [119,120]. Perhaps L-arginine will find a more effective role in the primary prevention of preeclampsia, like aspirin [11]. When taken as a nutritional supplement from early pregnancy onwards by women at high risk of preeclampsia the incidence of preeclampsia was significantly reduced [121].

Metformin, an oral hypoglycaemic agent, primarily used as a treatment of type-II diabetes or gestational diabetes [122], has attracted attention as a potential therapy for preeclampsia [123]. Though metformin may have preventative utility [124] it has yet to be investigated as an adjuvant to the acute management of preeclampsia.

### 4.2. Targeting oxidative stress

Following the observation that oxidative stress was a likely common pathway underlying the maternal endothelial dysfunction of preeclampsia [112,125,126] there was much interest in the therapeutic potential of antioxidants [127–129]. While the results of a small pilot study of vitamin C and E were promising [127], excitement was soon abated by larger trials that were profoundly negative [130,131] . Indeed, the lack of benefit of vitamin C and E as preventative therapies – not adjuvant therapies for established preeclampsia – largely turned attention away for antioxidant therapies and has stalled progress. Nonetheless, there are other promising adjuvant therapies that primarily act as antioxidants.

It has been long known that women with established pre-eclampsia are, on average, relatively selenium deficient [132]. A selenium deficient rat model expresses a preeclamptic phenotype [133] and selenium supplementation reduces cardiovascular oxidative stress implicated in hypertension [134]. Amongst other biological roles, such as thyroid hormone production, selenium is a sulphur analogue that reduces production of reactive oxygen species. It is a key component of glutathione peroxidase, and other ROS scavenging peroxidases and is essential to cellular and vascular health [135]. Promisingly, selenium given to women at increased risk of preeclampsia decreased circulating placental derived sFlt-1 [136], an antiangiogenic compound gaining recognition as a potential marker, and possible cause, of preeclampsia [137]. When given from early pregnancy, in a preventative manner, selenium supplementation can reduce the incidence of preeclampsia and pregnancy induced hypertension [138,139]. Whether selenium is useful in the acute setting, as an adjuvant therapy, has not yet been assessed. Excessive selenium can, however, cause toxicity (hepatotoxicity, gastrointestinal disturbance, hair and nail loss, fatigue, and mood disturbance) [135,140,141], may be teratogenic [140,142] and can impair neurological function [143].

Naturally occurring phytonutrients are gaining increasing attention for their antioxidant capacity and as safe treatment options for preeclampsia [144]. Unlike the 'passive' antioxidants vitamin C and E, phytonutrients act by enhancing endogenous antioxidant systems, recruiting numerous pathways and enabling gene amplification. For example, resveratrol, a natural polyphenol, is a potent inducer of antioxidant-coding genes, specifically the nuclear factor erythroid-like factor 2 antioxidant response element (NRF2/ARE), thereby enabling transcription and production of intracellular antioxidant and phase II enzymes [145,146]. When taken in addition to nifedipine by women with early onset pre-eclampsia, resveratrol afforded better blood pressure control, reduced the need for antihypertensive escalation and increased the interval between hypertensive crises [145]. While not designed to assess safety in detail, there were no adverse maternal or neonatal effects attributable to resveratrol in this study [145]. These early data certainly merit further assessment of resveratrol as an adjuvant therapy.

Similarly, melatonin, best known for its role in regulating circadian rhythm [147], has antioxidant properties through NRF2/ARE activation, mechanisms that mimic resveratrol [105]. By activating antioxidant regulatory genes, melatonin enhances expression of antioxidant response enzymes [148] and decreases placental and vascular inflammation [149,150], reducing high blood pressure [151]. Most recently, melatonin, as an adjuvant therapy, appeared effective in safely prolonging pregnancy for women in the acute setting of preeclampsia [105]. Importantly, adjuvant use of melatonin reduced maternal blood pressure, reduced the need for escalation of antihypertensive therapy and increased the diagnosis to delivery interval by a mean of six days [105]. Though modest, this prolongation of pregnancy provides support for antioxidant adjuvant therapy in the emergency management of preeclampsia.

Esomeprazole is a proton-pump inhibitor that has an extensive safety track record in pregnancy [152,153]. Serendipitously, it was observed that esomeprazole can reduce placental production of antiangiogenic compounds, such as sFlt-1, and afford endothelial protection *in vitro* through anti-oxidant pathways, resulting in vasodilation and lower blood pressure *in vivo* in a mouse model of preeclampsia [154]. As with other proton-pump inhibitors, the antioxidant effect of esomeprazole likely relates to endogenous activation of antioxidant pathways, specifically the NRF2/ARE system, and production of downstream antioxidants [155]. It was rapidly translated to a clinical trial as an adjuvant therapy in women with early onset preeclampsia [156]. While esomeprazole appeared to be associated with a decreased risk of placental abruption, it did not alter maternal circulating levels of antiangiogenic factors nor did it prolong pregnancy [157]. In the absence of dose escalation trials, the authors suggested a lack of clinical effect may relate to inadequate circulating levels of esomeprazole [157]. Given the promising *in vitro* work, further dose-response studies may be of value in investigating the efficacy and effectiveness of esomeprazole as an adjuvant therapy for preeclampsia.

## 5. Conclusion

Antihypertensive therapy is, and will remain, the central component in the emergency management of severe preeclampsia, reducing maternal morbidity and mortality. Magnesium sulphate is the sole drug of choice in the prevention and treatment of eclampsia. In the low resource setting magnesium sulphate is particularly valuable, being safe and effective. In the high resource setting, where, because of better access to care and better disease surveillance, the risk of eclampsia is much less there are opportunities to better rationalise the use of magnesium sulphate, limiting its use to only the most severe cases and to those women who have already fitted. The role of novel therapies to enable a targeted and disease specific approach to management will likely be the focus of future research into improving maternal and perinatal outcomes in preeclampsia.

## 6. Expert opinion

Genuine advances in the field of preeclampsia research are beginning to deliver meaningful improvements in maternal and perinatal outcomes the first time in decades. Importantly, these advances have been based on a better understanding of the pathogenesis of preeclampsia, specifically the role of placental dysfunction underlying the excessive release of angiogenic agents that induce maternal endothelial dysfunction and end-organ failure. In turn, these insights have led to (i) better prediction, (ii) improved prevention, (iii) better diagnosis and management, and (iv) the promise of future therapies.

### 6.1. Better prediction

*A* large body of research has been undertaken to develop sensitive and specific predictive algorithms that can be offered to all women in early pregnancy to identify those at high risk of developing preeclampsia. These algorithms have been particularly effective at the prediction of early-onset or severe preeclampsia [10,158,159] and now offer sufficient accuracy to be the basis of offering preventative therapy such as aspirin. However, routine screening of all women in early pregnancy is yet to be endorsed by learned Colleges and

government. This lag in policy is likely to cost lives through the lack of provision of preventative therapy.

### 6.2. Better prevention

The World Health Organisation endorses three approaches to prevent preeclampsia: low dose aspirin, calcium supplementation for those who are deficient, and tight blood pressure control for women with hypertension [5]. If started early in pregnancy, before 16 weeks gestation, low dose aspirin reduces the rate of early-onset preeclampsia by about 60%. Whether the addition of other agents, such as metformin [119,120], to low dose aspirin improves this further remains the focus of eagerly awaited on-going clinical trials. The prospect of eradicating early-onset preeclampsia through screening-targeted prevention is, finally, a realistic one.

### 6.3. Better diagnosis and management

In addition to sensitive and specific screening in early pregnancy, the ability to determine which women presenting with hypertension in later pregnancy will progress to severe disease has remained a challenge. Again, informed by an improved understanding of the role of angiogenic factors in the progression of the disease, improved triaging of those women at risk of severe disease is now a reality [156,159–162]. Specifically, an increased ratio of soluble FMS-like tyrosine kinase-1: placental growth factor (sFlt-1:PlGF) is used to identify the women most likely to progress to severe disease. This will allow more timely management with the appropriate antihypertensives – usually labetalol or nifedipine – and consideration of the need for escalated care. Such triaging offers the promise of better targeted care, and so better outcomes, an and significant cost savings [160]. Indeed, the routine use of sFlt-PlGF as a triaging tool in women presenting with possible preeclampsia is now recommended standard care in a number of countries including Germany and the UK [163]. Despite this, there is yet to be widespread uptake. As with early pregnancy screening, policy is significantly lagging evidence of best practice.

There are also likely to be opportunities to further improve the performance of biomarker triaging in women presenting with hypertension in pregnancy. Circulating levels of other angiogenic factors, such as activin and follistatin, are altered in women with preeclampsia [164] and these factors are known to play a central role in the resultant maternal endothelial damage [165–167]. Future studies should address the potential of these markers to add to the screening performance of sFlt-1 and PlGF.

### 6.4. The promise of future therapies

The future of the management of preeclampsia is focussed on safely prolonging pregnancy to improve maternal and perinatal outcomes. In this, it is likely that antihypertensive therapy has taken us as far as it can. Excitingly, the advances in our understanding of the pathological progression of preeclampsia now allow the development of disease pathway specific therapeutics. It is increasingly accepted that only by moving beyond solely controlling blood pressure to protect the maternal endothelium and organ function can we slow disease progression and safely prolong pregnancy [9]. Such an approach promises to afford improved fetal maturation and reduced morbidity associated with premature delivery [5]. Perhaps by adopting this upstream approach we can even reduce the long term cardiovascular and renal morbidity associated with preeclampsia. Of particular interest is the role of antioxidants in the management of preeclampsia. While endeavours to investigate targeted therapies and antioxidants have yet to yield practice-changing improvements in maternal and perinatal outcomes, there are many promising therapies on the near horizon [145,146,148]. Of particular interest will be compounds derived from plants such as green vegetables [144–146] and pomegranate [168]. What will be important to the timely evaluation of these new therapies will be to ensure that the pharmacokinetics are undertaken in pregnant women.

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# Chapter two

## *Melatonin for the management of preeclampsia: a review*

In the closing paragraphs of my first literature review I summarised the landscape of literature on future therapies for preeclampsia. From this, antioxidants stood out as one of the most promising approaches for disease specific and targeted treatment of preeclampsia. With an increased understanding of the role of oxidative injury in the placenta and consequently in maternal organs and blood vessels over the last twenty years, research has turned towards antioxidants as a possible therapy for preeclampsia. Though vitamin C and E proved to be unsuccessful therapies [50,51], scope remains for novel and alternate mechanisms of antioxidant therapy[51,52]. In particular, inducers of the nuclear factor E2-like related factor 2 (NFE2L2) antioxidant pathway have shown some, though limited results[53–55].

Nuclear factor E2-related factor 2 is an endogenous inducer of cellular antioxidants[56,57]. In physiological conditions, bioavailable levels of NFE2L2 remain consistent through cytosolic binding from KEAP-1, which prevents rapid proteasome degradation of NFE2L2[58,59]. Exposure to oxidative stress induces cysteine modifications within KEAP-1, allowing NFE2L2 to translocate to the nucleus[59,60]. By combining with small maf-proteins in the promoter region of antioxidant "safeguarding" genes, NFE2L2 stimulates the antioxidant response element (ARE) resulting in transcription of mRNA for a number of cellular antioxidants and phase two enzymes[56]. Namely, NADPH dehydrogenase (quinone) 1 (NQO1), glutathione S-transferase (GST) and the antioxidant heme oxygenase-1 (HO-1), rise in response to artificial administration of NFE2L2. Numerous studies have shown therapeutic benefits from NFE2L2 stimulation in maintaining endothelial health[53,57,61] and in the placenta[53,62,63].

At the time these PhD studies began, melatonin was under investigation by our group as a promising adjuvant therapeutic for preeclampsia. Best known for its role in modulating circadian rhythm, melatonin also has potent antioxidant activity via NFE2L2 activation. Melatonin reduces placental and endothelial dysfunction *in vitro* and, in a pilot study, clinically prolonged the diagnosis to delivery interval in women with preeclampsia[64–66]. Melatonin, and similar antioxidants, offer exciting possibilities for novel targets for the management of preeclampsia[55,67]. For this reason, and because prior to these studies there was no literature on sulforaphane in preeclampsia, this thesis includes a review the existing literature surrounding melatonin as a possible therapy for preeclampsia. Towards the end of the review opportunities to explore other antioxidants such as sulforaphane are raised, foreshadowing the work that, over three years of experimentation, form this thesis.

antioxidants

*Review*

# Melatonin for the Management of Preeclampsia: A Review

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**Abstract:** Preeclampsia is a disease specific to pregnancy characterised by new-onset hypertension with maternal organ dysfunction and/or fetal growth restriction. It remains a major cause of maternal and perinatal morbidity and mortality. For sixty years, antihypertensives have been the mainstay of treating preeclampsia and only recently have insights into the pathogenesis of the disease opened new avenues for novel therapies. Melatonin is one such option, an endogenous and safe antioxidant, that may improve the maternal condition in preeclampsia while protecting the fetus from a hostile intrauterine environment. Here we review the evidence for melatonin as a possible adjuvant therapy for preeclampsia, including in vitro evidence supporting a role for melatonin in protecting the human placenta, preclinical models, vascular studies, and clinical studies in hypertension and pregnancy.

**Keywords:** preeclampsia; melatonin; antioxidant; placental biology; mitochondrial function



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## 1. Introduction

Preeclampsia is a systemic maternal-fetal disorder specific to human pregnancy. It is characterised by new-onset hypertension after twenty weeks gestation and other end-organ damage, such as renal or liver impairment, haematological involvement, neurological excitability and/or fetal growth restriction. Preeclampsia is the leading cause of preterm delivery and is often accompanied by fetal compromise, in particular impaired fetal growth [1]. In addition to this, the knowledge that underlying preeclampsia is a dysfunctional placenta is why fetal growth restriction is increasingly included in definitions of preeclampsia [1].

Regarding placental dysfunction, impaired placental perfusion causes chronic and worsening hypoxic-reperfusion injury to the placenta across pregnancy. This injury underlies the excessive release of antiangiogenic factors into the maternal circulation [2,3]. In turn, these factors cause widespread maternal endothelial dysfunction that leads progressively to increased maternal systemic vascular resistance and activation of the maternal coagulation and immune systems [4,5]. If left untreated, this progressive vascular dysfunction leads to dangerously high blood pressures and stroke, systemic organ failure, and cerebral oedema and seizures. While many women who develop preeclampsia, particularly in the setting of late-onset disease, will have good outcomes, this condition remains the leading cause of maternal death and morbidity and a major cause of preterm birth that accounts for significant perinatal mortality and mortality worldwide [6–8]. Globally, preeclampsia accounts for nearly 75,000 maternal deaths and 700,000 neonatal deaths annually. Even in high-resource settings, preeclampsia accounts for upward of 15% of maternal deaths [6–8]. After delivery, the burden of disease for preeclamptic mothers continues. Women who have suffered preeclampsia during their pregnancy have an increased chance of developing cardiovascular compromise and ongoing related morbidity throughout their lives. This is likely due to, at least in part, residual endothelial dysfunction from significant vascular stress during the pregnancy period [9,10]. Similarly, the effects of preeclampsia on the fetus may be much more pronounced than those attributed to preterm delivery and growth

restriction. Epigenetic priming means that babies born to preeclamptic mothers are at an increased risk of a host of metabolic conditions throughout their lives [11,12]. So, while generally considered a maternal disease, preeclampsia at its core is a placental disease that imposes disease burden on both mother and fetus. Therapies targeting preeclampsia need to consider both patients.

In that regard, the management of preeclampsia depends on the gestation at onset, the severity—both maternal and fetal—and the rate of progression [1]. However, the ultimate treatment of preeclampsia is removal of the offending organ—the placenta. This is why preeclampsia remains a leading cause of prematurity because delivery of the placenta requires, of course, delivery of the fetus. Timing delivery is then a balance between the interests of the mother and the interests of the fetus. The interests of the woman with preeclampsia are always best served by delivery. Delivery prevents worsening hypertension and thereby avoids related complications including stroke, liver failure and kidney failure. However, early delivery may not best serve the baby, particularly if very preterm. Beyond neonatal demise, preterm delivery is a risk factor for a host of conditions that will affect a baby well into their adult life, including cerebral palsy, visual and hearing problems, respiratory difficulties, cardiovascular compromise, renal impairment, and learning and behavioural problems [13]. Even late preterm delivery (<37 weeks) increases the risk of lifelong cardiovascular and renal complications [14,15]. In this way, iatrogenic premature birth, though often necessary to save the life of the mother, also may come at significant cost to the baby [13]. So, at very early gestations, to offset those fetal risks, the maternal health risks are mitigated by controlling blood pressure with antihypertensives. This allows safer (for the mother) prolongation of the pregnancy to improve fetal maturity and reduce risks of neonatal mortality and morbidity [16].

Thus, antihypertensive treatment manages the high blood pressure to reduce maternal risks, particularly of stroke. This has been very successful. The use of antihypertensives in women with preeclampsia has greatly reduced rates of both maternal morbidity and mortality worldwide. However, antihypertensive treatment does not treat the underlying disease. Nor does it slow disease progression, although there is some debate about that. Its effect is very much limited to managing high blood pressure to reduce attendant maternal risks. That is not to say that antihypertensive treatment is without complication for both mother and baby, and the antihypertensive of choice remains contentious. Indeed, improvements over recent decades in maternal and perinatal outcomes in pregnancies complicated by preeclampsia have come mostly from the ability to deliver earlier than was previously possible, safe in the knowledge that better neonatal care has resulted in better outcomes for the preterm infant. Antenatal corticosteroids aside, there have been no real advances in the obstetric care of the woman with preeclampsia since the introduction of antihypertensives in the 1950s. Pharmacologically, the management of preeclampsia has been largely "treading water" [17].

However, this may be all set to change. Recent insights into the mechanisms underlying the maternal aspects of preeclampsia have offered the long-awaited promise of new treatments [17]. In particular, the recognition that maternal endothelial dysfunction due to placental vasoactive peptides [2,18] is a central feature of the disease has offered the promise of new, targeted therapies that might address the central causes of the hypertension rather than the hypertension per se [16,17]. Pathogenic mechanistic insights have led to the recognition that we must look to therapeutic approaches beyond antihypertensives if we wish to tackle the underlying disease processes that drive preeclampsia. Such developments have revolutionised research for preeclampsia and, for the first time in sixty years, soon we may be able to offer novel medical management to pregnant women with preeclampsia and their babies.

## 2. The Search for Novel Therapies

In 1989, Jim Roberts and colleagues suggested that preeclampsia might be due to widespread maternal endothelial dysfunction [19]. For the first time, a mechanism that

might explain the majority of the clinical features of the syndrome, not just the hypertension, had been proposed. In essence, Roberts suggested that disturbed vascular function, including altered tone and permeability, was the cause of hypertension, peripheral and cerebral oedema, and proteinuria [19,20]. The other features of preeclampsia such as liver injury, renal injury, thrombocytopenia and, ultimately, eclampsia itself were also thought to reflect progressive endothelial dysfunction across diverse target organs [19] (Figure 1). While our understanding of preeclampsia has evolved somewhat since then, Roberts' hypothesis was important because the recognition that endothelial dysfunction was a key mechanism underlying preeclampsia triggered the need to identify the cause(s) of that endothelial disturbance. It gave rise to the concept that preeclampsia is a two-step process: impaired placentation leading to progressive placental ischemia-reperfusion and oxidative injury, in turn causing the excessive release of vasoactive factors into the maternal circulation that induced endothelial dysfunction [4]. While not explaining all aspects of preeclampsia, it remains generally accepted that the maternal syndrome of preeclampsia is due, by and large, to widespread vascular endothelial dysfunction [20], and that the endothelial dysfunction was caused by substances released by a chronically injured placenta. The next significant advance was the identification of the candidate vasoactive substances that were causing the dysfunction. Three antiangiogenic agents have been proposed as major contributors—the soluble splice variant of the fms-like tyrosine kinase receptor-1 (sFlt1), the soluble cleavage product of the transforming growth factor (TGF-β1) coreceptor endoglin (sEng) and the proinflammatory cytokine member of the TGF-β super family activin A [2,5,21–23].

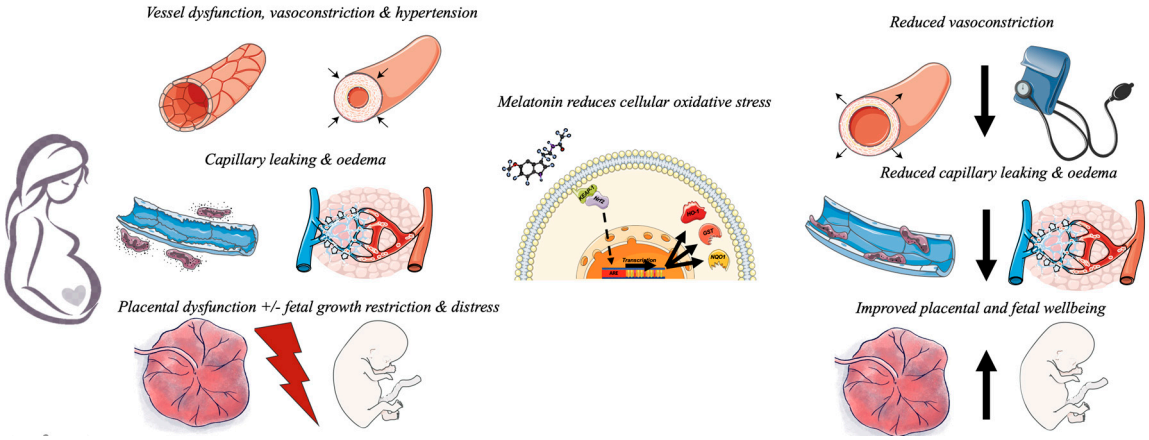


**Figure 1.** Preeclampsia involves widespread vascular dysfunction resulting in peripheral vasoconstriction which manifests as maternal hypertension. Capillary leakage results in oedema and impaired placental oxygenation results in placental dysfunction and fetal distress with, or without, fetal growth restriction. Melatonin is an antioxidant that reduces oxidative stress within the cells of the placenta and vasculature. Melatonin releases Nrf2 from intracellular binding by KEAP-1 allowing it to translocate to the nucleus of the cell. Here, Nrf2 activates the antioxidant response element of "safeguarding genes" resulting in transcription and translation of a number of antioxidant proteins. These proteins induce redox reactions to neutralise excessive intracellular reactive oxygen species that would otherwise cause damage to DNA and protein production essential for cell function. This allows melatonin to improve vascular function reducing vasoconstriction, reduce capillary leakage and improve placental function. Melatonin may also directly protect the developing fetal brain.

Circulating levels of both sFlt1 and sEng are many fold higher in women with preeclampsia than in those with a healthy pregnancy and levels correlate with disease severity [22,24]. Further, experimentally the administration of either, or both, sFlt1 and sEng to rodents induces many features of human preeclampsia including maternal hypertension, proteinuria, glomerular endotheliosis, thrombocytopenia, and elevated liver enzymes [22,25–27], as does activin [23]. Removal of sFlt1 by plasmapheresis also temporarily moderates the severity of hypertension in women with preeclampsia [28]. Hypoxic

insult induces post-transcriptional alternate splicing of mRNA for the membrane receptor Flt-1, to lose the transmembrane and intracellular signaling components of Flt-1 while preserving the extracellular ligand binding site [29]. In contrast, hypoxia directly triggers post-translational cleavage of the endoglin protein membrane receptor into its soluble form [22], which competitively binds TGF-β1. It is thought that sFlt1 and sEng induce endothelial dysfunction by either sequestering or antagonising pro-angiogenic factors that are vital for normal endothelial health such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) and TGF-β respectively. These three key proangiogenic compounds are essential for maintaining blood vessel integrity by inducing phosphorylation, and hence activation of nitric oxide (NO), a potent vasodilator essential in facilitating endothelial relaxation [30]. When competitive binding by sFlt-1 and sEng makes VEGF, PlGF and TGF-β1 unavailable, progressively worsening vascular dysfunction characteristic of preeclampsia ensues.

Similarly, circulating maternal levels of activin are 10–20-fold higher in women with preeclampsia compared to those with a healthy pregnancy, secondary to increased placental production driven by oxidative stress [31–33]. Much like sFlt and sEng, activin is antiangiogenic [34] and has been shown to inhibit endothelial proliferation and disrupt endothelial integrity in vitro [35,36]. Activin A binds to activin receptor II (ARII), which combines with type-1 activin receptor like kinase-4 (ALK-4) resulting in phosphorylation and nuclear translocation of post-receptor transcription factor Smad2/3 [23,36,37]. Excess activation of this pathway induces NADPH Oxidase 2 (Nox2) signaling and results in cellular accumulation of superoxide species and adhesive molecules. As with sFlt and sEng, the increased levels of activin in a preeclamptic woman lead to further vascular dysfunction, permeability and oedema, exacerbating the clinical syndrome of preeclampsia [23,31,38,39]. Activin also stimulates the release of endothelin, a potent vasoconstrictor, from the endothelium [40], consistent with it being able to cause hypertension. As would be required for activin to have direct effects on the endothelium, endothelial cells express both type I and II activin receptors and in late pregnancy activin itself can be immunolocalized to both the maternal and fetal vascular endothelium [41,42]. The likely cause of these increased activin levels in preeclampsia is placental oxidative stress, a key feature of the disease [23,32]. Of course, increased levels of activin in women with preeclampsia and possible placental mechanisms underlying those increased levels do not, by themselves, tease apart cause and effect. However, circumstantial evidence that excess circulating activin may indeed have a causative role is offered by the observation that in women who subsequently develop preeclampsia levels of activin are significantly increased as early as 8–13 weeks of pregnancy, many months before the clinical onset of hypertension [43]. It is also intriguing that levels of activin are increased in women with gestational diabetes, a condition with an increased incidence of preeclampsia [44]. In short, the result of the imbalance between these three antiangiogenic and pro-angiogenic factors such as PlGF and follistatin is increased endothelial oxidative stress—the likely final pathway underlying the systemic maternal endothelial dysfunction.

Not surprisingly, attention has now turned to therapies that might prevent the release of sFlt1, sEng or activin A, or perhaps more importantly, antagonize their antiangiogenic effects. Given that oxidative stress both increases the release of these antiangiogenic factors [32,40,45,46] and is a major mechanism by which they exert their damaging effects on vascular endothelium suggests that targeting oxidative stress, both within the placenta and in the maternal endothelium, may be an effective therapeutic approach [47–49]. In this regard, melatonin, an endogenous hormone, known to be safe in pregnancy and with potent antioxidant capacity is a promising agent [50].

## 3. Melatonin in Normal Pregnancy and Preeclampsia

Melatonin (5-methoxy-N-acetyltryptamine) is produced primarily by the pineal gland, providing circadian and seasonal timing cues. It is synthesized from serotonin through sequential acetyl transferase, to form *N*-acetylserotonin, and methylation to form mela-

tonin [51]. In addition to cueing the body clock, melatonin is also a powerful antioxidant, acting both directly as a highly effective scavenger of reactive oxygen and nitrogen species itself [52] and indirectly by stimulating a cassette of endogenous antioxidant enzymes including, but not limited to, glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase [52,53].

In human pregnancy, night time, but not daytime, maternal melatonin levels increase with advancing gestation, falling again postpartum [52]. The increasing levels at the end of pregnancy are thought to be important in the diurnal "training" of fetal physiology and behaviour. This sequential rise in melatonin across pregnancy may also play a role in stimulating labour, and melatonin has been proposed as a therapeutic adjuvant for induction of labour [54]. Intriguingly, night time levels of melatonin are lower in women with severe preeclampsia than in those with a healthy pregnancy [55]. In fact, the degree of night time melatonin deficiency correlates with preeclampsia disease severity [56]. The majority of circulating melatonin in pregnancy is thought to be of maternal pineal origin [51], much as it is outside of pregnancy. However, recent studies have identified that the expression of the two melatonin-synthesizing enzymes, aralkylamine *N*-acetyltransferase and hydroxyindole O-methyltransferase, and the two melatonin receptors, $MT_1$ and $MT_2$, are reduced in the preeclamptic placenta compared to healthy placentae from normotensive women [51]. As such, impaired placental production may underlie the reduced maternal levels of melatonin in women with preeclampsia and the normal biological effects of melatonin within the placenta may be reduced in preeclampsia. It is plausible that impaired melatonin activity, and therefore impaired endogenous antioxidant defenses, in the preeclamptic placenta contributes to the oxidative stress central to this disease. As such administration of melatonin to women with preeclampsia may reduce placental oxidative stress, reduce the production of sFlt1, sEng and activin, and improve placental function. Melatonin may also improve maternal endothelial function both directly, via endothelial melatonin receptors [57], and indirectly by reducing circulating levels of sFlt1, sEng, and activin A.

The antioxidant effects of melatonin have historically been attributed to activation of nuclear factor erythroid-like factor-2 (Nrf2), an endogenous inducer of cellular antioxidants. During homeostatic conditions the Nrf2 protein is bound to Kelch-like ECH associated protein 1 (KEAP-1) within the cytosol of the cell [58]. This process prevents proteosomal degradation of Nrf2 to ensure abundant Nrf2 remains ready to translocate to the nucleus of the cell during times of increased cellular oxidative stress. Oxidative stress triggers ubiquitination of KEAP-1 through directly modifying cysteine components of the protein structure, releasing Nrf2 into the cytosol of the cell [59]. In the nucleus Nrf2 binds small maf-proteins in the promotor region of the antioxidant response element of so-called safeguarding genes within the cell nucleus [59]. This stimulates increased transcription and translation of a host of antioxidant enzymes and phase two enzymes, namely NADPH dehydrogenase (quinone) 1 and glutathione S-transferase and the antioxidant heme oxygenase-1 [59]. These enzymes then undergo a series of redox reactions within the cytosol of the cell to neutralise, or "scavenge", damaging oxygen free radicals [60]. When the amount of damaging reactive oxidative species (ROS) overwhelm the capacity of this inbuilt antioxidant rescue mechanism, oxygen free radicals directly alter protein structure and damage cellular DNA, producing abnormal spliced proteins such as sEng and sFlt-1 and triggering inflammatory cascades to release cytokines such as activin A [60]. For good reason, stimulators of the Nrf2 pathway, such as resveratrol and sulforaphane, have received much attention for their impressive antioxidant capacity, and ability to both maintain endothelial health [49,61–63] and protect the placenta [48,49,64]. In fact, further evidence supports a role for the KEAP-1-Nrf2 pathway in protecting the fetus against complications from epigenetic priming that arise from a pregnancy overwhelmed by oxidative stress [11,12,65].

Not surprisingly given its antioxidant properties, melatonin has been shown to reduce placental production of antiangiogenic compounds from term placentae in vitro [47,66,67].

Melatonin also reduces trophoblastic debris from early trimester placentae exposed to preeclamptic serum [68]. In placental explants, melatonin reduced markers of oxidative stress induced by the superoxide generator xanthine/xanthine oxidase and increased production of Nrf2 and the downstream antioxidant enzyme heme oxygenase-1, suggesting the Nrf2 pathway was, at least in part, responsible for this effect [47]. Although melatonin did not alter cell production of markers of endothelial activation in endothelial cells, it did prevent disruption to the cell monolayer [47]. In isolated trophoblast cells, melatonin significantly decreased secretion of sFlt-1 [67]. While these studies did not identify a reduction in antiangiogenic compounds from placental explant tissue, the dose of melatonin used was equivalent to that in cell culture experiments. It is likely that higher doses are needed to penetrate explant tissue and induce a measurable effect. Further evidence of the endothelial protective capacity of melatonin was identified when melatonin prevented a rise in intracellular cell adhesion molecule-1 (ICAM-1) from endothelial cells exposed to trophoblast debris serum [66]. Melatonin also prevented a rise in nitrotyrosine in these placental explants exposed to preeclamptic serum [66].

An additional pathway by which melatonin mitigates placental oxidative stress may well begin in the organelles most reliant on oxygen supply and thus most affected by hypoxia: the mitochondria [69,70]. A role for disturbed mitochondrial dysfunction in preeclampsia was first recognised in the 1990s [71]. Mitochondrial dysfunction after hypoxic reperfusion injury is now accepted as the driver for ROS build-up in severe disease [72–76]. Mitochondria are responsible for multiple functions, including respiration and production of cellular energy, homeostatic regulation of ROS and the intrinsic pathway of apoptosis. Mitochondria form a dynamic network within the cell and constantly undergo repeated fission and fusion events whereby multiple mitochondria fuse to a large single mitochondria (fusion) or split into multiple smaller mitochondria (fission) [77–79]. The balance of this process is essential for cellular homeostasis and ensures maintenance of healthy mitochondrial structure with stability of the matrix membrane which hosts the five complexes of the electron transport chain [78]. Low oxygen tensions, as in preeclampsia, induce abnormal fission and fusion dynamics, generating small mitochondria with low motility [80]. Hypoxic insult induces mitochondrial permeabilization and breakdown and, when extensive, can initiate pathways of mitophagy and intrinsic cellular apoptosis [75]. The mitochondrial electron transport chain, essential for production of ATP for cellular energy, is reliant on the presence of oxygen for oxidative phosphorylation of ADP into ATP [81]. During respiration, electrons move through a series of complexes, allowing the formation of a proton gradient in the intermembrane space which then allows passive diffusion of hydrogen ions through the final complex ATP synthase where ADP is phosphorylated into ATP [80,81]. Without oxygen to act as an electron accepter, as occurs in an oxygen starved-preeclamptic placenta, electrons are unable to flow along the electron transport chain and instead leak into the matrix space [82]. Here, electrons react with oxygen to form charged superoxide species: the damaging ROS of preeclampsia. To a degree, this process occurs even in the presence of oxygen to allow homeostatic regulation of the ROS necessary for physiological cellular processes [82]. However, when ROS formation exceeds antioxidant enzyme production, the electron transport chain becomes a source of the very ROS that drive placental antiangiogenic protein formation in preeclampsia [82]. Indeed, mitochondrial function is disturbed in preeclampsia [71,72,83] suggesting that targeting the mitochondria offers an attractive therapeutic option to reduce oxidative stress in this disease. Again, melatonin appears a suitable candidate for that therapy [70,84,85].

Melatonin is highly expressed in the mitochondria of placental trophoblasts [86,87] and is responsible for the production of key antioxidant enzymes in this organelle [70]. Indeed, melatonin is known to protect mitochondrial function [88], reduce electron leakage and ameliorate ROS production of the electron transport chain [89,90], particularly in the face of hypoxic-reperfusion injury [84,91,92]. In the placenta of obese women, placentae characterised by trophoblastic ROS production similar to that of preeclampsia, melatonin significantly improves the function of the electron transport chain [93]. Specifically, in the

term placental syncytiotrophoblast of obese women, melatonin improves the spare respiratory capacity, a marker of cellular reserve, an important feature for placentae exposed to oxygen deficient environments [93]. Melatonin also increases the maximal respiration and correspondingly reduces the placental production of superoxides. These findings indicate that melatonin may act as a protective buffer for the placental mitochondrial electron transport chain against the damaging effects of hypoxic-ischaemic reperfusion injury and the resultant superoxides. Whether melatonin exerts this action via antioxidant effects, such as activation of the Nrf2 antioxidant response element pathway, or by directly modulating the components of the electron transport chain remains unclear. This is certainly worthy of study. However, a similar compound to melatonin, sulforaphane, has action at the level of the mitochondria and this appears to be via potent Nrf2 antioxidant activity as well as by modulating mitochondrial fission and fusion and the complexes of the electron transport chain [94]. It would be worth investigating the capacity and mechanisms of action of melatonin in improving mitochondrial function in preeclamptic placentae or suitable in vitro hypoxic-reperfusion models of injury.

In addition to its beneficial effects in the placenta, melatonin also has reparative actions on the endothelium. Studies of melatonin on vascular cells have primarily focussed on human umbilical vein endothelial cells (HUVECs). In these cells, a microarray analysis demonstrated that melatonin significantly modulates expression of genes involved in apoptosis, cell differentiation and proliferation [95]. In an in vitro hypoxia-reoxygenation model in endothelial cells, melatonin treatment prevented hypoxic—reperfusion injury by preventing a rise in ROS and corresponding impaired cell migration and proliferation in a dose-dependent manner, without negatively affecting cell viability [96]. More recently, melatonin has been shown to have anti-ROS activity in toxic environments of oxidative stress and hypoxia, with reduced endothelial cell proliferation and tube formation [97]. These studies provide supporting evidence that melatonin displays antiangiogenic effects by suppressing the proliferation of endothelial cells, an effect achieved by the downregulation of hypoxia inducible factor 1$\alpha$ (HIF-1$\alpha$), ROS and vascular endothelial growth factor. Suppressing these pathways is an important step in ameliorating the excessive production of the sEng and sFlt-1 seen in the preeclamptic placenta.

Though melatonin certainly freely crosses the placenta, this is not a concern. In fact, melatonin has also showed promise for the management of fetal growth restriction (FGR), a condition that goes hand in hand with preeclampsia and can be, broadly, viewed as the fetal manifestation of placental insufficiency, much as preeclampsia is the maternal manifestation. Evidence from animal models of impaired placentation and FGR support a role for melatonin in improving placental function and fetal outcomes. For example, following early pregnancy nutritional restriction in the sheep, oral maternal administration of melatonin improved uteroplacental blood flow—both uterine and umbilical—and fetal weight [98]. Melatonin is also able to protect the fetal brain and normalize early neurodevelopment in a fetal sheep model of FGR using umbilical cord occlusion [99]. Improved fetal growth was also afforded by maternal melatonin in both a nutritionally restricted rat model of FGR [100] and in a rat model where FGR was imposed by transient occlusion of the utero-ovarian arteries in mid-pregnancy [92]. In each of these models the administration of melatonin was associated with decreased placental oxidative stress and increased antioxidant enzymes [65,98,100]. Studies of melatonin for FGR have shown that oral melatonin taken by women during pregnancy crosses the placenta into the fetal circulation where it may protect the fetal brain from the harmful effects of oxidative stress [101]. In a pilot FGR trial where 8 mg melatonin per day was given to 12 women with severe FGR, a 200-fold increase in both maternal and fetal melatonin levels without maternal or fetal adverse effects was seen [102]. The clinical utility of melatonin as a neuroprotective therapy to improve neonatal outcomes in the setting of FGR is under ongoing investigation [103]. This neuroprotective feature is an appealing feature of melatonin for conditions of placental oxidative stress such as FGR and preeclampsia, killing two birds with one stone as it were.

In summary, melatonin has beneficial effects both within the placenta and on the maternal endothelium. It improves endothelial cell health by reducing inflammatory activation and antiangiogenic factor secretion [66], most likely through improved mitochondrial function, and increases expression of antioxidant enzymes in trophoblasts [67]. So what is the evidence for melatonin as an antihypertensive?

## 4. Melatonin as an Antihypertensive

Melatonin is an antihypertensive, reducing blood pressure in experimental animal models of hypertension, in healthy individuals, and in patients with established hypertension [7,57,104–106]. In fact in early animal studies, removal of the pineal gland, responsible for endogenous melatonin production, resulted in hypertension [107,108] with exogenous administration of melatonin reversing this vasoactive effect [109]. These initial studies provided evidence that melatonin was involved in cardiovascular regulation and prompted further investigation. Since then, a number of studies have identified that exogenous melatonin modifies blood flow, with effects varying depending on the vasculature.

The mechanisms underlying melatonin's antihypertensive actions remain to be fully elucidated [57], but are likely to involve some or all of: central effects, systemic anti-inflammatory, antioxidant, and lipid lowering effects, direct effects on the myocardium and direct effects on the vascular endothelium [57]. In pregnancy melatonin increases umbilical blood flow in sheep [110], uterine artery blood flow in cows [111] and decreases cerebral blood flow in young rats [112]. These observations are consistent with improved placental function. Interestingly, in a chronic nitric oxide (NO) inhibited rat model of hypertension, melatonin treatment for 5 days significantly reduced basal mean arterial pressure [113]. A chronic intermittent hypoxic rat model induced endothelial dysfunction in the aorta by decreasing relaxation when exposed to the endothelium-dependent vasodilator acetylcholine [114]. This was mitigated by melatonin. Such findings were attributed to an increase in NO availability and increased protein expression of endothelial NO synthase (eNOS) in the aorta [114]. In this study, melatonin also decreased mRNA expression in the aorta of endothelial dysfunction markers including vascular cell adhesion molecule-1 (VCAM-1), ICAM-1 and E-selectin. While useful in understanding the effect of melatonin on the cardiovascular system, none of these animal models were models of preeclampsia. Melatonin is believed to directly activate receptors located on endothelial and vascular smooth muscle cells, and through its antioxidant properties, indirectly modulate vascular tone [57]. In the vasculature, melatonin receptors have conflicting effects, inducing vasoconstriction via the receptor $MT_1$ and vasodilation via $MT_2$, as first demonstrated in isolated rat caudal arteries [115]. It is the relative distribution differences of the melatonin receptors that elicits differential vascular responses in different blood vessels, and often selectively potentiates the vasoconstrictor response to serotonin. For example, in pigs, melatonin appears to reduce vasoconstriction in the coronary artery and increase vasoconstriction in the pulmonary artery, while vasoconstricting the rat coronary artery [116]. In contrast, melatonin induces vasodilation in rabbit aorta, iliac and renal vasculature [117,118] and the rat aorta [119]. Conversely, melatonin increases vasoconstriction in the coronary artery of pigs, but only if serotonin is present [120]. With such variability, the studies of the vascular actions of melatonin have revealed a substantial heterogeneity of effects.

Only recently were the mechanisms behind melatonin-induced vasodilation explored. Now, we understand that melatonin can affect arterial blood pressure and blood flow to tissues and organs by modulating the diameter of the vasculature [57,121,122]. This function likely occurs via directly activating the $MT_1$ and $MT_2$ receptors located on endothelial and vascular smooth muscle cells, and indirectly by its antioxidant properties to effect vascular tone. $MT_1$ and $MT_2$ receptors have been localized to a variety of arterial beds in humans, including the aorta, and coronary and cerebral arteries. Interestingly, melatonin receptors $MT_1$ and $MT_2$ are not expressed in all blood vessels so melatonin only exerts these potent vasoactive effects in specific regions of the vasculature [57]. The role of melatonin in the

cardiovascular system at large has been summarized elsewhere and will not be covered in detail in this review [123].

Evidence that melatonin modifies production of NO is, unsurprisingly, vascular bed specific. In porcine coronary vascular smooth muscle, melatonin inhibited NO-induced increases in cGMP and artery relaxation via the $MT_2$ receptor [124]. Interestingly, conflicting findings also revealed that melatonin exerts neuroprotective effects by suppressing NO production and enhancing activity of the endogenous antioxidant superoxide dismutase following oxidative injury [125,126]. Melatonin also increases NO availability, thereby inducing vasodilation of the mesenteric artery of healthy [127,128] and hypertensive rats [129].

These findings also translated to studies in human blood flow and vascular distribution. After human ingestion, melatonin reduced renal blood flow velocity and vascular conductance, enhanced forearm blood flow and vascular conductance, while not changing middle cerebral artery blood flow [121]. The decrease in renal blood flow could be eliminated by $\alpha$-adrenergic receptor antagonism, indicating that melatonin is augmenting sympathetic outflow to the kidney. The effect on forearm blood flow, and lack of effect on middle cerebral artery blood flow was hypothesised to be due to a difference in melatonin receptor expression. An earlier study also demonstrated a lack of effect after a bolus injection of melatonin on cerebral blood flow, as measured in the basilar artery [130]. Once again, these studies support the concept that the relative distribution of the melatonin receptors influences the vascular effects of melatonin. Furthermore, in patients with three-vessel coronary disease, one month of oral melatonin treatment resulted in decreased plasma levels of VCAM and ICAM, while increasing plasma NO levels, providing further evidence that melatonin can protect against endothelial dysfunction [131].

In addition to increasing NO availability via $MT_1/MT_2$ receptors in endothelial cells, melatonin activates large conductance $Ca^{2+}$-activated $K^+$ ($BK_{Ca}$) channels of smooth muscle cells, the role of which is in relaxation [128]. However, as the effects of melatonin are highly variable depending on the vasculature, the mechanisms explored in the mesenteric artery may not be translated to other vascular beds. It is hypothesised that melatonin, via its receptors, changes calcium and potassium channel regulation, and may also directly activate guanylate cyclase [132]. This is supported by an in vitro study that demonstrated melatonin-induced relaxation was only partially inhibited when NOS, the enzyme responsible for NO production, and guanylate cyclase were blocked [133]. In this chronic NO inhibited rat model of hypertension, melatonin treatment for 5 days significantly reduced basal mean arterial pressure. These rats had been administered the NO synthase (NOS) blocker L-NAME for 14 days, indicating that a mechanism beyond NO must be responsible for the observed antihypertensive effect of melatonin [113]. Though the conflicting nature of data regarding vasoactive effects of melatonin in animal vasculature limits the applicability to human populations, it certainly offers proof of concept that melatonin has the potential to directly modulate the vasculature. These mechanistic explorations may explain the hypotensive effect observed in humans [47,57,104–106].

Recently, a small open-label study sought to assess whether melatonin may allow safe prolongation of pregnancy in women with early-onset (<34 weeks gestation) preeclampsia by improving maternal endothelial dysfunction [47]. The administration of 30 mg daily of melatonin to 20 women with early-onset preeclampsia was associated with a six day increase in diagnosis to delivery interval and a reduced need for escalation of antihypertensive therapy. Consistent with those clinical outcomes, melatonin reduced placental and endothelial dysfunction [47]. Certainly this small study was open to bias, with small numbers, lack of blinding and use of historical controls. However, it was the first report of the use of melatonin therapeutically in preeclampsia. The correlation between both clinical and biochemical outcomes also supports a possible role for melatonin in restraining the otherwise unbridled placental and vascular oxidative stress of preeclampsia. While promising, these findings now require confirmation by a large randomised controlled trial before melatonin can be recommended for clinical use [134].

Pending further clinical trial assessment, melatonin has a number of attributes that would make it a particularly attractive therapy for preeclampsia (Figure 1). The safety of melatonin in pregnancy is well documented. It readily crosses the human placenta [135] and, even in high doses it has few, if any, adverse effects. Numerous animal studies have shown no maternal or fetal adverse effects from exogenous administration of melatonin [99,113,136]. Pilot studies of melatonin for women with growth restricted pregnancies (8 mg daily) [137], preeclampsia (30 mg daily) [47], and in 160 women undergoing in vitro fertilisation (8–16 mg daily) did not identify adverse outcomes from melatonin [138]. In a long-term contraceptive trial, women who took 75 mg melatonin a day for months reported no adverse effects [139] further supporting the safety profile of melatonin. The beneficial effects of melatonin for preeclampsia may extend beyond the mother. Current trials are investigating the potential for melatonin to protect the fetus in hostile intrauterine environments such as intra uterine growth restriction [103]. Research is underway to assess whether melatonin may offer benefit in augmenting induction of labour [54], preventing blood loss after caesarean section [140] and post-partum haemorrhage (IRCT2015050919037N9). In humans, melatonin also has anticonvulsant activity [141], which may be useful in the primary prevention of eclampsia.

## 5. Beyond Melatonin: Other Antioxidants for Preeclampsia

Melatonin is not alone in holding potential therapeutic utility as an antioxidant for the management of preeclampsia. Indeed, much excitement surrounded antioxidants upon the discovery that oxidative stress is likely a key player in the pathogenesis of the disease. This abated somewhat when the results from large clinical trials of Vitamin C and E were negative [142,143]. However, these therapies act only to directly scavenge ROS so do not carry the same potency as Nrf2-activators which upregulate a plethora of antioxidant enzymes and utilise innate cellular processes of signal amplification. Promising in vitro data [144,145] for esomeprazole saw it was rapidly evaluated in a clinical trial to delay delivery in women with preeclampsia [146]. That the clinical trial did not confirm any benefit of esomeprazole in diagnosed preeclampsia, despite preclinical observations, is a timely reminder of the need for thorough clinical trial assessments of new therapies. Potential benefit for high-risk women when given esomeprazole in the first trimester is under investigation. Resveratrol, found in red wine, is an inducer of the Nrf2 antioxidant pathway and has been shown to improve the health of trophoblasts and vascular cells in vitro [49,64,147]. Clinically, resveratrol improves the antihypertensive efficacy of nifedipine in managing preeclampsia [148]. A formal clinical trial of resveratrol would certainly be worthwhile. Another promising therapeutic candidate that addresses oxidative stress pathways is sulforaphane, a naturally occurring Nrf2 inducer found in cruciferous vegetables, particularly broccoli seed. Sulforaphane has been shown to have similar actions to melatonin in syncytiotrophoblast mitochondria, improving the resilience of the electron transport chain to both the hypoxic and oxidative mechanisms of injury of preeclampsia [94]. Sulforaphane directly reduces placental production of antiangiogenic proteins that trigger vascular dysfunction in preeclampsia [48]. As well as reducing inflammation and oxidative stress in vascular cells [20], sulforaphane protects omental blood vessels taken from pregnant women at the time of caesarean section against "preeclamptic-like" injury [63]. Not only does sulforaphane reduce sensitivity to vasoconstrictors, it also improves vasorelaxation in these injured blood vessels and, in supraphysiologic doses, can act as a direct vasodilator [63]. Clinical investigations of sulforaphane, as a broccoli extract formula, to treat established preeclampsia are underway and the results of that are eagerly awaited [149]. If the dose-finding studies are any indicator, sulforaphane looks very promising indeed. Even as a single dose, sulforaphane transiently reduced diastolic blood pressure and circulating sFlt1 levels in women with pregnancy hypertension at term [150].

## 6. Conclusions

Though essential in reducing stroke risk, antihypertensive therapy has reached the limit of its utility in the management of preeclampsia. Instead, we must now put to use our increased understanding of the pathogenesis of the disease to guide rational drug therapy. Specifically, antioxidants to mitigate the oxidative stress that underpins preeclampsia are likely an appropriate solution to protect the placenta and maternal endothelium. Melatonin is a safe, endogenous hormone with impressive antioxidant and antihypertensive effects that may make it a useful adjuvant for the management of preeclampsia. These antioxidant effects have been elucidated in placenta cells, in mitochondria, and in vascular cells, all key contributors to the pathogenesis of preeclampsia. The vasoactive effects of melatonin appear to be vessel specific in animals, though in humans it appears hypotensive and may reduce maternal blood pressure such that the inevitable delivery of the fetus can be safely delayed. Perhaps most excitingly, melatonin may offer benefits that extend beyond maternal care to protect the fetal brain in hostile intrauterine environments, such as FGR. Melatonin has been investigated for a host of other disorders related to pregnancy (summarized in Table 1). None of these studies raised safety concerns, even in high doses. Collectively, these findings highlight that melatonin is an exciting candidate for an adjuvant therapy for preeclampsia. Preliminary clinical trial evidence is promising and further, more fulsome, clinical evaluation is certainly warranted. An abundance of in vitro, animal-based and clinical evidence supports a role for melatonin in the management of preeclampsia, and indeed other disorders of pregnancy. Only through ongoing investigation of naturally occurring antioxidants, such as melatonin, can we hope to safely prolong pregnancy in severe preeclampsia and perhaps, for the first time in fifty years, offer a way to improve, and save, the lives of pregnant women with preeclampsia and their babies.

**Table 1.** Summary of clinical trials investigating melatonin for pregnancy outcomes.

| | Number of Participants | Intervention | Primary Outcome | Study Type |
|---|---|---|---|---|
| Swarnamani, K. et al., 2020 [54] | 774 women undergoing induction of labour | Four doses of 10 mg of melatonin or placebo | Requirement for caesarean section | Double-blind randomised placebo-controlled trial |
| Palmer K. R. et al., 2019 [103] | 336 women with FGR pregnancy between 23 + 0 and 31 + 6 weeks | 30 mg per day or placebo | Neurodevelopment (difference of 5 points in the cognitive domain of the Bayley-III) | Triple-blind, parallel, randomised placebo-controlled trial |
| Khezri, M. et al., 2019 [140] | One hundred and twenty women undergoing caesarean section | 3 or 6 mg of melatonin or placebo 20 min before caesarean section | Change in haemoglobin level | Double-blind randomised trial |
| Fernando, S. et al., 2018 [138] | One hundred and sixty women undergoing their first cycle of IVF or ICSI | 2, 4 or 8 mg of melatonin or placebo twice daily | Clinical pregnancy rate | Double-blind randomised placebo-controlled trial |
| Hobson S.R. et al., 2016 [47] | Twenty women with preeclampsia | 10 mg of melatonin three times daily | Diagnosis to delivery interval | Single-arm, open-label study |
| Alers N.O. et al., 2013 [137] | 12 women with FGR pregnancies < 34 wks | 4 mg melatonin twice daily | Biomarkers placental and circulating oxidative stress | Single-arm, open-label study |

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# Chapter three

## *Sulforaphane improves endothelial function and reduces placental oxidative stress in vitro*

Similar to melatonin, the plant extract, NFE2L2 inducer and anti-inflammatory agent sulforaphane may reduce placental and vascular oxidative stress in preeclampsia[68–71]. Sulforaphane is a naturally occurring organosulphur antioxidant found in cruciferous vegetables, particularly broccoli seeds and sprouts, with a well-documented safety profile[71–76].

Sulforaphane induces phase two-enzymes NQO1 and GST by activating cytosolic transcription and nuclear translocation of NFE2L2 and was first identified in broccoli when consumption of this vegetable demonstrated impressive induction of NQO1 and GST relative to plasma sulforaphane[77,78]. Identification of sulforaphane as one of the most potent inducers of NFE2L2 *in vitro* led to further molecular evaluation of this compound. *In vitro* assays have demonstrated that sulforaphane preserves NFE2L2 stability and potentiates NFE2L2 accumulation within the cell by inducing cysteine modifications to the molecular structure of KEAP-1[79,80]. Sulforaphane also attenuates proteosomal degradation of NFE2L2 by impairing ubiquitination and increasing phosphorylation of NFE2L2[79]. Broccoli sprout extract rich in sulforaphane has received much attention in the literature for endothelial protective and anti-inflammatory effects[71–76,81]. However, broccoli extract has never been investigated in pregnant women and nor as a possible therapy for preeclampsia.

This study was designed to assess whether the antioxidant and anti-inflammatory compound sulforaphane could mitigate endothelial and trophoblast dysfunction from oxidative stress *in vitro*, with a view to developing a novel adjuvant therapy for preeclampsia.

***Hypothesis***

That sulforaphane will protect endothelial and placental cells against inflammatory insult, via activation of the NFE2L2 antioxidant pathway (Figure 1).

***Aims***

1. To investigate whether sulforaphane protects endothelial cells against preeclamptic-like injury.
2. To investigate whether sulforaphane reduces placental production of damaging compounds implicated in preeclampsia after hypoxic and hyperoxic injury.
3. To investigate whether sulforaphane exerts these effects via activation of the NFE2L2 antioxidant pathway.

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# Sulforaphane improves endothelial function and reduces placental oxidative stress *in vitro*

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ABSTRACT

*Introduction:* The maternal endothelial dysfunction characteristic of preeclampsia arises, in part, from excessive placental production of anti-angiogenic factors, including soluble Flt-1, soluble endoglin and activin A, inducing oxidative stress. We assessed whether the antioxidant and NRF2-activator sulforaphane could mitigate endothelial and trophoblast dysfunction *in vitro*.

*Methods:* We induced dysfunction in human umbilical vein endothelial cells (HUVECs) with TNF-α, assessing endothelial activation and dysfunction (endothelin-1, vascular cell adhesion molecule; VCAM1, intracellular adhesion molecule; ICAM1, e-selectin and endothelial permeability) in the presence or absence of sulforaphane. We also assessed the effects of sulforaphane in mitigating hypoxic and hyperoxic injury in term placental explants by measuring secretion of anti-angiogenic factors. To assess the role of NRF2 we silenced NRF2 in HUVECs and primary trophoblast cells.

*Results:* Sulforaphane reduced TNF-α mediated HUVEC secretion of endothelin-1, VCAM1, ICAM1 and E-selectin, and prevented increased endothelial permeability. In placental explants, sulforaphane reduced the secretion of soluble Flt-1, soluble endoglin and activin A. Sulforaphane induced activation and nuclear translocation of NRF2 in HUVECs, inducing heme oxygenase 1. NRF2 silencing blocked some but not all of sulforaphane's effects in HUVECs. NRF2 silencing did not prevent sulforaphane's inhibition of trophobast secretion of soluble Flt-1 or activin A.

*Conclusion:* In reducing placental and endothelial oxidative stress, sulforaphane may offer a new adjuvant therapeutic approach for the treatment of preeclampsia.

## 1. Introduction

Preeclampsia remains a significant cause of maternal and perinatal morbidity and mortality, and is a leading cause of iatrogenic preterm birth [1]. The mainstay of care of women with preeclampsia is controlling maternal blood pressure to safely allow prolongation of pregnancy to improve fetal maturation [1,2]. This focus of management on the hypertension has not substantially changed in over 50 years [3]. However, over the last decade, insights into the mechanisms underlying the hypertension and maternal organ dysfunction have identified promising new avenues for other approaches to management [3].

In particular, inadequate placentation from the beginning of pregnancy is thought to result in sustained and progressive ischemic-reperfusion injury to the placenta across pregnancy [4,5]. This injury in turn underlies the excessive placental release of anti-angiogenic vasoactive substances, such as soluble fms-like tyrosine kinase-1 (sFlt-1), soluble endoglin (sEng) and activin A [6,7], that, via oxidative stress, induce systemic inflammation and endothelial damage, vasoconstriction, hypertension and other end-organ damage [7–11]. Recognition of inflammation and endothelial damage as key contributors to the development of preeclampsia has better enabled more precise pharmacological targeting as potential adjunctive therapeutic approaches [3].

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By protecting the endothelium, reducing systemic inflammatory stress and minimizing placental production of vasoactive compounds, halting disease progression is, perhaps, a realistic prospect.

Nuclear factor erythroid 2–related factor 2 (NRF2) is an endogenous inducer of cellular antioxidants [12]. Under physiological conditions bioavailable levels of NRF2 are regulated through cytosolic binding to Kelch-like-ECH-associated protein-1 (KEAP-1) by preventing its proteasomal degradation [12,13]. Exposure to oxidative stress induces modifications in KEAP-1, allowing NRF2 to translocate to the cell nucleus. There, NRF2 stimulates the antioxidant response element (ARE) leading to the transcription of a cassette of cellular antioxidants such as heme oxygenase-1, (HO-1), NAD(P)H dehydrogenase (NQO1) and glutathione S-transferase (GST) [12,14]. Exogenous stimulation of NRF2 has been shown to restore endothelial health and attenuate the damaging effects of vasoactive compounds in preeclampsia [12]. This suggests that the administration of a NRF2 activator may be an effective adjunctive therapy for preeclampsia [12,14].

Sulforaphane, a naturally occurring organosulfur present in cruciferous vegetables, is a NRF2 inducer that exerts both antioxidant and anti-inflammatory actions via the Nrf2/ARE pathway [15,16]. By inducing cysteine modifications to KEAP-1, sulforaphane preserves NRF2 stability, promotes cytosolic transcription, and facilitates nuclear translocation of NRF2 [13,17]. In various small animal models, sulforaphane has been shown to protect endothelial function and improve retinal, renal and liver function following ischaemic-reperfusion injuries [18–21]. It is also a promising therapy in cardiovascular disease such as chronic hypertension and atherosclerosis [22,23].

We hypothesize that sulforaphane may have utility as an adjunctive therapy for preeclampsia. Here we used *in vitro* models of preeclampsia to assess the endothelial and placental protective capacity of sulforaphane.

## 2. Materials and methods

### 2.1. Ethics

The Monash Health Human Research Ethics Committee approved the collection of placentae, umbilical cords and serum and informed written consent was obtained from each patient prior to collection. Healthy singleton term human placentae and cords for human umbilical vein endothelial cell (HUVEC), and trophoblastexplant and villous cytotrophoblast culture were collected at the time of caesarean section. Exclusion criteria were: smoking, alcohol and drug abuse, multiple pregnancies, < 38 weeks, BMI > 35, pre-existing hypertension, renal disease, diabetes, thyroid dysfunction, metabolic syndrome or preeclampsia in a previous pregnancy. Exclusions were made for the following medications; antihypertensive, aspirin, non-steroidal anti-inflammatory drugs or thyroid medications. Serum was obtained from women diagnosed with preeclampsia according to SOMANZ guidelines, [2] immediately prior to delivery.

### 2.2. Human umbilical vein endothelial cell culture

HUVECs were isolated from umbilical cords collected from healthy term pregnancies (n = 5–7) at the time of caesarean section, as previously described [24]. Briefly, cells were cultured in M199 medium (Gibco-BRL), 10% fetal calf serum (FCS), epidermal and fibroblast growth factors (10 ng/ml), heparin (90ug/ml), 1% streptomycin sulphate (100 μg/ml) and amphotericin B (0.25 μg/ml; Invitrogen). Flasks were coated with 0.2% gelatin before seeding with isolated HUVECs. Cells were incubated and expanded in 5% $CO_2$, 70% $N_2$, and 25% $O_2$ at 37 °C with cultures for experimentation taken from second and third passages.

### 2.3. Markers of endothelial cell activation

The markers of endothelial cell activation were quantified in HUVECs treated with sulforaphane (Sigma-Aldrich, St. Louis, MO, S6317-5MG; 5 μM, 10 μM, 20 μM) and/or TNFα (1 ng/ml, Thermo Fisher Scientific PHC3015) for 24 h, via flow cytometry, expressed as mean flow index (MFI). Briefly, HUVECs were mechanically scraped from their flasks prior to staining with one of the following antibodies, with synonyms, antibody dilutions and catalogue numbers shown in parentheses: CD54-Pacific Blue (intracellular adhesion molecule; ICAM1, 1:100, 353109), CD106-PE (vascular cell adhesion molecule; VCAM1, 1:50, 305805) CD62E-APC (E-selectin, 1:100, 336011) and staining was compared against their appropriate isotype controls. Antibodies and isotype controls were obtained from Biolegend (San Diego, CA). HUVECs were incubated on ice with either antibodies or isotype controls for 20 min prior to washing and fixing in 1% paraformaldehyde in FACS buffer. The markers of endothelial cell activation were then analyzed on a BD FACS Canto II (BD Biosciences, San Jose, CA). Analysis was performed using FlowJo cytometric analysis software (Tree Star, Oakland, OR).

### 2.4. Endothelin-1 and 8-isoprostane production by HUVECs

Endothelin-1 (ET-1) levels were determined in the supernatants of HUVECs treated with sulforaphane (Sigma-Aldrich, St. Louis, MO, S6317-5MG; 5 μM, 10 μM, 20 μM) and/or TNFα (1 ng/ml) for 24 h via ELISA (DET100, R&D systems, Minneapolis, MN). Level of 8-isoprostane, a marker of oxidative stress, was measured in the same culture supernatants by enzyme immunoassay (Caymen Chemicals, Ann Arbor, MI). All assays were performed according to manufacturer's instructions

### 2.5. Endothelial cell permeability

Integrity of the endothelial cell monolayer following TNFα treatment was assessed via a FITC-dextran based permeability assay. HUVECs were plated in gelatinized polycarbonate transwell inserts (Corning, Oneonta, NY; 0.4 μM, 6.5 mm, 24 well plate) at a density of 50,000 cell insert, and incubated at 37 °C for 3 days to form a monolayer. Cells were then treated with recombinant TNFα at an optimized dose (100 ng/ml) in the presence or absence of sulforaphane (20 μM) for 24 h. At the end of the treatment period, culture media was removed from both the upper and lower chambers, and 1 mg/ml FITC-dextran (Sigma-Aldrich; MW 40,000) in culture media was added to the upper chamber, whilst fresh culture media was added to the lower chamber. Cells were then incubated for 1 h at 37 °C. Media was removed from the lower chamber and diluted 1:20 with PBS in a black-walled 96-well plate before fluorescence readings were obtained at 485 nm excitation and 535 nm emission.

Endothelial cell permeability following treatment with serum from women with preeclampsia (5% serum in M199 Medium supplemented with 1% antibiotics and 1% L-Glutamine) was measured using the 24-well *in vitro* vascular permeability assay kit (Merck Millipore, Billerica, MA). The assay was performed according to manufacturer's instructions. Endothelial cells treated with serum from gestation matched healthy pregnant women were used as controls.

### 2.6. Placental explant culture

Immediately after collection, several cotelydons were removed from the placenta and rinsed in chilled 1x Hank's Balanced Salt Solution (HBSS) to remove blood. Samples were submerged in 1x HBSS in a petri dish while villous tissue was gently dissected from major connective tissue and vasculature. Placental villous explants (50 mg) were then cultured on a 6 well plate with Medium 199 (Life Technologies, Carlsbad, CA) supplemented with 1% Antibiotics (Life Technologies)

and 1% L-Glutamine (Life Technologies).

One group of placental explants (n = 6) was treated with xanthine/xanthine oxidase (X/XO) (Sigma-Aldrich; 2.3 mM X and 15 mU/ml XO), to induce oxidative stress, in the presence or absence of sulforaphane (5 μM, 10 μM and 20 μM). Placental explants treated with media only were used as negative controls. These explants were cultured in 5% $O_2$ at 37 °C for 24 h. Another group of placental explants (n = 6) were exposed to 1% $O_2$ in the presence or absence of sulforaphane (5 μM, 10 μM and 20 μM) at 37 °C for 24 h. Placental explants exposed to 5% $O_2$ were used as negative controls. After 24 h of incubation, culture media was removed and stored at −80 °C with 0.005% butylated hydroxytoluene (BHT, Sigma-aldrich, St. Louis, MO; W218405) to prevent radical formation and degradation of 8-isoprostane.

### 2.7. Placental villous cytotrophoblast culture

Cotelydons were randomly excised from placentae (n = 6), the basal plate removed and samples washed in ice-cold 1x HBBS to remove residual blood and encourage villous segmentation. Samples were placed in a petri dish and submerged in 1x HBSS. Villi (20–25 g) were separated from vessels and exposed to three digestions in low glucose DMEM (Life technologies; 316000083) with 7.5 ml of 2.5% trypsin (Life Technologies; 15090046), 0.19 g of Dispase Grade II (Life Technologies; 17105–041) and 150uL of bovine DNase I Grade II (Sigma-Aldrich; 10104159001) for 15 min in a 37 °C water bath. Between each digestion supernatant was collected, filtered through a cell strainer and held at room temperature until all incubations were completed. Samples were centrifuged at 350 g, 4 °C for 5 min, the supernatant discarded and the pellet resuspended in complete trophoblast media (DMEM/F12 Life Technologies; 11330-057, 1% antimycotic-antibiotic Life Technologies 15; 140-122 and Life Technologies; 15240-062). Percoll gradient separation (5–70% GE Healthcare; 17-0891-01) was used with centrifugation at 1200 g, 4 °C for 20 min to isolate a layer of villous cytotrophoblasts. These were diluted in complete trophoblast media and plated at 0.7 million cells per well on a 24 well plate. All samples were incubated at 37 °C in 8% oxygen for 24 h to encourage adhesion and proliferation.

Samples were randomly divided into two treatment groups. The first X/XO (2.3 mM X and 15 mU/m XO) in the presence or absence of sulforapahane (20 μM) in 8% oxygen. The second group was placed in 1% oxygen in the presence or absence of sulforaphane (20 μM). Negative controls either received sulforaphane or not and did not undergo treatment. Samples were incubated at 37 °C in their respective oxygen incubators for a further 24 h.

### 2.8. Small interfering RNA transfection

HUVECs and placental explants were reverse transfected with single stranded small interfering RNA (siRNA) directed towards NRF2, scrambled sequence (negative control) or GAPDH (positive control) with the use of Lipofectamine RNAimax Reagent (all from Life Technologies, Carlsbad, CA), according to manufacturer's instructions. All experiments were completed within 4 days of transfection as described below. Samples were returned to 8% oxygen, 37 °C and incubated for a further 24 h.

### 2.9. Assays

Commercial ELISAs were used in accordance with manufacturer guidelines. Levels of sFlt-1 (R&D Systems, Minneapolis, Human VEGF R1/Flt1 DVR100B dilution: 1:25) and sEng (R&D Systems, Minneapolis Human Endoglin/CD105 DNDG00 dilution: neat) were tested in the supernatant of hypoxic samples while concentrations of 8-isoprostane (Cayman, Ann Arbour, 8-isoprostane 516,351 dilution 1:50) and activin A (R&D Systems, Minneapolis DAC00B dilution: 1:2) were evaluated in the supernatant of X/XO treated samples.

### 2.10. Protein extraction

HUVEC and placental supernatant was collected and stored at −80 °C with 0.005% BHT (Sigma-Aldrich) to prevent radical formation and degradation of 8-isoprostane. Protein was extracted using physical cell scraping in the presence of lysis buffer (made in house) and protease inhibitor (Sigma-Aldrich; 11697498001). Centrifugation was used to separate cellular debris and the resulting supernatant was stored at −80 °C prior to analysis with western blot.

### 2.11. Western blot

HUVEC cultures were assessed for NRF2 activation and antioxidant response by determining nuclear NRF2 translocation and HO-1 protein levels. Confirmation of siRNA knockdown was also performed using western blot for HO-1. Nuclear and cytoplasmic fractions were isolated using NE-PER reagents (Thermofisher) Protein levels were quantified using BCA. Western blots were performed as previously described [8]. Membranes were blocked with 5% skim milk in 1 x HBBS with 0.1% Tween-20 for 1 h prior to probing with antibodies. The primary antibody HO-1 (ab52947, 1:2000, Abcam) was diluted in blocking buffer and incubated at 4 °C overnight. Membranes were incubated in secondary anti-rabbit antibody (sc-2004, 1:10000, Abcam) with blocking buffer for one hour at room temperature, prior to imaging (biorad ChemiDoc, Western ECL substrate, 170–5060). Membranes were then washed and incubated in primary beta actin antibody for cytosoloic fractions (sc-47778, 1:5000, Abcam) or HDAC1 for nuclear fractions (ab7028, 1:2000, Abcam) and secondary anti-mouse (sc-2005, 1:20000, Abcam) each for one hour at room temperature.

### 2.12. Statistical analyses

Statistical analyses were performed using one-way ANOVA followed by multiple comparisons with a Tukey post-hoc test. Groups were considered to be significantly different if $P < 0.05$. All data were analysed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA).

## 3. Results

### 3.1. Sulforaphane increased NRF2 protein levels, NRF2 nuclear translocation and HO-1 protein levels in HUVECs

Treatment of HUVECs with TNF-α did not induce NRF2 nuclear translocation (Fig. 1A) or total NRF2 (Fig. 1B). Treatment of HUVECs with sulforaphane increased nuclear translocation of NRF2 3-fold with a corresponding 4-fold increase in total NRF2, compared with untreated and TNF-α alone treated HUVECs (Fig. 1A & C; $p < 0.01$, Fig. 1B & C; $p < 0.0001$). This was associated with an almost 4-fold increase in HO-1 protein levels compared to TNF-α treatment alone (Fig. 1D $p = 0.04$), confirmed by western blot (Fig. 1E).

### 3.2. Sulforaphane ameliorated endothelial cell dysfunction in vitro

Treatment of HUVECs with TNFα significantly increased the expression of ICAM1, VCAM1 and E-selectin ($P < 0.001$; Fig. 2A–C). These effects were mitigated by sulforaphane in a dose-dependent manner (Fig. 2A–C; $p < 0.01$). TNF-α treatment of HUVECs also increased expression of ET-1 ($p = 0.004$). Only the highest dose of sulforaphane (20 μM) significantly mitigated this effect (Fig. 2D, $p = 0.04$). At 20 μM sulforaphane also attenuated both TNF-α and preeclamptic serum stimulated increases in endothelial cell monolayer permeability (Fig. 2E; $p = 0.001$ & Fig. 2F; $p = 0.04$, respectively, expressed as fold change from control).

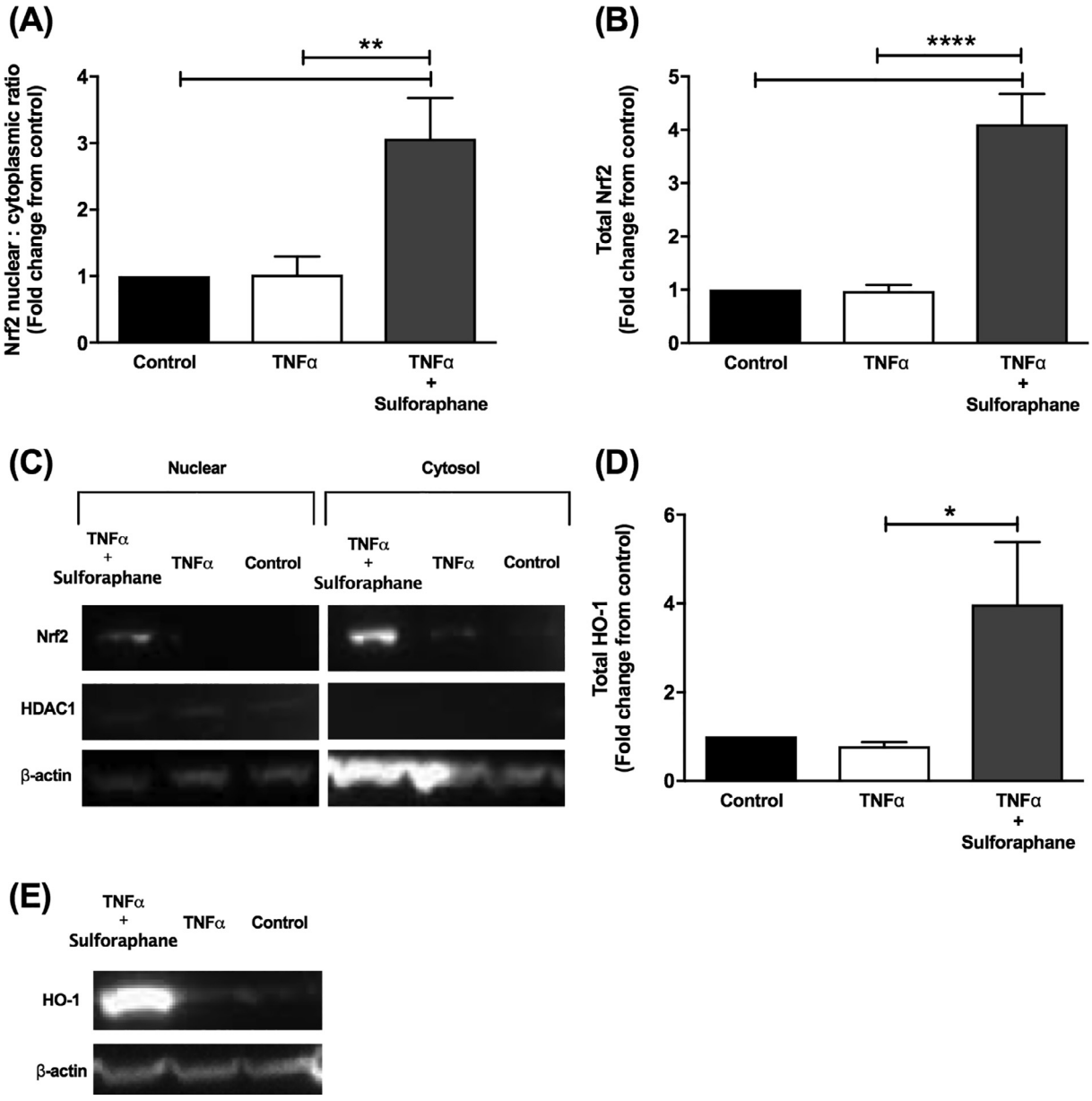


**Fig. 1.** Nrf2 and HO-1 protein expression in HUVECs treated with sulforaphane. (A) Nrf2 nuclear translocation and (B) total Nrf2 protein expression were measured 6 h after HUVECs were treated with media only, TNF-α (100 ng/ml) or TNF-α with 20 μM sulforaphane. (C) Representative western blot image for Nrf2 are shown with white space indicating noncontiguous lanes from the same blot. (D) Total HO-1 expression measured 24-hours after treatments and a (E) representative western blot image. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. n = 6 cell lines from independent placental donors.

### *3.3. Nrf2 knockdown blocked some of the endothelial cell effects of sulforaphane*

To determine the involvement of NRF2 as a mediator of sulforaphane on *in vitro* endothelial function, we silenced NRF2 with siRNA. In the presence of NRF2 silencing sulforaphane neither increased HO-1 in HUVECs (Fig. 3A & B) nor prevented TNF-α induced increased endothelial monolayer permeability (Fig. 3C, expressed as fold change from control). NRF2 silencing prevented the TNF-α-induced increase in ICAM-1 (Fig. 3D) but it did not block sulforaphane's mitigation of TNF-α induction of VCAM-1 and E-selectin (Fig. 3E & 3F respectively; all $p < 0.05$). Silencing NRF2 blocked the TNF-α-induced increases in ET-1 and the oxidative stress marker 8-isoprostane, preventing any injury and therefore any assessment of sulforaphane (Fig. 3G & H respectively).

### *3.4. Sulforaphane altered placental explant and isolated trophoblast secretion of vasoactive compounds*

We then sought to evaluate the effects of sulforaphane on the placental secretion of the vasoactive compounds sFlt-1 and soluble sEng following hypoxic injury (1% $O_2$). Both compounds are elevated in the serum of preeclamptic women and have been implicated in the endothelial dysfunction that underlies disease progression. Placental explant culture in 1% $O_2$ significantly increased sFlt-1 ($p = 0.04$) and sEng, ($p = 0.005$) secretion (Fig. 4A and 4B), an effect prevented by sulforaphane at 20 μM, but not at lower concentrations (sFlt-1: Fig. 4A, $p = 0.047$) and (sEng: Fig. 4B, $p = 0.02$).

Mimicking oxidative injury, exposure of placental explants to X/XO significantly increased activin A secretion ($p = 0.004$), an effect mitigated by sulforaphane treatment at 10 μM and 20 μM concentrations (Fig. 4C $p = 0.03$, $p = 0.047$).

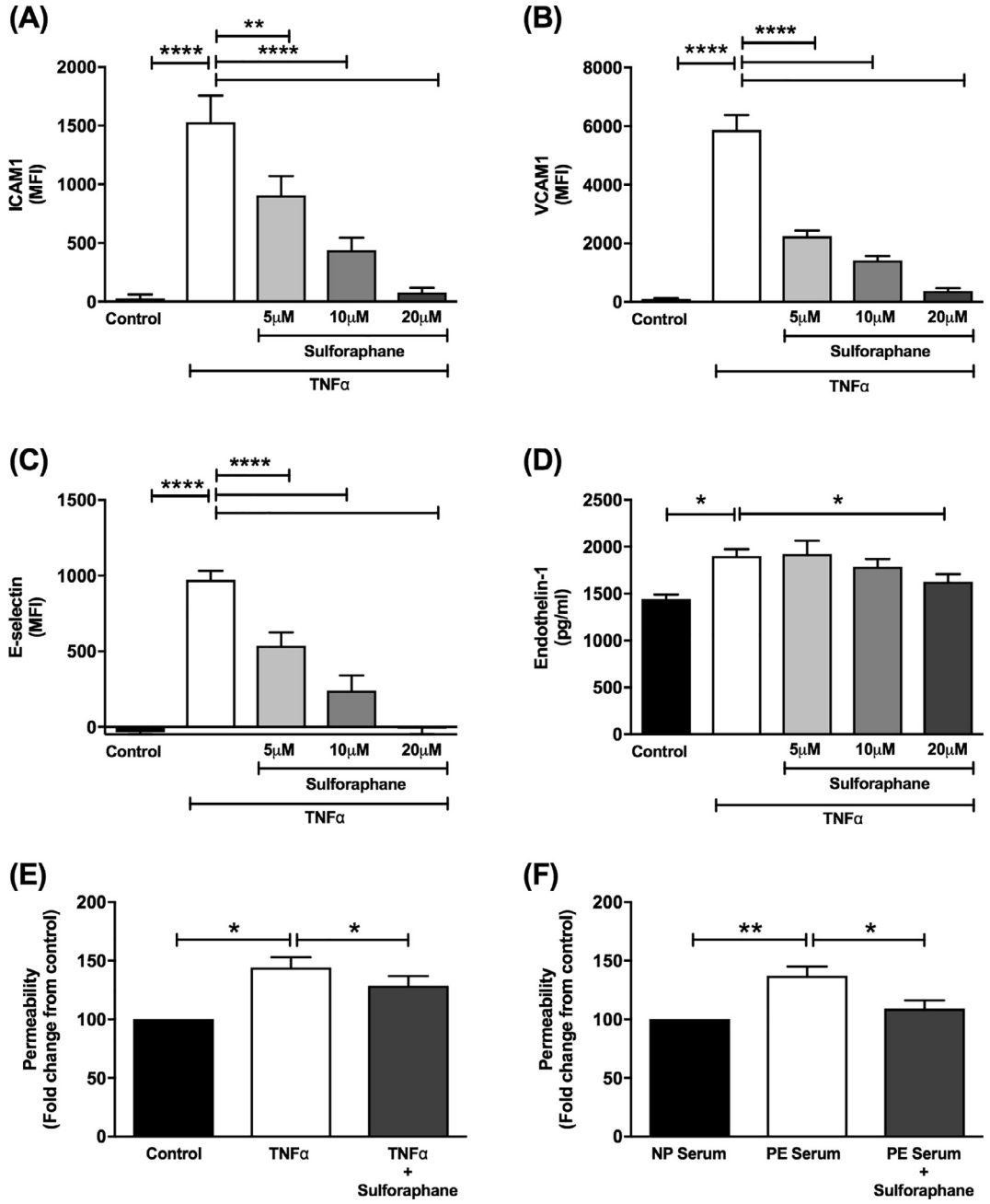


**Fig. 2.** Effect of sulforaphane treatment on hallmarks of endothelial dysfunction *in vitro*. Expression of (A) ICAM1, (B) VCAM1 and (C) E-selectin measured 6 h after HUVECs were treated with either media only, TNF-α (1 ng/ml figures a-d or 100 ng/ml figures e-f) or TNF-α with 3 different doses of sulforaphane (5 μM, 10 μM, 20 μM) as measured using flow cytometry. Protein levels of (D) endothelin-1 in culture supernatants collected 24-hours after treatment was measured using ELISA. (E) FITC-dextran permeability through HUVEC monolayers treated with either media only, TNF-α (100 ng/ml) or TNF-α and 20 μM sulforaphane following 24 h of treatment, expressed as fold change from control. (F) FITC-dextran permeability in HUVEC monolayers 24 h after treatment with either serum from normal pregnant women (NP serum), with serum from preeclamptic women (PE serum) or with PE serum and 20 μM sulforaphane, expressed as fold change from control. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. n = 5–7 cell lines from independent placental donors.

We then used isolated villous cytotrophoblasts to explore the effect of sulforaphane in ameliorating production of sFlt-1, sEng, activin A and 8-isporostane. Incubation in 1% $O_2$ did not significantly increase trophoblast production of sFlt-1 (Fig. 5A) or sEng (Fig. 5B). However, administration of sulforaphane (20 μM) significantly reduced sFlt-1 secretion, compared to normoxic controls (p = 0.0006) and hypoxic culture (p = 0.0003). There were no effects of sulforaphane on sEng secretion (p = 1.0).

Treatment of isolated trophoblasts with X/XO significantly increased secretion of both activin A (p = 0.006) and 8-isoprostane (p = 0.009). Sulforaphane (20 μM) prevented the increase in activin A (p = 0.02) but not 8-isoprostane.

To explore whether the effects of sulforaphane in villous cytotrophoblasts were mediated via the NRF2 pathway we used single stranded silencing RNA directed at NRF2 to block this pathway. Despite NRF2 silencing, sulforaphane continued to decrease sFlt-1 secretion from trophoblasts compared to both normoxic (p = 0.04) and hypoxic (p = 0.001) cultures. Similarly, NRF2 silencing did not block sulforaphane mitigation of X/XO-induced activin A secretion (p = 0.01). Paradoxically, we observed a rise in soluble endoglin (p = 0.007) when sulforaphane was administered to NRF2 silenced cells under hypoxic conditions (Fig. 5).

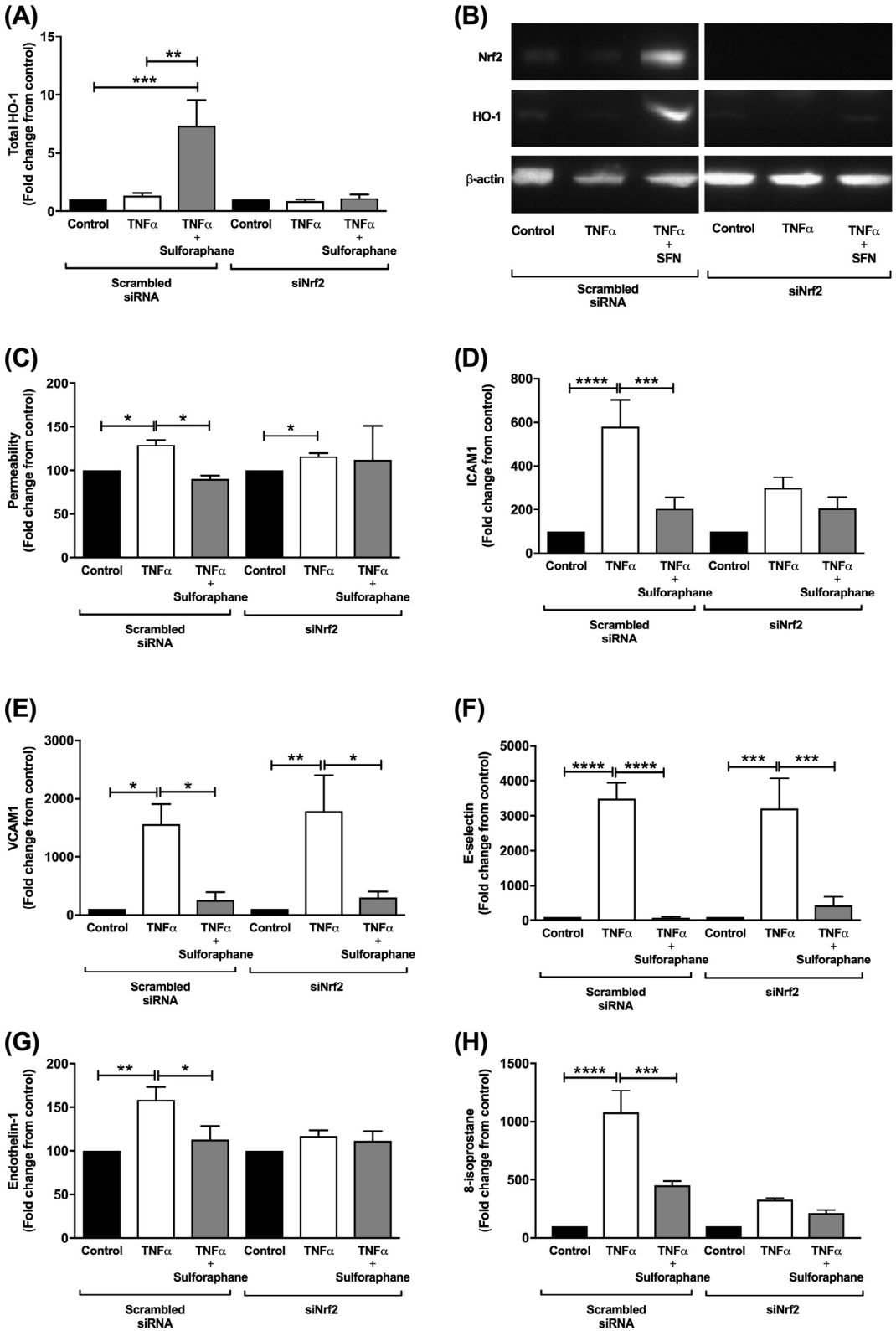


**Fig. 3.** Nrf2 knockdown. HUVECs were transfected with small interfering RNA directed towards Nrf2 (siNrf2) and treated with either media only, TNF-α only (1 ng/ml figures a,b, d-f and 100 ng/ml figure c) or TNF-α with 20 μM sulforaphane. HUVECs transfected with small interfering RNA containing a scrambled sequence (scrambled siRNA) and then exposed to the treatments were used as controls. (A) Total HO-1 protein expression in HUVECs 24-hours after treatment measured using western blot. (B) Representative images for HO-1 western blot. Additionally, after 24-hours FITC-dextran permeability though (C) HUVEC monolayers was measured, expressed as fold change from control. Expression of (D) ICAM1, (E) VCAM1 and (F) E-selectin was measured 6 h after treatment using flow cytometry. Levels of (G) endothelin-1 and (H) 8-isoprostane in HUVEC culture supernatants were measured 24 h after treatment using ELISA and EIA respectively. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. n = 5–6 cell lines from independent placental donors.

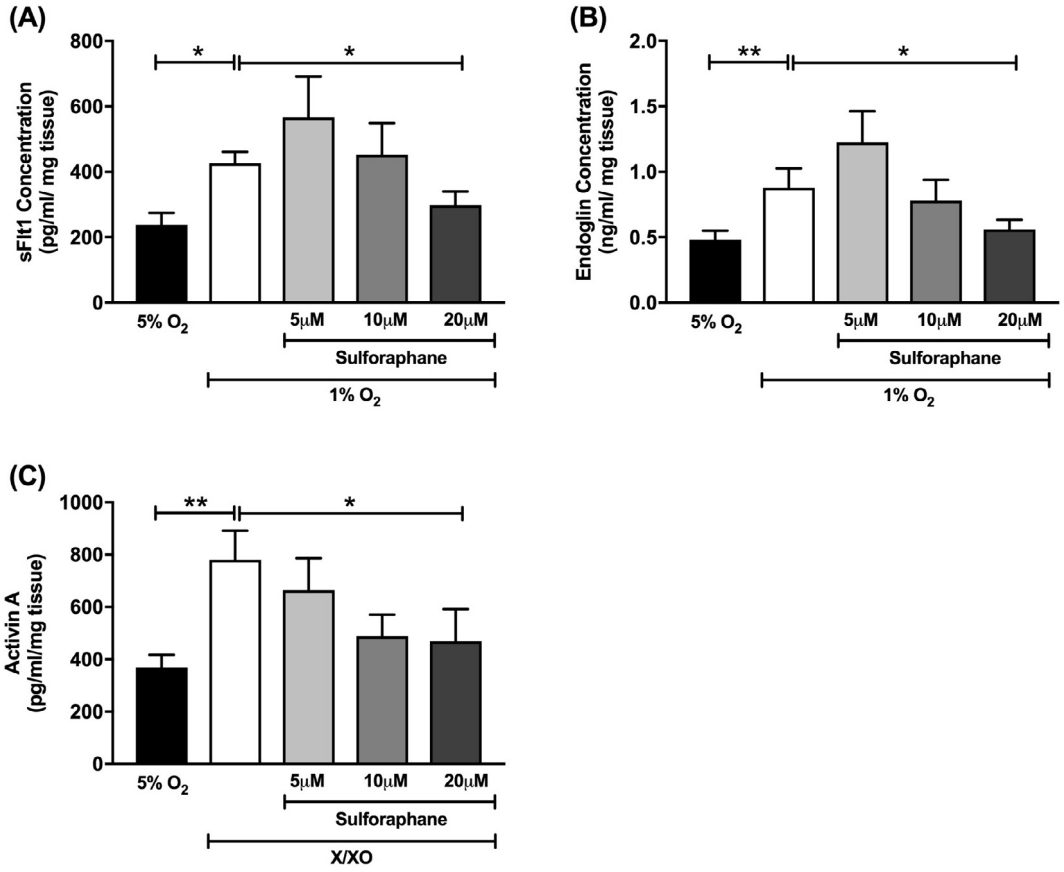


**Fig. 4.** Effect of sulforaphane treatment on cultured placental explants *in vitro*. Explants were incubated for 24 h in 5% oxygen 37˚c in either media only or media with X/XO solution, or in 1% oxygen 37˚c for 24 h, in the presence or absence of sulforaphane (5 μM, 10 μM and 20 μM). Levels of (A) sFlt-1 and (B) sEng were quantified using ELISA from culture media of hypoxic samples after 24 h of treatment. (C) The level of activin A quantified using ELISA from culture media of X/XO treated samples after 24 h of treatment. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. n = 6 explants from independent placental donors.

We saw no significant changes in HO-1 protein expression in any treatment group (data not shown).

## 4. Discussion

Here we have shown that sulforaphane is able to protect endothelial cells and reduce placental secretion of vasoactive compounds. Specifically, in endothelial cells sulforaphane reduced TNF-α induced endothelial monolayer permeability and the secretion of ET-1, ICAM, VCAM and E-selectin. In placental explants, sulforaphane reduced secretion of factors known to be upregulated by preeclampsia, including sFlt-1, sEng and activin A. While NRF2 silencing blocked the effects of sulforaphane in HUVECs, it did not prevent the sulforaphane-induced decrease in trophoblast secretion of sFlt-1 or activin A, suggesting that some of sulforaphane's actions operate through pathways other than NRF2.

Following the observation that oxidative stress was a likely common pathway causing the maternal endothelial dysfunction underlying preeclampsia, [25–27] there has been much interest in antioxidants as preventative therapies [28–31]. While the results from small clinical trials were promising [32], large randomized clinical trials of vitamin C and E failed to show benefit [33,34]. As vitamin C and E act in a downstream manner, reducing intracellular superoxide and inducing endothelial nitric oxide synthase (eNOS) activation, [35] it is possible that this post-transcriptional approach to antioxidant defense is too late in the disease cascade. Rather than an exogenous antioxidant approach, we suggest that promoting and harnessing endogenous defenses may be more effective.

This is not a new suggestion. Statins are known to induce the NRF2 pathway [36] and reduce markers of preeclampsia [37]. A pilot clinical trial suggested that pravastatin was safe in early pregnancy and may be an effective preventative. Results of larger trials are awaited. Resveratrol is another NRF2 activator that we, [38] and others, [39] have shown can reduce trophoblast secretion of anti-angiogenic factors sFlt-1, sEng and activin A, and can mitigate endothelial dysfunction *in vitro*. However, it was recently reported that resveratrol may adversely affect fetal pancreatic development [40]. Given these safety concerns we explored another NRF2 regulator, sulforaphane. Despite clinical evidence of the safety and efficacy of sulforaphane, this compound has yet to be evaluated in pregnant women or in models of preeclampsia [41–43]. Therefore, as a first step our intent was to evaluate sulforaphane *in vitro*, using concentrations of sulforaphane similar to therapeutic *in vitro* levels [41–43].

We confirmed that sulforaphane improves endothelial health in HUVECs treated with TNFα. Others have shown that sulforaphane reduced TNFα-induced expression of VCAM and ICAM in human endothelial cell types [44,45]. As with resveratrol, [38] sulforaphane mitigated the placental secretion of sFlt-1, sEng and activin A. This prompted us to undertake experiments with isolated villous cytotrophoblasts. However, simulating hypoxic-reperfusion placental injury by hypoxia (1% oxygen) did not increase sFlt-1 or sEng secretion from villous cytotrophoblasts as it did in placental explants [46,47]. This has been reported by others [48]. We believe that this is most likely due to abnormal syncytial formation under hypoxic conditions [49]. However, sulforaphane did significantly reduce the secretion of sFlt-1 and activin A. Taken together, our observations suggest that sulforaphane may

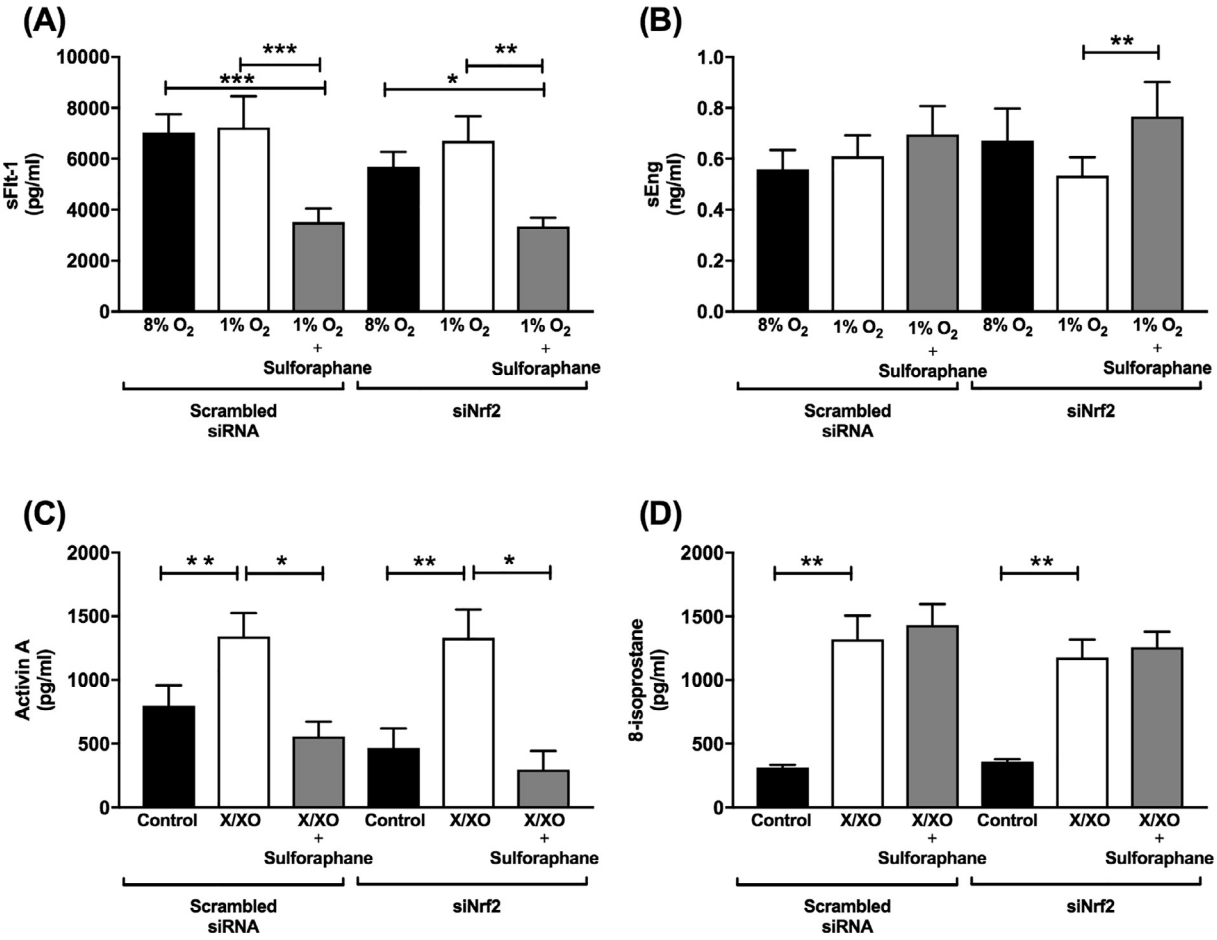


**Fig. 5.** Effect of sulforaphane on treated placental villous cytotrophoblasts. Trophoblasts were incubated for 24 h in 8% oxygen at 37˚c before silencing with single stranded mRNA silencing sequence directed at Nrf2 or scrambled negative sequence. After 48 h, samples were treated with either X/XO and returned to 8% oxygen at 37˚c, or moved to a hypoxic environment of 1% oxygen at 37˚c, in the presence of absence of sulforaphane (20 μM). At 72 h levels of (A) sFlt-1 and (B) sEng were quantified in hypoxic supernatant and (C) activin A and (D) 8-isoprostane measured in X/XO treated culture media. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$. n = 6 placentae.

offer promise as a preventative and adjuvant treatment for pre-eclampsia and that clinical trials would be worthwhile.

A novel aspect to our study is that we silenced the NRF2 pathway to explore the involvement of NRF2 in sulforaphane's effects. As we did, others have shown that the sulforaphane-induced decrease in adhesion molecules expression was independent of NRF2 [44,45]. We also found that sulforaphane suppression of sFlt-1 and activin A secretion from villous cytotrophoblasts was maintained after NRF2 knockdown. This suggests that sulforaphane confers placental protection through mechanisms beyond the NRF2 pathway. Whether NRF2 silencing results in cell death has yet to be confirmed but we observed no difference in protein levels between treatment groups (data not shown) suggesting that this was not the case. The role of NRF2 signaling and HO-1 protein abundance in villous cytotrophoblasts remains unclear [39]. Studies advocating antioxidant-mediated cytoprotection via the NRF2/HO-1 pathway did not use villous cytotrophoblasts [50]. In fact, HO-1 activation in primary cytotrophoblasts did not confer cellular protection, nor did silencing HO-1 increase secretion of antiangiogenic compounds [51]. Therefore, in villous cytotrophoblasts, sulforaphane may act via alternate antioxidant pathways such as Rho A/ROCK and NF-κB signaling [45].

Sulforaphane influences TGF-β signaling, inhibiting the pro-inflammatory 2/3 pathway and stimulating Samd1/5/8 to reduce immune activation [52,53] and improve cellular function, independently of NRF2. That sEng secretion from cytotrophoblast cells increased under hypoxic conditions in the presence of sulforaphane following NRF2 knockdown further implicates TGF-β signaling. As a co-receptor for the TGF-β receptor, endoglin mediates a pro-angiogenic effect via the TGF-βRII/ALK1/Smad1/5/8 signaling cascade, a mechanism involved in vascular proliferation and migration [54,55]. This pathway is strongly linked to angiogenic maintenance through activation of eNOS and vessel dilation. Though the exact mechanisms remain unclear, sEng is believed to induce Smad 2/3 anti-angiogenic signaling, resulting in inflammation and endothelial dysfunction [55]. As such, by accentuating TGF-β 1/5/8 signaling, sulforaphane may induce a positive feedback mechanism to increase endoglin synthesis [56]. This mechanism is important given that TGF-β signaling has been implicated in structural abnormalities of preeclamptic placentae [57].

In summary, we have shown that sulforaphane is both endothelial protective, largely though not solely via NRF2 mediated antioxidant actions, and has protective effects in reducing the placental production of antiangiogenic compounds. However, the lack of ability of sulforaphane to mitigate all injury, in either endothelial cells or trophoblast, likely reflects the multiple pathways underlying such injuries, only some of which are targeted by sulforaphane. Similarly, that some of the actions of sulforaphane are not blocked by NRF2 silencing suggests other, as yet unknown, mechanisms of action. It is clear that the development of novel preventative and therapeutic agents in pre-eclampsia remains dependent on further elucidation of the fundamental pathogenesis of the disease. That said, we suggest that the data reported here support further study of sulforaphane as a potential adjuvant therapy for preeclampsia.

## Conflict of interest

The authors have no conflicts of interest to declare.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.preghy.2019.02.002.

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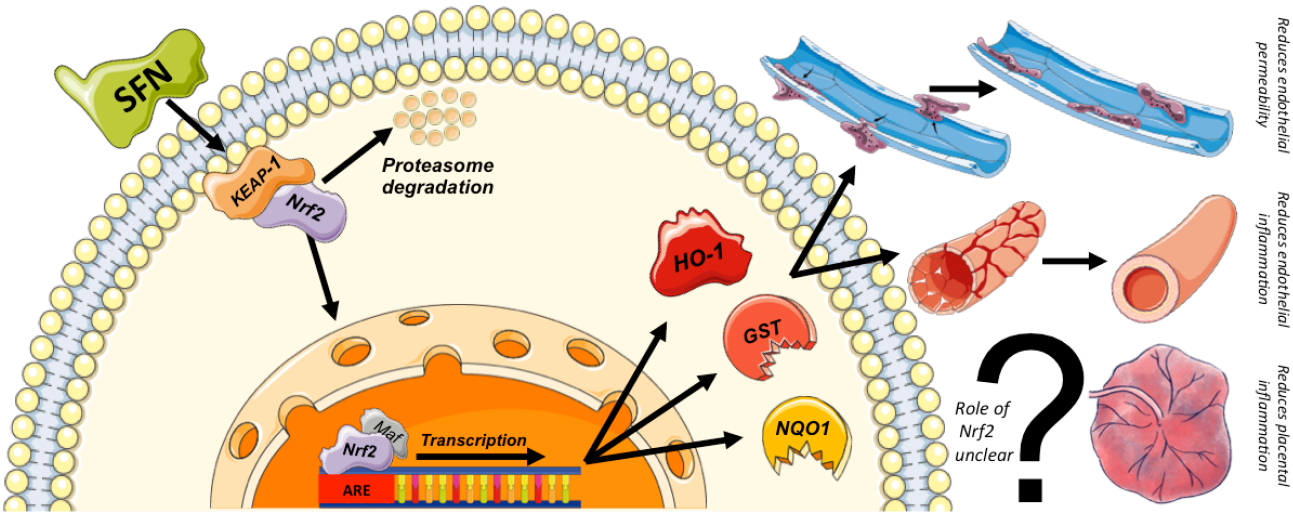


*Figure 1 Effect of sulforaphane on endothelial cells and the placenta.*

Figure 1. Sulforaphane up regulates NFE2L2 in human umbilical vein endothelial cells (HUVECs), reducing monolayer permeability and inflammation. Sulforaphane reduces placental inflammation however the mechanism behind this is unclear.

# Chapter four

## *Sulforaphane improves syncytiotrophoblast mitochondrial function after in vitro hypoxic and superoxide injury*

The studies in *Chapter three* demonstrated, broadly, the capacity of sulforaphane to target and mitigate placental oxidative stress. However, those studies did not have scope to fully explore the mechanism(s) underlying these effects. In the studies described in this second experimental chapter hypoxic and superoxide injury were explored more closely[11], specifically within placental mitochondria[22,82]. A role for disturbed mitochondrial dysfunction in preeclampsia was first recognised in the 1990s[23] and mitochondrial dysfunction after hypoxic reperfusion injury is now accepted as the driver for ROS build up in severe disease[22,82–84,85]. Mitochondrial dysfunction underlies the increased placental secretion of antiangiogenic factors that triggers the systemic inflammation and endothelial dysfunction of preeclampsia[86,87]. Already it is known that freshly isolated placental tissue from preeclamptic women have impaired mitochondrial function compared to that of normotensive women[21]. However, the effect of hypoxic-reperfusion injury has never been explored in the syncytiotrophoblast of cultured placental cells.

Mitochondria form an interconnected, dynamic network undergoing repeated fission and fusion events whereby multiple mitochondria fuse to a large single mitochondrion (fusion) or split into multiple smaller mitochondrion (fission)[88–90]. The balance of this process is essential for cellular homeostasis and ensures maintenance of healthy mitochondrial structure with stability of the matrix membrane which hosts the five complexes of the electron transport chain, as shown in figure 2[89]. Low oxygen tensions induce abnormal fission and fusion dynamics, generating small mitochondria with low motility[91]. Hypoxic insult induces mitochondrial permeabilization and breakdown and, when extensive, can initiate pathways of mitophagy and

intrinsic cellular apoptosis[84]. The integrity of the mitochondrial membrane is crucial for respiration through the electron transport chain and consequential oxidative phosphorylation of ADP into ATP for cellular energy[89,92]. During respiration, electrons flow along the mitochondrial electron transport chain promoting efflux of hydrogen ions from the mitochondrial matrix to the intermembrane space[89,92]. Consequently, protons flow down an electrochemical gradient through the final complex, ATP synthase, which phosphorylates ADP into the ATP needed for cellular energy and function[93]. However, the respiration process is dependent on the presence of oxygen. In normoxic conditions, hydrogen combines with oxygen and accepts four electrons at complex IV. In hypoxia, insufficient oxygen impairs electron flow resulting in an accumulation of electron ions near complex II and limiting formation of the necessary proton gradient for ATP phosphorylation[24,91]. Problematically, these electrons can leak through the membrane into the matrix [94]. Reperfusion injury then overwhelms the chain with excessive oxygen molecules that combine with free electrons forming oxygen anions, and resulting in the excessive ROS production that underpins preeclampsia[20,25,85].

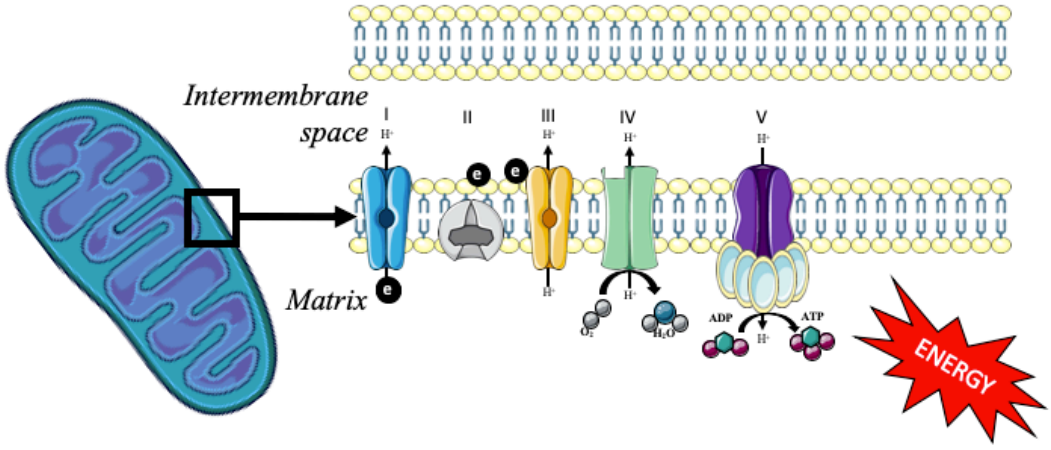


*Figure 2. Mitochondrial electron transport chain*

Figure 2. Electrons flow through the electron transport chain creating a proton gradient that enables synthesis of ATP from ADP at complex IV.

An antioxidant that acts to stabilise the mitochondria and improve ATP production and electron flow may offer a useful adjuvant therapy for preeclampsia. One such compound is sulforaphane. It is known that sulforaphane can stabilise fission/fusion[95] dynamics. However, whether it can improve respiration is yet to be evaluated. In fact, the response of the syncytiotrophoblast mitochondria to hypoxic reperfusion injury is also unknown. The effect of hypoxic and reperfusion injury on the placental syncytiotrophoblast, under both chronic and acute conditions and whether sulforaphane can mitigate or prevent this injury, is investigated in this chapter. Finally, preeclamptic placentae were collected and the effect of sulforaphane on respiration in preeclamptic placental cells was established.

***Hypothesis***

Hypoxia and superoxide induce trophoblast mitochondrial dysfunction in both acute and chronic settings. Sulforaphane is able to protect against these effects.

***Aims***

1. To investigate mitochondrial dysfunction in placental trophoblasts after hypoxic and superoxide injury in acute and chronic settings.
2. To investigate the capacity of sulforaphane to mitigate mitochondrial dysfunction in placental trophoblasts after injury, and in the placenta of women with preeclampsia.

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# Sulforaphane improves syncytiotrophoblast mitochondrial function after *in vitro* hypoxic and superoxide injury

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ABSTRACT

*Introduction:* Placental mitochondrial dysfunction contributes to the oxidative stress that underlies preeclampsia. Here, we assessed whether sulforaphane (SFN) could improve syncytiotrophoblast mitochondrial function after *in vitro* hypoxic and superoxide injury.

*Methods:* Placental cytotrophoblasts were isolated from healthy term placentae (n = 12) and incubated for 48 h in 8% $O_2$ ± 1 μM SFN before acute (4hrs) or chronic (24hrs) hypoxic (1% $O_2$), or superoxide (xanthine/xanthine oxidase) injury. Cytotrophoblasts were also isolated from preeclamptic placentae (n = 5) and cultured in 8% $O_2$ ± 1 μM SFN. Mitochondrial respiration was measured using the Seahorse MitoStress XF assay. Cells were stained with mitotracker red to assess mitochondrial membrane health and mitochondrial gene expression assessed using RT-qPCR.

*Results:* SFN prevented significant reductions in syncytiotrophoblast mitochondrial maximal respiration, spare respiratory capacity, basal respiration and ATP production following acute hypoxia. Chronic hypoxia only reduced maximal and spare respiratory capacity. SFN prevented these negative changes and increased respiration overall. Alternatively, acute superoxide injury significantly increased mitochondrial maximal respiration and spare respiratory capacity. SFN treatment further increased basal respiration following superoxide injury and prevented significant decreases in ATP production and coupling efficiency. In preeclamptic placentae, SFN significantly increased mitochondrial maximal respiration, spare respiratory capacity, basal respiration and ATP production, and decreased proton leak. SFN up-regulated mRNA expression of mitochondrial complexes and corrected an up-regulation in fission gene expression observed after hypoxic-superoxide injury. Finally, preliminary results suggest SFN prevented hypoxia-induced impairment of mitochondrial membrane structure.

*Discussion:* SFN mitigated hypoxia and superoxide induced changes to syncytiotrophoblast mitochondrial function *in vitro,* and improved mitochondrial respiration in trophoblast cells from preeclamptic placentae.

## 1. Introduction

Preeclampsia is a pregnancy specific disorder characterised by hypertension with associated maternal organ dysfunction and/or fetal growth restriction [1]. Severe preeclampsia remains a leading cause of maternal and perinatal morbidity and mortality [1,2]. Though much remains unclear about the pathophysiology of preeclampsia, it is generally accepted that inadequate spiral artery remodelling early in pregnancy results in repeated and progressive hypoxic reperfusion injury to the placenta that results in significant placental oxidative stress [3–5]. This in turn triggers excessive release of a number of anti-angiogenic and pro-inflammatory compounds that impair maternal vasculature function [6–8]. The resulting vascular damage manifests as hypertension, oedema and the end organ injury recognised clinically as the syndrome of preeclampsia.

Over the past decade, better insights into the placental injury that underlies the clinical manifestation of preeclampsia has offered opportunities for the development of novel therapies [9,10]. In particular, there is increasing attention on placental mitochondria as a therapeutic target [11,12]. Mitochondria are key modulators of cellular reactive

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oxygen species (ROS) homeostasis [13]. Accordingly, mitochondrial dysfunction induced by sustained hypoxia-reperfusion injury is thought to be a major contributor to the excessive placental oxidative stress that underpins severe preeclampsia [11,12,14,15].

Protecting placental mitochondria from hypoxic and superoxide injury may be an effective approach to the prevention or management of preeclampsia. SFN is a naturally occurring organosulfur antioxidant present in cruciferous vegetables that may mitigate the pathogenic mechanisms driving preeclampsia. SFN has a well-documented safety profile [16] and, as a nutritional supplement, is particularly appealing for use in pregnancy [9]. In fact, animal studies suggest SFN may have fetal-protective effects [17]. SFN has long been recognised as a potent antioxidant via activation of the ROS scavenging NFE2L2/ARE pathway [18] and, more recently, has been shown to protect human retinal pigment cell mitochondria after induction of apoptosis with staurosporine, by stabilising fission/fusion dynamics [19]. However, the unique cell structure of the syncytiotrophoblast and the innately low oxygen tension of the placenta [20,21] means response to hypoxic reperfusion injury in this cell type is unlikely to reflect that of any other cell population. Whether SFN can improve trophoblast mitochondrial function in the setting of hypoxia-reperfusion has not yet been reported. Here, we investigated the effects of *in vitro* hypoxic and superoxide injury on mitochondrial structure and function in primary human syncytiotrophoblasts, and whether SFN was able to mitigate any injury.

## 2. Materials and methods

### 2.1. Ethics and tissue collection

This study was approved by the Monash Health Human Research and Ethics Committee (HREC: 01067B). Healthy term placentae from singleton pregnancies were collected, with informed written consent, at the time of elective caesarean section. Exclusion criteria included multiple pregnancy, maternal medical conditions, any medication, any history of preeclampsia and/or fetal growth restriction, any smoking, alcohol or drug use during pregnancy. Patient demographics are presented in Table 1 [22]. Placentae were also collected, with informed written consent, from women with established early-onset (<34 weeks) preeclampsia, as defined by Australian national clinical guidelines [1].

**Table 1**
Population demographics.

| | Normotensive placentae (n = 12) | Preeclamptic placentae (n = 5) |
|---|---|---|
| Gravidity | 2.5(1.25–2.75)[c] | 1(1–2.5)[c] |
| Parity | 2(1–3)[c] | 1(1–1.5)[c] |
| Gestational age (wks) | 39.04 (0.63)[a] | 30.33(2.38)[a] |
| Maternal age (yrs) | 33.75(4.07)[a] | 31(2.76)[a] |
| Country of birth | | |
| *Asia* | 9(75%)[b] | 0(0%)[b] |
| *Australia* | 3(25%)[b] | 5(100%)[b] |
| Antenatal medications | | |
| *Multi vitamin* | 12(100%)[b] | 5(100%)[b] |
| *Iron* | 4(33%)[b] | 1(20%)[b] |
| *Antihypertensive* | 0(0%)[b] | *Labetalol:* 5(100%)[b] *Nifedipine:* 4(80%)[b] *Hydralazine:* 1 (20%)[b] |
| Drugs | | |
| *Cigarettes* | 0(0%[b] | 2(40%)[b] |
| *Alcohol* | 0(0%)[b] | 0(0%)[b] |
| Antenatal admissions | (8.3%)[b] | 5(100%)[b] |
| *Diagnoses* | Braxton Hicks contractions | Preeclampsia |
| Blood pressures >140/90 mmHg | 0(0%)[b] | 5(100%)[b] |
| Screened for diabetes | 12(100%)[b] | 3(60%)[b] |
| *Unknown* | 0(0%)[b] | 2(40%)[b] |
| Antenatal steroids | 0(0%)[b] | 5(100%)[b] |
| Magnesium sulfate | 0(0%)[b] | 2(40%)[b] |
| Delivery mode | | |
| *C-section repeat* | 9(75%)[b] | 1(20%)[b] |
| *C-section primary* | 3(25%)[b] | 4(80%)[b] |
| *Labour* | 0(0%)[b] | 0(0%)[b] |
| Birth weight | 3396.25(372.15)[a] | 1319.75(413.82)[a] |
| Placental weight | 660.72(130.39)[a] | 190.3(76.9)[a] |
| *Unknown* | 0(0%)[b] | 1(20%)[b] |
| Baby's sex | | |
| *Female* | 4(33%)[b] | 2(40%)[b] |
| *Male* | 8(67%)[b] | 3(60%)[b] |
| Delivery to processing time (mins) | 28.38(7.89)[a] | 32.2 (7.69)[a] |

[a] Mean (standard deviation.
[b] Number (%).
[c] Median (interquartile range).

### 2.2. Trophoblast isolation

Placental trophoblasts were isolated as previously described [23,24]. Briefly, placental tissue was dissected and vigorously washed in chilled 1 x phosphate buffered solution (PBS) to remove blood. The fetal plate and decidua basalis were cut away and villous tissue scraped to remove any visible vasculature. Villous tissue then underwent three 20-min incubations in digestion buffer; 1 x Hank's Balanced Salt Solution (HBSS), 1 M HEPES, 7.5% sodium bicarbonate, 0.25% trypsin (Merck, Boston, MA) and 0.01% DNase I Grade II (Sigma-Aldrich, St Louis, MO) at 37 °C with agitation. Supernatant containing cells was immediately filtered and centrifuged to remove debris (21 °C, 10 min 1000 *g*). Supernatant was then resuspended in 1 x HBSS and gently layered onto 5–70% Percoll gradients (Sigma-Aldrich). Gradients were centrifuged with no brakes (21 °C, 25 min 1200 *g*). Layers from 50% to 30% were collected, diluted 4 x in complete trophoblast media; (Life-Technologies Noble Park, VIC), 10% Fetal Bovine Serum (ThermoFisher, Noble Park, VIC) and 1% antimycotic antibiotic (Life Technologies), and centrifuged to remove Percoll (21 °C, 10 min 1000 *g*). Cells were diluted 1:5 in trypan blue and a cell count manually performed using a haemocytometer.

### 2.3. Antibody purification

Cells were then diluted to a concentration of $10^7$/ml in complete trophoblast media and anti- CD9 antibody (MAB1880, In Vitro Technologies, Victoria, Australia) added at a concentration of 1 μg/ml [25]. After 10 min incubation on rotation at 4 °C, cells were washed in 2 x volume of 0.01% BSA/PBS and centrifuged (21 °C, 5 min 1000 *g*) to form a cell pellet. Dyanabeads™ coated in Goat AntiMouse IgG (ThermoFisher) were prepared according to manufacturer guidelines and cells incubated with dynabeads (4 °C on rotation, 30 min) at a concentration of 50 μl of dynabeads per $10^7$ cells in glass vials. Vials were placed on a magnet for 2 min and supernatant collected. A cell count was manually performed with a 1:5 dilution using trypan blue and a haemocytometer. Cells were centrifuged to form a pellet (21 °C, 10 min 1000 *g*) and resuspended in complete trophoblast media at a concentration of $10^7$/ml and plated at optimised densities on fibronectin (ThermoFisher) coated plates.

### 2.4. Treatment and hypoxic injury

Cells were incubated in 8% $O_2$, 37 °C, 5% $CO_2$ for 24 h to allow for adhesion. After 24 h, cells were either incubated in a loading dose of 1 μM sulforaphane (SFN) (Sigma-Aldrich) or remained in standard culture media, for a further 24 h. Cells were then given a second dose of 1 μM sulforaphane or not and were placed in one of five conditions; 1) 8% $O_2$ for 24 h, 2) 1% $O_2$ for 24 h, 3) 8% $O_2$ for 20 h and 1% $O_2$ for 4 h, 4) 2.3 mM xanthine and 0.015 U/ml xanthine oxidase (X/XO, Sigma-Aldrich) as previously described [26] for 24 h and, 5) 8% $O_2$ for 20 h and X/XO for 4 h. Preeclamptic cells were incubated in 8% $O_2$, 37 °C, 5% $CO_2$ for 24 h. Cells were then treated with 1 μM sulforaphane and incubated for a further 48 h in 8% $O_2$, 37 °C, 5% $CO_2$. Cells were incubated in a hypoxic

cell chamber flushed with nitrogen gas and supplemented with 5% $CO_2$ and the required oxygen tension. While hypoxic workstations were not available at our facility, all treatments were performed quickly and bench time did not exceed 10 min.

### 2.5. Assessment of mitochondrial respiration

We assessed mitochondrial function in incubated, fused syncytiotrophoblasts, 72 h after primary isolation. To avoid disrupting the syncytium in normal mitochondrial extraction protocols, whole cell respiration was assessed using the XFp Extracellular Flux Analyser (Seahorse Bioscience, Mulgrave, Victoria, Australia) [27,28]. Cytotrophoblasts were plated at a density of $8 \times 10^5$ on seahorse XFp plates (Agilent, Santa Clara, CA), as previously described [27]. Mitochondrial respiration was assessed using the Seahorse XFp analyser and Mito Stress Test Kit according to manufacturer guidelines. Oxygen consumption rate (OCR) was measured at baseline to determine basal respiration and after injection of optimised doses of oligomycin (15 μM), carbonyl cyanide p-trifluoromethoxy-phenyldydrazone (FCCP, 1 μM), and antimycin A/rotenone (10 μM) to determine non-mitochondrial respiration, maximal respiration, spare respiratory capacity, ATP production, coupling efficiency and proton leak (mito stress test). Cells underwent trypsinisation (15-min, 0.25% trypsin in EDTA, 37 °C) before being spun at 1200 g for 5 min. The supernatant was discarded, the pellet washed in 50 μL of PBS and resuspended in RIPA buffer. Cells were incubated for 30 min at 4 °C with agitation to induce lysis. Cells underwent centrifugation for 20 min and the protein lysate collected and measured using a bichinchonic acid assay (ThermoFisher). Readings were taken using a spectrometer at a wavelength of 460 nm and were used to normalise respiration data according to the Bradford method. Raw values for mitochondrial respiration were normalised to protein concentration/sample. Data are expressed relative to the control group.

### 2.6. Gene expression

Cells for RNA collection were incubated at a density of $2 \times 10^6$ per well on 12 well plates in complete trophoblast media. Following 72 h of incubation, including the respective injury, cells were trypsinised (0.25% trypsin in EDTA, 37 °C) for 10 min. Cells were centrifuged (21 °C, 10 min 1000 *g*) and the pellet resuspended in RNAlater. RNA and protein were extracted using the Paris Kit (ThermoFisher) and 1 μg of RNA reverse transcribed into cDNA (Qiagen RT-PCR QuantiTech, Melbourne, Victoria, Australia). Gene expression for markers of mitochondrial injury *(BAX:BLC2, NFE2L2)*, density *(tRNA:B2M)*, biogenesis *(NRF1, PCG1)*, complexes *(MTCO1, MTCO2, MTCO3)*, fission *(MFF, DNML1, FIS1)*, fusion *(OPA1, MFN1, MFN2)* and energy metabolism *(SIRT1, SIRT3)* was quantified using BioMark© Dynamic Array with Real-Time qPCR and TaqMan© probes (supplementary file 1). Gene data were analysed using the $^{\Delta\Delta}$CT, being normalised to *RN18S* housekeeping gene. Data are expressed relative to the control group.

### 2.7. Detection of mitochondrial membrane potential ($\Delta\Psi_m$)

Mitochondrial localisation was assessed by staining live cells (n = 3 placentae) after 72 h of incubation, with the mitochondrial dye Mitotracker Deep Red (MTDR) and tetramethylrhodamine methyl ester perchlorate (TMRM) to assess changes in mitochondrial membrane potential (MMP).

Cells were plated at a density of $4.5 \times 10^5$ on Sarstedt Chamber Slide 8 well (946170802 Sarstedt, Numbrecht, Germany) and incubated for 72 h. Cells were then stained for 15 min at 37 °C with 2 mM TMRM (T668, ThermoFisher 1:1000) and MTDR (M22426, ThermoFisher, 1:1000) in 300 μl of complete trophoblast media. A 1-h live imaging time-lapse was obtained using an Olympus FV1200 confocal microscope (Olympus, Notting Hill, Victoria, Australia). All images were taken at uniform acquisition exposure-time conditions, converted to binary and changes in florescence were measured in arbitrary units using image J software (NIH, MD, USA). The ratio of TMRM/MTDR intensity in mitochondria was reported. A low ratio of TMRM/MTDR indicates reduced mitochondrial membrane potential.

### 2.8. Statistical analyses

Mitochondrial respiration, normalised gene expression and mitochondrial membrane potential data were analysed using two-way ANOVA with the independent variables being injury and SFN treatment. Post hoc analysis was Holm-Sidak correction for multiple comparisons. Preeclamptic samples with/without SFN treatment were assessed using a paired Student's t-test. A $p \leq 0.05$ was deemed statistically significant. All analysis was performed using GraphPad Prism 7.0 (San Diego, CA).

## 3. Results

### 3.1. Mitochondrial respiration

We first investigated the effect of acute (4hrs) and chronic (24hrs) hypoxic injury on syncytiotrophoblast mitochondrial respiration and whether SFN pre-treatment could prevent adverse effects (Fig. 1, representative traces: Supplementary file 2.). Acute hypoxic injury non-significantly reduced maximal respiration (Fig. 1A; $P_{INT}$0.07) and significantly reduced spare respiratory capacity (Fig. 1B; $P_{INT}$0.03), basal respiration (Fig. 1C; $P_{INJURY}$0.03) and ATP production (Fig. 1D; $P_{INT}$0.03) and coupling efficiency (Fig. 1E; $P_{INT}$0.02) compared to the normoxic controls, by ~30%–70%. SFN pre-treatment increased overall maximal respiration (Fig. 1A; $P_{TREAT}$0.002) and basal respiration (Fig. 1C; $P_{TREAT}$0.01) and prevented hypoxia-induced decreases in spare respiratory capacity (Fig. 1B; $P_{TREAT}$0.001), ATP production (Fig. 1D; $P_{TREAT}$0.003) and coupling efficiency (Fig. 1E; $P_{TREAT}$0.04). There was no effect on proton leak.

Compared to acute hypoxia, chronic (24hrs) hypoxia had fewer effects on mitochondrial respiration (Fig. 1G-L). Only maximal respiration and spare respiratory capacity were reduced (60%, Fig. 1G; $P_{INJURY}$0.05 and 70%, Fig. 1H; $P_{INJURY}$0.01, respectively) compared to the normoxic controls. SFN increased overall maximal respiration (Fig. 1G; $P_{TREAT}$0.05), spare respiratory capacity (Fig. 1H; $P_{TREAT}$0.02), and basal respiration (Fig. 1I; $P_{TREAT}$0.02). Neither 24hrs of hypoxic injury nor SFN altered ATP production, proton leak or coupling efficiency (Fig. 1J-L).

The effects of 4hrs and 24hrs of superoxide stress, induced by X/XO, on mitochondrial respiration are presented in Fig. 2. Acute X/XO injury resulted in a different profile of injury to acute hypoxia. Compared to normoxic controls, maximal respiration, spare respiratory capacity and basal respiration were significantly increased in X/XO treated cells (Fig 2A; 85%, $P_{INJURY}$<0.0001; Fig 2B; 320% $P_{INJURY}$<0.0001; Fig. 2C; $P_{INJURY}$<0.0001). Overall, SFN significantly increased basal respiration (Fig. 2C; $P_{TREAT}$0.006), although this was independent of superoxide injury (Fig. 2C; $P_{INT}$0.36). Despite this increase in respiration after acute superoxide injury there was a 60% reduction in ATP production in untreated cells, compared to normoxic controls (Fig. 2D; $P_{INT}$0.0001). Pre-treatment with SFN doubled ATP production in superoxide injured cells (Fig. 2D; $P_{TREAT}$<0.0001). Not surprisingly, coupling efficiency was reduced by 30% after acute exposure to X/XO (Fig. 2E) and proton leak increased 2.7-fold (Fig. 2F). SFN pre-treatment prevented these derangements (Fig. 2E; $P_{INT}$0.001, $P_{TREAT}$0.002 and Fig. 2F; $P_{INT}$<0.0001, $P_{TREAT}$<0.0001).

Exposure to X/XO for 24hrs induced an injury profile that mirrored that of chronic hypoxia, with a reduction in maximal respiration after injury (Fig 2G; 50%, $P_{INJURY}$0.001) that was non-significantly mitigated with SFN (Fig 2G; 30%, $P_{TREAT}$0.07). Chronic X/XO also reduced spare respiratory capacity by 65%, an effect prevented by SFN (Fig. 2H; $P_{INJURY}$0.02, $P_{TREAT}$0.04). SFN increased basal respiration irrespective of

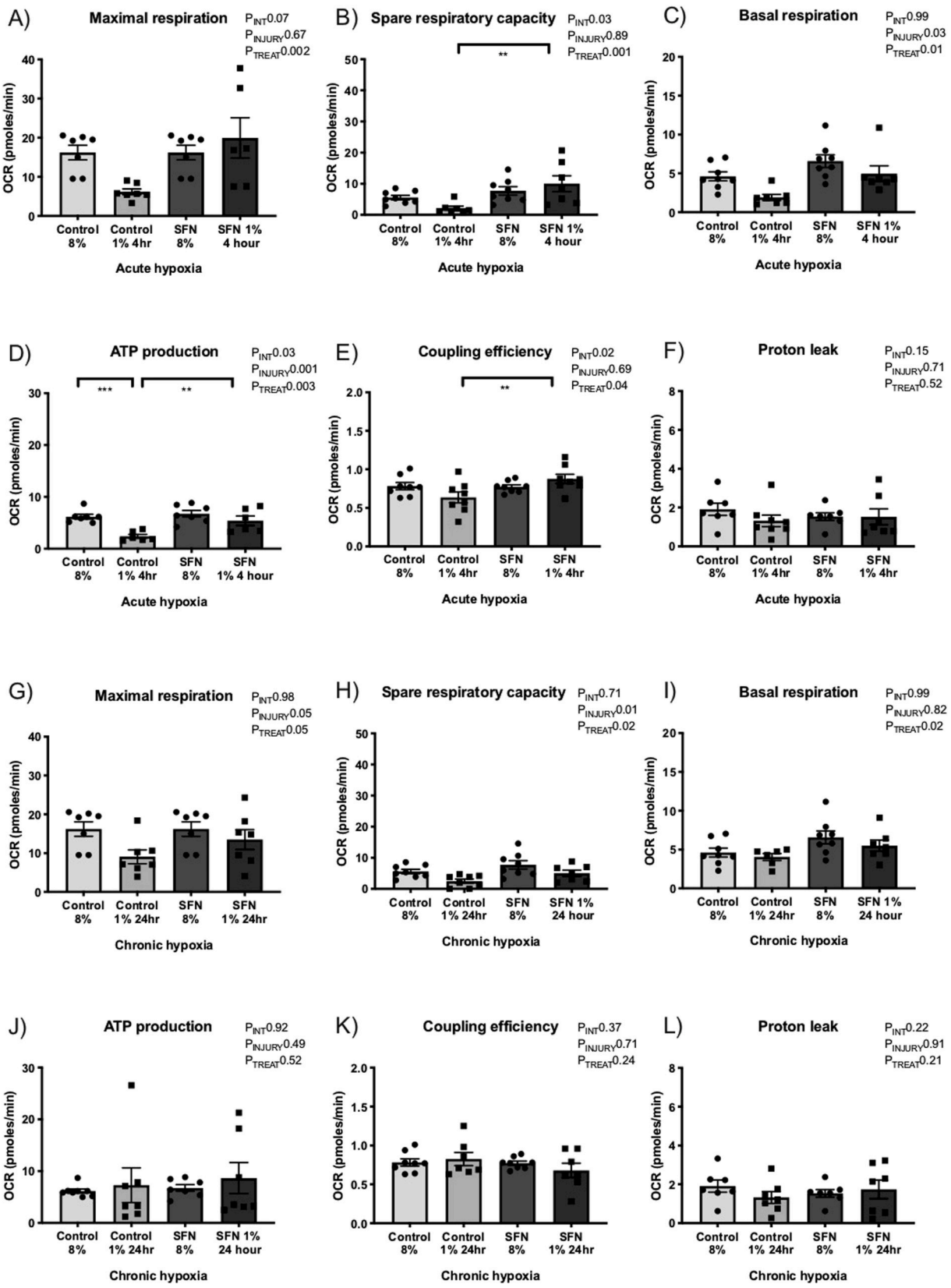


**Fig. 1. Mitochondrial Respiration following acute and chronic hypoxic insult.** Parameters of mitochondrial respiration, including maximal respiration (A & G), spare respiratory capacity (B & H), basal respiration (C & I), ATP production (D & J), coupling efficiency (E & K) and proton leak (F & L) following 4-h acute (A–F) and 24-h chronic (G–L) exposure to hypoxia ± 1 μM SFN. Data are presented as means ± SEM. Untreated normoxic and hypoxic groups are represented in light grey and SFN treated groups in dark grey. n = 8/treatment. Statistical analysis, Two-Way ANOVA with Holm-Sidak correction for multiple comparisons. Significance $P \leq 0.05$. Lines represent significance with post-hoc analysis, the asterisk indicates the degree of significance: * <0.05, ** <0.01, ***<0.001, ****<0.0001.

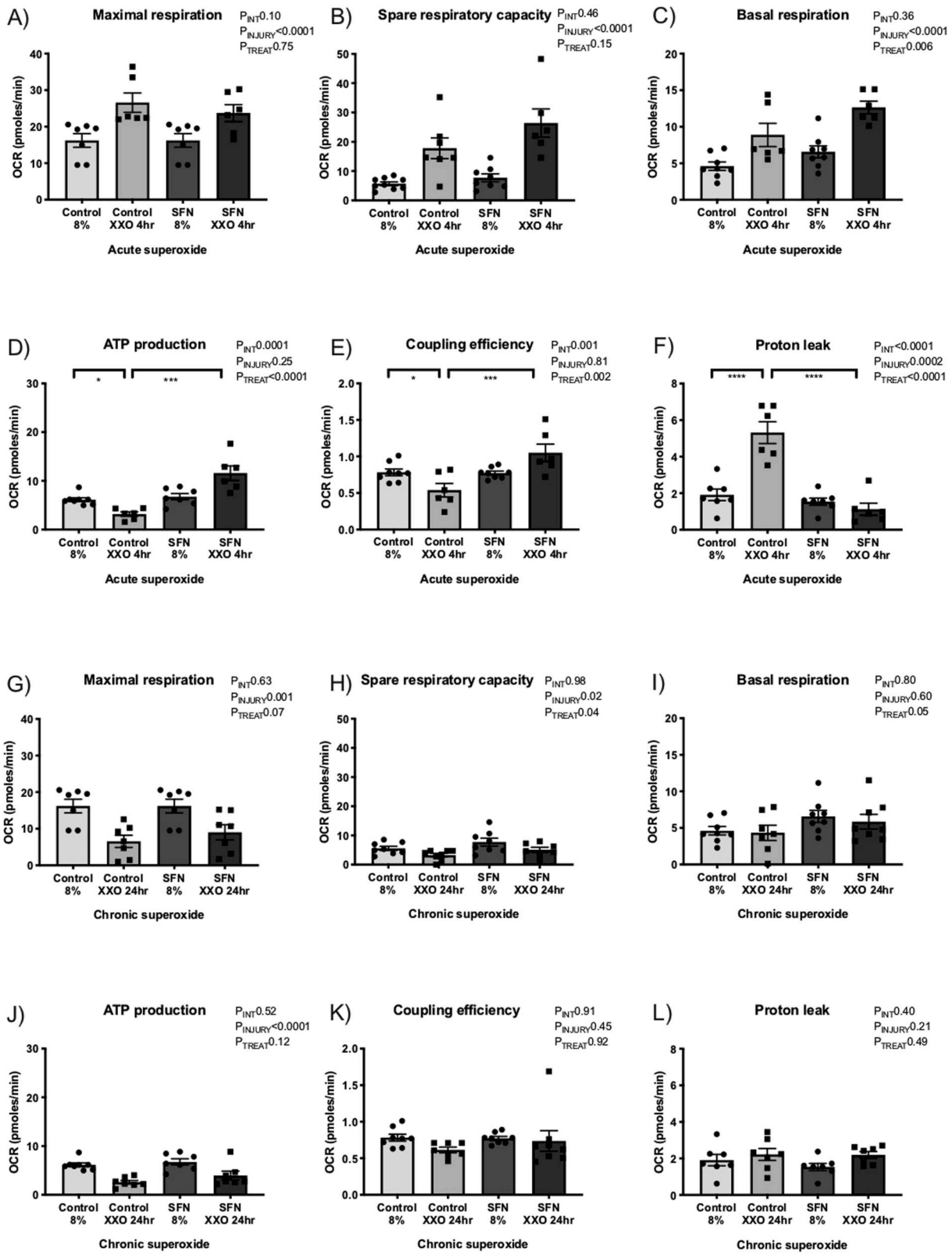


**Fig. 2. Mitochondrial Respiration following acute and chronic superoxide insult.** Parameters of mitochondrial respiration, including maximal respiration (A & G), spare respiratory capacity (B & H), basal respiration (C & I), ATP production (D & J), coupling efficiency (E & K) and proton leak (F & L) following 4-h acute (A–F) or 24-h chronic (G–L) xanthine/xanthine oxidase (X/XO) exposure ± 1 μM SFN. Data are presented as means ± SEM. n = 8/treatment. Statistical analysis, Two-Way ANOVA with Holm-Sidak correction for multiple comparisons. Significance $P \leq 0.05$. Lines represent significance with post-hoc analysis, the asterisk indicates the degree of significance: * <0.05, ** <0.01, ***<0.001, ****<0.0001.

injury (Fig 2I$P_{TREAT}$0.05). Overall, ATP production was reduced following chronic X/XO exposure (Fig. 2J; $P_{INJURY}$<0.0001), irrespective of SFN pre-treatment. Proton leak and coupling efficiency were unchanged.

Next, we explored effects of SFN on mitochondrial respiration in trophoblast cultures derived from preeclamptic placentae (Fig. 3). In preeclamptic placentae, SFN significantly increased maximal respiration (Fig 3A; 50%, p = 0.01), spare respiratory capacity (Fig 3B; 200%, p < 0.0001), basal respiration (Fig 3C; 50% p = 0.0025), and ATP production (Fig 3D; 40% p = 0.003) under normoxic (8% $O_2$) conditions. SFN significantly decreased proton leak by 40% (Fig 3F; p = 0.049) while coupling efficiency was unaffected (Fig 3E; p = 0.09).

### 3.2. Gene expression

Gene expression data are presented in Table 2. In summary of the significant findings, neither acute hypoxia nor SFN pre-treatment altered mRNA expression of the injury marker *HIF1a*. However, compared to untreated cells, SFN pre-treatment increased *NFE2L2* mRNA expression 62-fold ($P_{INT}$<0.0001, $P_{TREAT}$<0.0001). SFN pre-treatment also increased expression of the mitochondria biogenesis marker *NRF1* ($P_{TREAT}$0.047). Similarly, SFN increased mRNA expression of mitochondrial complex three *(MTCO3,* $P_{TREAT}$0.024*),* though not complexes one *(MTCO1)* and two *(MTCO2)*. Hypoxia decreased mRNA expression of the mitochondrial fission gene *MFF* ($P_{INJURY}$<0.0001), most significantly in SFN pre-treated hypoxic cells compared to normoxic controls and the hypoxic injured cells ($P_{INT}$0.0006). Hypoxic injury reduced *OPA1* mRNA expression ($P_{INJURY}$0.02), which was increased with SFN pre-treatment ($P_{INT}$0.038, $P_{TREAT}$0.034), although there was no effect from either acute hypoxia or SFN on other fusion genes (*MFN1, MFN2*) or modulators of energy metabolism (*SIRT1, SIRT3)*.

Chronic hypoxia (24hrs) reduced mRNA expression of injury marker *HIF1α* ($P_{INJURY}$0.03) and this effect was not changed with SFN pre-treatment. Though there was no affect from chronic hypoxic injury, SFN treatment increased mRNA expression of *NFE2L2* ($P_{TREAT}$0.04) and biogenesis marker *NRF1* ($P_{TREAT}$0.04). SFN increased mRNA expression of all three mitochondrial complexes (*MTCO1*: $P_{TREAT}$0.046, *MTCO2*:

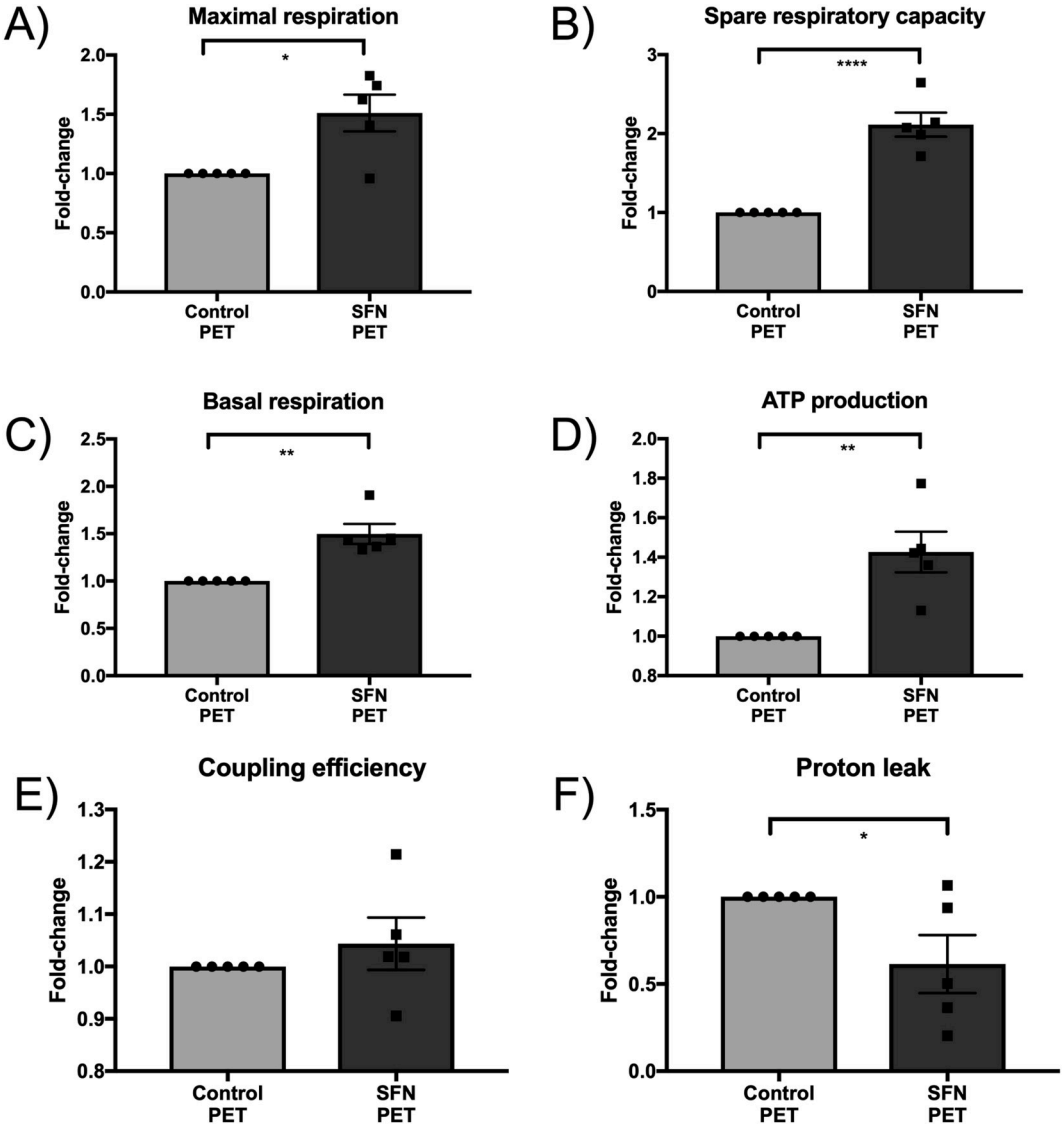


**Fig. 3. Mitochondrial Respiration in Syncytiotrophblasts of Preeclamptic Placentae.** Parameters of mitochondrial respiration, including maximal respiration (A), spare respiratory capacity (B), basal respiration (C), ATP production (D), coupling efficiency (E) and proton leak (F) in cytotrophoblasts from preeclamptic placentae (n = 5) incubated ± 1 μM SFN for 48 h. Untreated controls are represented in the dark grey bars and SFN treatment in the light grey. Data are presented as means ± SEM. n = 5/treatment. Statistical analysis, paired T-Test. Significance P ≤ 0.05. The asterisk indicates the degree of significance: * <0.05, ** <0.01, ***<0.001, ****<0.0001.

**Table 2**
Gene expression data.[a].

| | | | Norm | Norm SFN | Injury | Injury SFN | $P_{INT}$ | $P_{INJURY}$ | $P_{TREAT}$ |
|---|---|---|---|---|---|---|---|---|---|
| Hypoxia (4h) | **Injury** | *BAX: BCL2* | 1.29 ± 0.13 | 1.23 ± 0.15 | 0.85 ± 0.14 | 0.97 ± 0.09 | 0.49 | **0.02** | 0.82 |
| | | *tRNA* | 1.00 ± 0.16 | 1.28 ± 0.21 | 1.02 ± 0.21 | 1.15 ± 0.19 | 0.062 | 0.30 | 0.79 |
| | | *HIF1a* | 1.00 ± 0.13 | 1.18 ± 0.15 | 0.73 ± 0.14 | 0.12 ± 0.18 | 0.54 | 0.22 | 0.11 |
| | | ***NFE2L2*** | **1.00 ± 0.15$^{abc}$** | **1.48 ± 0.28$^{a}$** | **0.67 $^{a}$±0.11$^{bd}$** | **62.72 ± 2.3$^{cd}$** | **<0.0001** | **<0.0001** | **<0.0001** |
| | **Density** | *tRNA: B2M* | 1.43 ± 0.36 | 1.57 ± 0.23 | 2.63 ± 0.84 | 2.1 ± 0.58 | 0.49 | 0.11 | 0.35 |
| | **Biogenesis** | *NRF1* | 1.00 ± 1.10 | 1.41 ± 0.21 | 0.89 ± 0.6 | 1.23 ± 0.18 | 0.81 | 0.40 | **0.047** |
| | | *PACG1* | 1.00 ± 0.23 | 0.90 ± 0.22 | 0.84 ± 0.26 | 1.06 ± 0.25 | 0.51 | 0.98 | 0.80 |
| | **Complexes** | *MTCO1* | 1.00 ± 0.4 | 1.44 ± 1.3 | 1.01 ± 0.10 | 1.06 ± 0.12 | 0.34 | 0.38 | 0.24 |
| | | *MTCO2* | 1.00 ± 0.13 | 1.63 ± 0.39 | 1.04 ± 0.11 | 1.14 ± 0.13 | 0.74 | 0.65 | 0.37 |
| | | *MTCO3* | 1.00 ± 0.13 | 1.68 ± 0.35 | 1.03 ± 0.12 | 1.31 ± 00.14 | 0.22 | 0.92 | **0.024** |
| | **Fission** | ***MFF*** | **1.00 ± 0.35$^{a}$** | **1.28 ± 0.59** | **0.88 ± 0.32 $^{b}$** | **0.32 ± 0.14$^{ab}$** | **0.0006** | **<0.0001** | 0.33 |
| | | *DNML1* | 1.00 ± 0.10 | 1.32 ± 0.17 | 0.93 ± 0.11 | 1.17 ± 0.19 | 0.77 | 0.45 | 0.06 |
| | | *FIS1* | 1.00 ± 0.10 | 1.16 ± 0.15 | 0.83 ± 0.10 | 1.03 ± 0.16 | 0.88 | 0.26 | 0.17 |
| | **Fusion** | ***OPA1*** | **1.00 ± 0.4** | **1.21 ± 0.17** | **0.59 ± 0.23$^{a}$** | **1.19 ± 0.21$^{a}$** | **0.038** | 0.31 | **0.034** |
| | | *MFN1* | 1.00 ± 0.14 | 1.13 ± 0.20 | 0.61 ± 0.10 | 1.04 ± 0.21 | 0.42 | 0.17 | 0.10 |
| | | *MFN2* | 1.00 ± 0.12 | 1.19 ± 0.19 | 0.51 ± 0.09 | 1.11 ± 0.13 | 0.22 | 0.97 | 0.05 |
| | **Energy metabolism** | *SIRT1* | 1.00 ± 0.19 | 1.60 ± 0.37 | 0.650 ± 0.11 | 0.860 ± 0.16 | 0.386 | 0.07 | 0.16 |
| | | *SIRT3* | 1.00 ± 0.12 | 1.29 ± 0.15 | 1.00 ± 0.19 | 1.19 ± 0.19 | 0.87 | 0.63 | 0.10 |
| | | | **Norm** | **Norm SFN** | **Injury** | **Injury SFN** | $P_{INT}$ | $P_{INJURY}$ | $P_{TREAT}$ |
| Hypoxia (24h) | **Injury** | *BAX:BCL2* | 1.29 ± 0.13 | 1.23 ± 0.15 | 1.26 ± 0.16 | 1.13 ± 0.14 | 0.83 | 0.64 | 0.52 |
| | | *tRNA* | 1.00 ± 0.16 | 1.28 ± 0.21 | 1.41 ± 0.24 | 1.62 ± 0.29 | 0.87 | 0.29 | 0.11 |
| | | *HIF1a* | 1.00 ± 0.13 | 1.18 ± 0.15 | 0.75 ± 0.09 | 0.87 ± 0.14 | 0.95 | **0.03** | 0.35 |
| | | ***NFE2L2*** | 1.00 ± 0.15 | 1.48 ± 0.28 | 0.76 ± 0.11 | 1.11 ± 0.24 | 0.73 | 0.12 | **0.04** |
| | **Density** | *tRNA: B2M* | 1.43 ± 0.36 | 1.57 ± 0.23 | 3.55 ± 2.13 | 1.37 ± 0.30 | 0.74 | 0.73 | 0.88 |
| | **Biogenesis** | ***NRF1*** | 1.00 ± 1.10 | 1.41 ± 0.21 | 1.03 ± 0.6 | 1.22 ± 0.22 | 0.71 | 0.85 | **0.04** |
| | | *PCG1* | 1.00 ± 0.23 | 0.90 ± 0.22 | 0.71 ± 0.13 | 1.06 ± 0.28 | 0.27 | 0.68 | 0.51 |
| | | ***BCL2*** | **1.00 ± 0.06$^{ab}$** | **1.61 ± 0.24$^{a}$** | **0.69 ± 0.06$^{b}$** | **0.85 ± 0.12** | 0.11 | **0.0009** | **0.003** |
| | **Complex** | ***MTCO1*** | 1.00 ± 0.4 | 1.44 ± 1.3 | 0.97 ± 0.07 | 1.25 ± 0.17 | 0.66 | 0.55 | **0.05** |
| | | ***MTCO2*** | 1.00 ± 0.13 | 1.63 ± 0.39 | 1.00 ± 0.08 | 1.28 ± 0.16 | 0.55 | 0.54 | **0.03** |
| | | ***MTCO3*** | **1.00 ± 0.13** | **1.52 ± 0.35** | 0.95 ± 0.09 | 1.13 ± 0.14 | 0.60 | 0.43 | **0.05** |
| | **Fission** | *MFF* | 1.00 ± 0.09 | 1.28 ± 0.59 | 0.96 ± 0.09 | 1.26 ± 0.24 | 0.99 | 0.11 | 0.12 |
| | | *DNML1* | 1.00 ± 0.10 | 1.32 ± 0.17 | 0.96 ± 0.08 | 1.20 ± 0.18 | 0.96 | 0.80 | **0.02** |
| | | *FIS1* | 1.00 ± 0.10 | 1.16 ± 0.15 | 0.68 ± 0.08 | 0.96 ± 0.17 | 0.37 | 0.12 | 0.04 |
| | **Fusion** | *OPA1* | 1.00 ± 0.4 | 1.21 ± 0.17 | 0.95 ± 0.14 | 1.12 ± 0.20 | 0.89 | 0.66 | 0.24 |
| | | *MFN1* | 1.00 ± 0.14 | 1.13 ± 0.20 | 0.96 ± 0.16 | 0.99 ± 0.20 | 0.95 | 0.86 | 0.43 |
| | | *MFN2* | 1.00 ± 0.12 | 1.19 ± 0.19 | 0.87 ± 0.12 | 0.99 ± 0.19 | 0.83 | 0.29 | 0.31 |
| | **Energy metabolism** | ***SIRT1*** | 1.00 ± 0.19 | 1.60 ± 0.37 | 0.76 ± 0.11 | 1.03 ± 0.25 | 0.96 | 0.80 | **0.02** |
| | | *SIRT3* | 1.00 ± 0.12 | 1.29 ± 0.15 | 1.5 ± 0.19 | 1.67 ± 0.36 | 0.78 | **0.003** | 0.31 |
| | | | **Norm** | **Norm SFN** | **Injury** | **Injury SFN** | $P_{INT}$ | $P_{INJURY}$ | $P_{TREAT}$ |
| X/XO (4h) | **Injury** | *BAX: BCL2* | 1.29 ± 0.13$^{a}$ | 1.23 ± 0.15 | **2.48 ± 0.74$^{ab}$** | **0.61 ± 0.08$^{b}$** | **0.002** | 0.32 | **0.001** |
| | | *tRNA* | 1.00 ± 0.16 | 1.28 ± 0.21 | 1.34 ± 0.61 | 1.09 ± 0.37 | 0.39 | 0.82 | 0.96 |
| | | *HIF1a* | 1.00 ± 0.13 | 1.18 ± 0.15 | 1.4 ± 0.36 | 1.53 ± 0.35 | .90 | 0.10 | 0.51 |
| | | ***NFE2L2*** | **1.00 ± 0.15$^{a}$** | 1.48 ± 0.28 | **1.6 ± 0.43$^{b}$** | **3.31 ± 0.50$^{ab}$** | 0.06 | **0.0009** | **0.002** |
| | **Density** | *tRNA: B2M* | 1.43 ± 0.36 | 1.57 ± 0.23 | 1.37 ± 0.64 | 0.81 ± 0.23 | 0.39 | 0.31 | 0.61 |
| | **Biogenesis** | ***NRF1*** | 1.00 ± 1.10 | 1.41 ± 0.21 | 1.05 ± 0.40 | 1.90 ± 0.22 | 0.26 | 0.59 | 0.35 |
| | | *PCG1* | **1.00 ± 0.23$^{a}$** | 0.90 ± 0.22 | **2.4 ± 0.96$^{b}$** | **6.41 ± 1.84$^{ab}$** | 0.006 | <0.0001 | 0.008 |
| | **Complex** | ***MTCO1*** | **1.00 ± 0.4$^{a}$** | 1.44 ± 1.3 | **0.60 ± 0.14$^{b}$** | **2.05 ± 0.46$^{ab}$** | **0.001** | 0.14 | **0.0009** |
| | | ***MTCO2*** | 1.00 ± 0.13 | 1.63 ± 0.39 | **0.74 ± 0.23$^{a}$** | **1.86 ± 0.50$^{a}$** | **0.005** | 0.06 | 0.07 |
| | | ***MTCO3*** | **1.00 ± 0.13$^{a}$** | **1.52 ± 0.35$^{a}$** | 1.01 ± 0.28 | 1.52 ± 0.52 | 0.99 | 0.14 | 0.14 |
| | **Fission** | *MFF* | 1.00 ± 0.09 | 1.28 ± 0.59 | 1.11 ± 0.35 | 1.13 ± 0.33 | 0.56 | 0.95 | 0.50 |
| | | *DNML1* | 1.00 ± 0.10 | 1.32 ± 0.17 | 1.26 ± 0.23 | 1.52 ± 0.51 | 0.68 | 0.12 | 0.10 |
| | | *FIS1* | 1.00 ± 0.10 | 1.16 ± 0.15 | 1.04 ± 0.39 | 1.10 ± 0.33 | 0.67 | 0.43 | 0.30 |
| | **Fusion** | *OPA1* | 1.00 ± 0.4 | 1.21 ± 0.17 | 0.91 ± 0.34 | 0.82 ± 0.26 | 0.99 | 0.95 | 0.38 |
| | | *MFN1* | 1.00 ± 0.14 | 1.13 ± 0.20 | 1.11 ± 0.34 | 0.18 ± 0.41 | 0.68 | 0.36 | 0.32 |
| | | *MFN2* | 1.00 ± 0.12 | 1.19 ± 0.19 | 1.05 ± 0.32 | 1.01 ± 0.26 | 0.90 | 0.58 | 0.34 |
| | **Energy metabolism** | *SIRT1* | 1.00 ± 0.19 | 1.60 ± 0.37 | 1.58 ± 0.53 | 1.62 ± 0.68 | 0.74 | 0.32 | 0.27 |
| | | ***SIRT3*** | **1.00 ± 0.12$^{a}$** | **1.29 ± 0.15** | **0.19 ± 0.05$^{ab}$** | **1.12 ± 0.29$^{b}$** | **0.045** | **0.31** | **0.003** |
| | | | **Norm** | **Norm SFN** | **Injury** | **Injury SFN** | $P_{INT}$ | $P_{INJURY}$ | $P_{TREAT}$ |
| X/XO (24h) | **Injury** | *BAX: BCL2* | 1.29 ± 0.13$^{a}$ | 1.23 ± 0.15 | 0.91 ± 0.16 | 0.56 ± 0.12 | 0.38 | **0.003** | 0.21 |
| | | *tRNA* | 1.00 ± 0.16 | 1.28 ± 0.21 | 1.04 ± 0.22 | 1.03 ± 0.24 | 0.52 | 0.65 | 0.55 |
| | | *HIF1a* | 1.00 ± 0.13 | 1.18 ± 0.15 | 0.96 ± 0.12 | 1.01 ± 0.13 | 0.71 | 0.54 | 0.48 |
| | | ***NFE2L2*** | **1.00 ± 0.15$^{ab}$** | 1.48 ± 0.28 | **2.05 ± 0.13$^{a}$** | **2.2 ± 0.16** | 0.49 | **0.0009** | 0.21 |
| | **Density** | *tRNA: B2M* | 1.43 ± 0.36 | 1.57 ± 0.23 | 1.04 ± 0.27 | 0.99 ± 0.31 | 0.79 | 0.18 | 0.87 |
| | **Biogenesis** | ***NRF1*** | 1.00 ± 1.10 | 1.41 ± 0.21 | **0.95** ± 0.15 | 1.05 ± 0.21 | 0.45 | 0.23 | 0.32 |
| | | *PCG1* | **1.00 ± 0.23$^{ab}$** | 0.90 ± 0.22 | **24.8 ± 9.26$^{ac}$** | **4.06 ± 1.74$^{bc}$** | 0.008 | 0.007 | 0.0008 |
| | | ***BCL2*** | 1.00 ± 0.06 | 1.61 ± 0.24 | **1.13 ± 0.13$^{a}$** | **2.65 ± 1.11$^{a}$** | 0.28 | 0.17 | **0.02** |
| | **Complex** | ***MTCO1*** | **1.00 ± 0.4$^{a}$** | **1.44 ± 1.30** | **0.90 ± 0.10$^{b}$** | **6.90 ± 3.0$^{ab}$** | **0.004** | **0.006** | **0.001** |
| | | ***MTCO2*** | **1.00 ± 0.13 $^{a}$** | **1.63 ± 0.39** | **1.05 ± 0.12 $^{b}$** | **8.68 ± 3.9 $^{ab}$** | **0.006** | **0.006** | **0.002** |
| | | ***MTCO3*** | **1.00 ± 0.13$^{a}$** | **1.52 ± 0.35** | **0.86 ± 0.10$^{b}$** | **7.06 ± 0.32$^{ab}$** | **0.014** | **0.02** | **0.004** |
| | **Fission** | *MFF* | 1.00 ± 0.09 | 1.28 ± 0.59 | 1.27 ± 0.18 | 2.34 ± 0.66 | 0.21 | 0.008 | 0.03 |
| | | *DNML1* | 1.00 ± 0.10 | 1.32 ± 0.17 | 0.88 ± 0.23 | 1.06 ± 0.24 | 0.75 | 0.34 | 0.20 |
| | | *FIS1* | 1.00 ± 0.10 | 1.16 ± 0.15 | 0.58 ± 0.16 | 0.60 ± 0.11 | 0.69 | 0.003 | 0.61 |
| | **Fusion** | ***OPA1*** | **1.00 ± 0.4$^{ab}$** | 1.21 ± 0.17 | **0.49 ± 0.11$^{a}$** | **0.48 ± 0.09$^{b}$** | **0.78** | **0.0007** | 0.56 |

(*continued on next page*)

**Table 2** (*continued*)

| | | Norm | Norm SFN | Injury | Injury SFN | $P_{INT}$ | $P_{INJURY}$ | $P_{TREAT}$ |
|---|---|---|---|---|---|---|---|---|
| | *MFN1* | 1.00 ± 0.14 | 1.13 ± 0.20 | 0.67 ± 0.17 | 0.56 ± 0.15 | 0.55 | **0.03** | 0.95 |
| | *MFN2* | 1.00 ± 0.12 | 1.19 ± 0.19 | 0.73 ± 0.13 | 0.66 ± 0.14 | 0.48 | **0.03** | 0.72 |
| **Energy metabolism** | *SIRT1* | 1.00 ± 0.19 | 1.67 ± 0.37 | 1.33 ± 0.21 | 1.83 ± 0.33 | 0.88 | 0.12 | 0.43 |
| | ***SIRT3*** | **1.00 ± 0.12[ab]** | 1.29 ± 0.15 | **0.33 ± 0.15[a]** | **0.30 ± 0.07[b]** | 0.45 | **<0.0001** | 0.54 |

[a] Placental cells after injury with either hypoxia (1% $O_2$ 4 h or 24hrs) or Xanthine/Xanthine Oxidase (X/XO 4hrs or 24hrs), or in control conditions (8% $O_2$) in the presence or absence of sulforaphane (SFN). Data are expressed as delta change ± SEM. Two-Way ANOVA with Holm-Sidak correction for multiple comparisons. Statistical significance ($P < 0.05$). Alphabetical superscripts represent significance with post-hoc analysis. Corresponding letters indicate a significant difference between groups.

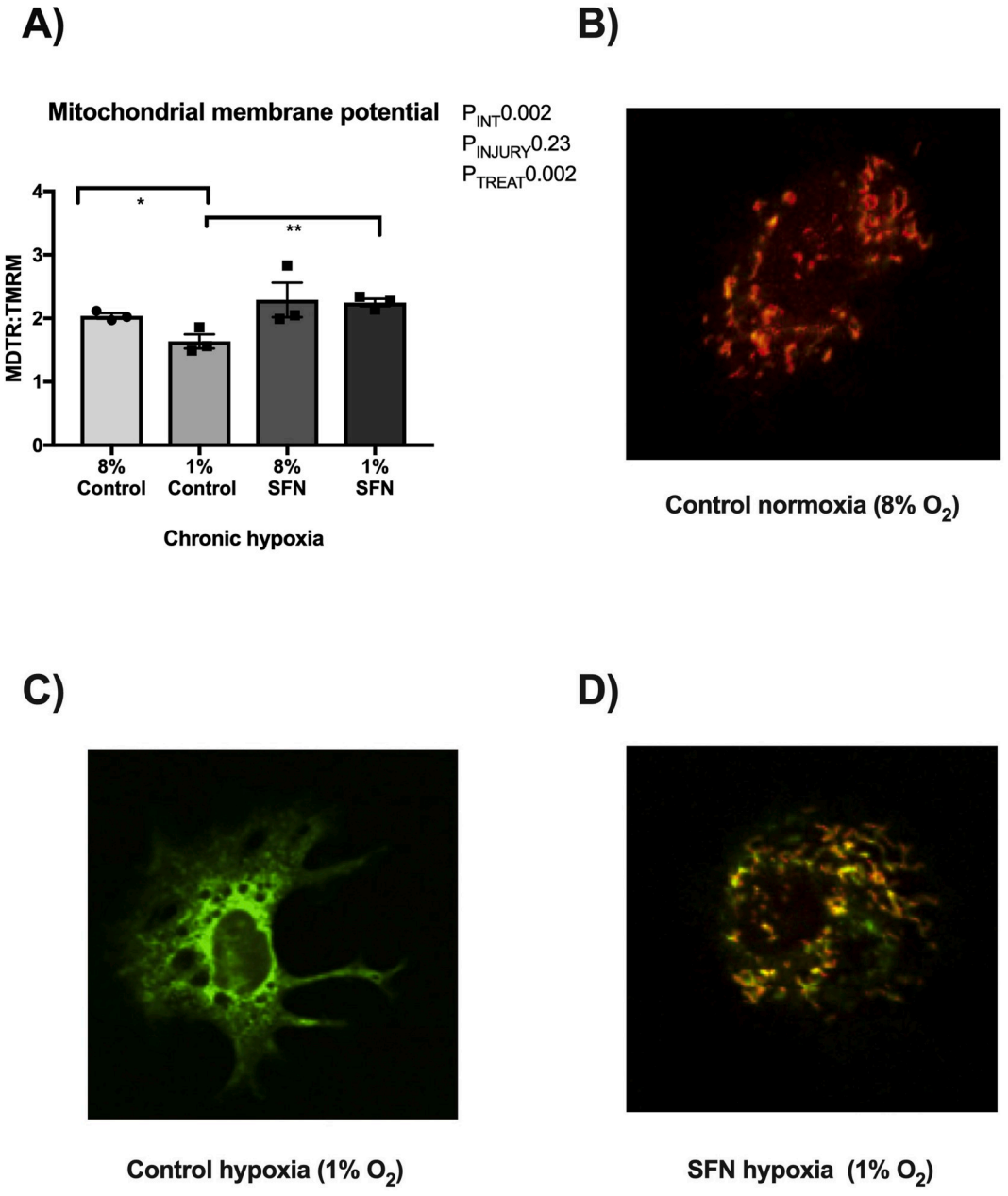


**Fig. 4. Live-cell Imaging for Assessment of Mitochondrial Membrane Potential.** MDTR:TMRM ratio (A) in untreated and SFN-treated cytotrophoblasts exposed to 24-h of hypoxia (1% $0_2$). Untreated cells are represented in the dark grey and SFN treated in the light grey. n = 8/treatment. Statistical analysis, Two-Way ANOVA with Holm-Sidak correction for multiple comparisons. Significance $P \leq 0.05$. Represented images of merged red TMRM and green MDTR staining are shown for (B) normoxic (8% $O_2$) control, (C) hypoxic (1% $O_2$) control and (D) SFN treated hypoxic (1% $O_2$) cells. Lines represent significance with post-hoc analysis, the asterisk indicates the degree of significance: * <0.05, ** <0.01.

$P_{TREAT}$0.03, *MTCO3*: $P_{TREAT}$0.047), though this occurred in the absence of any effect on mRNA expression from hypoxia. mRNA expression of fission gene *DNML1* was increased with SFN pre-treatment ($P_{TREAT}$0.02) as was the energy homeostasis modulator gene *SIRT1* ($P_{TREAT}$0.02), though chronic hypoxia had no effect on either. *SIRT3,* was increased in chronic hypoxia ($P_{INJURY}$0.003) and there was no effect from SFN pre-treatment.

Acute superoxide injury (4hrs 1% O2 + X/XO) increased cell death (*BAX:BCL2* ratio) 2.5-fold, which was prevented by SFN pre-treatment of the cells ($P_{INT}$0.002, $P_{TREAT}$0.001). Acute X/XO increased mRNA expression of *NFE2L2* ($P_{INJURY}$0.0009) and biogenesis gene *PCG1* ($P_{INJURY}<0.0001$), however mRNA expression was again higher in SFN treated X/XO injured cells for both *NFE2L2* ($P_{TREAT}$0.002) and *PLG1* ($P_{TREAT}$0.0008, $P_{INT}$0.006). Energy metabolism modulator gene *SIRT3* was down-regulated in acute X/XO by 80%, which was prevented by SFN pre-treatment ($P_{INT}$0.045, $P_{TREAT}$0.003). mRNA expression of complexes one and two were reduced following acute X/XO, which was prevented by SFN pre-treatment (*MTCO1*: $P_{INT}$0.0001, $P_{TREAT}$0.0009; *MTCO2*: $P_{INT}$0.005).

Chronic X/XO (24hrs + X/XO) increased *NFE2L2* mRNA expression 2-fold ($P_{INJURY}$0.0009) and SFN did not increase this further. Chronic hypoxia up-regulated mRNA expression of the biogenesis gene *PCG1* 24.5-fold. Though in SFN treated X/XO injured cells this remained higher than control ($P_{INJURY}$0.007), it was significantly less than that of X/XO control ($P_{INT}$0.008, $P_{TREAT}$0.0008). Chronic superoxides down-regulated mRNA expression of fusion genes *OPA1* ($P_{INJURY}$0.0007), *MFN1* ($P_{INJURY}$0.03) and *MFN2* ($P_{INJURY}$0.03). The energy modulator gene *SIRT3*, was also down-regulated ($P_{INJURY}<0.0001$) by chronic X/XO *SIRT1*. There was no effect from SFN pre-treatment on any fusion gene investigated. In chronic superoxide injury, SFN treatment resulted in a 7-fold increase in mRNA expression of mitochondrial complexes one (*MTCO1*:$P_{INT}$0.004, $P_{TREAT}$ 0.001) and three (*MTCO3:*$P_{INT}$0.014, $P_{TREAT}$ 0.004) and an 8-fold increase in complex two (*MTCO2:* $P_{INT}$0.006, $P_{TREAT}$ 0.002).

### 3.3. Density

To explore whether altered mitochondrial fission and fusion was accounting for the normalised respiration observed in the 24hr groups, we next looked at mitochondrial density markers (*tRNA:B2M* ratio, Table 2). There were no significant differences in mitochondrial density.

### 3.4. Detection of mitochondrial membrane potential ($\Delta\Psi_m$)

Next, we imaged chronic hypoxic cells to gain preliminary assessment of the mitochondrial membrane integrity (n = 3/treatment). Hypoxic cells had a lower TMRM/MTDR ratio than normoxic controls (Fig. 4A; $P_{INT}$0.002), indicating a modest reduction in mitochondrial membrane potential. This effect was prevented by SFN (Fig. 4A; $P_{TREAT}$0.0002, $P_{INT}$0.002).

## 4. Discussion

Here, we have shown that SFN is able to mitigate syncytiotrophoblast mitochondrial dysfunction induced by either hypoxia or superoxides *in vitro*. SFN also appears to improve the function of trophoblast mitochondria from preeclamptic placentae and even enhance mitochondrial respiration in control conditions. These observations suggest that SFN may be useful as either a preventative or adjuvant therapy for preeclampsia.

That acute hypoxia impaired mitochondrial respiratory function and reduced ATP production in syncytiotrophoblasts was not surprising. The acute depletion of oxygen renders insufficient oxygen molecules to act as the electron accepter at complex IV [4,13,29]. This results in the accumulation of electrons in the electron transport chain that then leak into the matrix where they combine with molecular oxygen to form superoxides, creating the oxidative stress that is characteristic of pre-eclampsia [4,13,29]. To investigate the role of oxidative stress, we quantified expression of *NFE2L2* mRNA, a major activator of intracellular ROS scavenging pathways. Though acute hypoxia alone did not alter the mRNA expression of *NFE2L2* an interaction between SFN and hypoxia greatly increased *NFE2L2* expression in treated cells. This increase in antioxidant pathways is a likely mechanism underlying the ability of SFN to rescue mitochondrial respiratory function during acute hypoxia.

Impaired electron flow induced by hypoxia also prevents the phosphorylation of ADP to ATP, reducing cellular energy production [30–32]. In an attempt to rescue ATP production in acute hypoxia, mitochondrial fission-fusion dynamics is skewed to fission, creating more but smaller organelles [30–32]. This reduces mitochondrial respiratory efficiency and increases proton leak by damaging the mitochondrial membranes [31]. Some of these changes were evident in our study. We saw a reduction in the expression of the pro-fusion gene *MFN1* but we did not see increases in fission genes or in proton leak. Nonetheless, under acute hypoxia SFN skewed mitochondrial dynamics away from fission towards biogenesis and fusion, as evidenced by increased mRNA expression of biogenesis gene *NRF1* and fusion genes *MFN2* and *OPA1* and a reduction in the fission gene *MFF*. These changes may explain the capacity of SFN to protect mitochondrial respiration acutely [33].

After 24hrs of hypoxia, syncytiotrophoblast mitochondrial respiration appeared to have largely adapted to hypoxic challenge. Only maximal and spare respiratory capacity remained reduced. Such mitochondrial adaptation has been reported by others [28]. The improved respiration in SFN pre-treated cells was likely secondary to an increase in all three cytochrome C oxidase subunits (*MTCO-1, MTCO-2, MTCO-3*), key components of the electron transport chain. Nonetheless, while resting ATP production was maintained in cells exposed to chronic hypoxia this may have been at the expense of mitochondrial membrane health [34], as evidenced by our preliminary data, which showed impaired membrane potential on live imaging. These observations will need to be followed-up with higher powered studies before conclusions can be drawn.

Acute oxidative stress, created by X/XO in the presence of low oxygen, induced a pattern of mitochondrial dysfunction quite distinct from that of hypoxic injury alone. Specifically, although baseline respiration was unchanged, both maximal and spare respiratory capacity increased. This may relate to the uncoupling effect of superoxides facilitating proton leakage from the electron transport chain, reducing the electrochemical gradient and diverting energy away from oxidative phosphorylation [35,36]. Such uncoupling would increase respiration and proton leak without increasing ATP production, as was observed in this study. The dramatic increase in mRNA expression of the antioxidant pathway, transcription activator *NFL2E2*, and likely corresponding reduction in cellular ROS in SFN pre-treated cells may explain the lack of proton leak and maintained ATP synthesis observed in these cells, despite acute superoxide injury. Instead, the increase in respiration detected may result from increased electron transport efficiency from an up-regulation in cytochrome C oxidase subunits I and II (*MTCO-1 MTCO-2*). After 24 h of superoxide injury this effect was lost and we saw a reduction in maximal and spare respiratory capacity, with ATP-linked respiration normalised.

We expected that the effect of chronicity of treatment on mitochondrial derangement would be explained by increased fission in the 24-h group. However, gene analysis of both chronic and acute X/XO treated cells suggested a tendency towards mitochondrial fission at both timepoints, though this was certainly more profound at 24 h. Interestingly, this corresponded with an increase in biogenesis gene *PCG1*, which was less pronounced in SFN pre-treated cells. Increased biogenesis may explain the normalisation of respiration observed at 24 h. When cells were pre-treated with SFN a pro-fusion state was maintained and the rise in *PCG1* was much less pronounced. Instead, SFN increased

mRNA expression of all three cytochrome C oxidase subunits (*MTCO-1, MTCO-2 MTCO-3*) after 24 h of superoxide injury. As at 4 h, this may explain the improved respiration in SFN treated X/XO cells.

That SFN significantly improved respiration in these mitochondria is consistent with our findings from the injury experiments and supports the possibility that SFN may be useful clinically to improve placental bioenergetics.

Overall our observations suggest that, at least *in vitro*, SFN is able to maintain mitochondrial respiration, optimise fission/fusion dynamics and protect mitochondrial membrane structure from both hypoxic and oxidative stress. However, our study has a number of limitations. Firstly, our *in vitro* model uses a preventative approach, treating cells with SFN before initiating injury, rather than a treatment approach. We chose this model as proof-of-principle and to allow SFN, a transcription activator [23], to alter gene expression prior to initiation of insult. Further studies are required to build on our proof-of-principle findings, to explore whether SFN can recover established injury. Promisingly, we were able to show SFN improves mitochondrial respiration in placentae of women with severe preeclampsia. In this study we were unable to identify whether sulforaphane can modulate syncytial formation in culture and this would be an interesting component for future investigations.

## 5. Conclusion

Acute hypoxia and acute superoxides caused distinct impairment of mitochondrial function, supporting the concept of repeated, alternating, hypoxic and superoxide injury causing significant placental injury in preeclampsia. We suggest that much of this injury in severe pre-eclampsia occurs at the level of the mitochondria, as have others. Significantly, we have provided evidence that the naturally occurring antioxidant SFN may prevent disturbances to mitochondrial function, in both chronic and acute settings. Therefore, we recommend further clinical assessment of SFN as a potential novel therapy for preeclamptic pregnancies.

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## Declaration of competing interest

The authors have no conflicts of interest to declare.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2020.05.005.

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# Chapter five

## *Sulforaphane improves vascular reactivity in mouse and human vessels after "preeclamptic-like" injury*

In the studies described in this chapter the effects of sulforaphane on the systemic vascular dysfunction of preeclampsia were explored. Specifically, these studies assess the effect of sulforaphane on the vasculature using an *ex vivo* model of preeclampsia.

The capacity of a vessel to relax to cumulative doses of endothelial dependent vasodilators is a marker of vessel health with reduced endothelial-dependant relaxation a measure of damage, as seen in women with preeclampsia[96,97]. Classical relaxation can occur via three different pathways: nitric oxide (NO), prostacyclin ($PGI_2$) and endothelium-dependent hyperpolarisation (EDH). NO-mediated vasorelaxation occurs via passive diffusion from the endothelium into smooth muscle cells. Here, NO binds and activates the enzyme guanylate cyclase. This then induces cleavage of cGTP to form cGMP, which activates cAMP, opening $Ca^{2+}$ channels. This inhibits phosphorylation of myosin by myosin-light-chain-kinase, therefore inducing vasorelaxation by preventing the cross-bridge cycle of constriction. In a similar mechanism, when activated, the prostacyclin receptor (IP) increases cAMP production. Finally, EDH is an additional mechanistic phenomenon first discovered when vasodilation was observed to persist even in the absence of NO and $PGI_2$ signalling[98,99]. Endothelial hyperpolarisation occurs after the activation of intermediate and/or small-voltage potassium-mediated calcium-channels ($IK_{Ca}$ and $SK_{Ca}$) resulting in depletion of intracellular calcium, causing vasodilation[100]. Vessel damage can also be identified through increased sensitivity to common vasoconstrictors. In human samples, a cumulative dose response to the potent vasoconstrictors thromboxane $A_2$ agonist U46619 and endothelin-1 can identify enhanced

constriction. Arteries exposed to "preeclamptic injury" would be expected to constrict more readily to endogenous vasoconstrictors and show a reduced response to vasodilation.

A model of preeclamptic vessel injury using both murine (mesenteric) and human (omental) vessels was created in this chapter. Placental explants were isolated from preeclamptic placentae and incubated in media for twenty-four hours. Placental trophoblasts were also collected and incubated in 1% $O_2$ for twenty-four hours to induce dysfunction. This media, rich in antiangiogenic compounds sFlt-1 and sEng was used to induce dysfunction in human (explant media) and mouse (trophoblast media) vasculature. Human omental arteries taken from women at the time of caesarean section were incubated in explant media, or placebo, +/- sulforaphane for three hours to assess for vascular dysfunction and determine if sulforaphane prevents this dysfunction. Mouse arteries were incubated for twenty-four hours in trophoblast media before vascular reactivity was measured. Others have used preeclamptic serum to induce myometrial artery dysfunction[96] and have used trophoblast media to induce dysfunction in mouse vasculature[101]. Vascular dysfunction secondary to sFlt-1 is commonly due to the endothelial specific effects of impaired NO production[102]. Whether sulforaphane could act as a direct vasodilator was investigated by exposing arteries directly to sulforaphane in a cumulative dose-response.

***Hypothesis***

Sulforaphane protects human and mouse arteries from "preeclamptic" injury, while directly inducing vasorelaxation in human and mouse arteries.

***Aims***

1. To investigate whether sulforaphane can protect human and mouse arteries from preeclamptic like injury.
2. To assess whether sulforaphane can act as a direct vasodilator in human and mouse arteries.

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# Sulforaphane improves vascular reactivity in mouse and human arteries after "preeclamptic-like" injury

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ABSTRACT

*Introduction:* The widespread maternal endothelial dysfunction that underlies the manifestations of preeclampsia is thought to arise from excessive placental production of antiangiogenic factors and enhanced oxidative stress. Therefore, we assessed whether the natural antioxidant sulforaphane could improve vascular function.
*Methods:* Cell viability of human umbilical vein endothelial cells (HUVECs) was assessed after 24 or 48 h in normoxia (20% $O_2$) or hypoxia (1% $O_2$) with or without sulforaphane. To model vascular dysfunction associated with preeclampsia, mouse mesenteric arteries were incubated in trophoblast conditioned media (TCM), and human omental arteries incubated in preeclamptic explant media (PEM) with or without sulforaphane. Both media are rich in antiangiogenic compounds associated with preeclampsia. TCM was generated from primary cytotrophoblast cells from term placentae of normotensive, while PEM was generated from explants from pre-eclamptic women. Reactivity was assessed by wire myography. sulforaphane's actions as a vasodilator were also investigated.
*Results:* Under conditions of hypoxia, sulforaphane improved HUVEC viability. In mouse mesenteric arteries, sulforaphane reduced contraction evoked by potassium ($p < 0.001$), phenylephrine and endothelin 1 (all $p < 0.001$). Sulforaphane also inhibited $Ca^{2+}$-induced contraction ($p = 0.014$). Sulforaphane prevented TCM-induced augmentation of phenylephrine and angiotensin II-mediated contraction of mouse mesenteric arteries. In human omental arteries, sulforaphane induced vasodilation ($p < 0.001$), and prevented PEM-induced endothelial dysfunction by restoring arterial sensitivity to the endothelium-dependent vasodilator bradykinin ($p = 0.008$).
*Discussion:* Sulforaphane causes relaxation in arteries and protects against arterial dysfunction induced by placental-derived antiangiogenic factors, which are known to contribute to the preeclampsia.

## 1. Introduction

Preeclampsia is a multi-system disorder characterised by new onset hypertension after 20 weeks' gestation with associated maternal organ dysfunction and/or fetal growth restriction [1]. It remains a leading cause of maternal and perinatal morbidity and mortality worldwide. Though much remains unclear regarding the pathophysiology of pre-eclampsia, the last twenty years have seen significant advances in our understanding of the disease [2]. Early in pregnancy incomplete spiral artery remodelling results in inadequate blood flow to the placenta and subsequent progressive and sustained hypoxic ischaemic injury throughout pregnancy [3]. The oxidative stress from this insult causes inflammation and excessive placental release of several inflammatory cytokines and vasoactive compounds into maternal circulation [3]. In turn, these compounds, such as soluble fms-like tyrosine kinase-1 (sFlt-1) [4], soluble endoglin [5,6] and activin A [7,8], induce dysfunction of the maternal vascular endothelium [9,10]. As the endothelium becomes damaged, its protective vasodilator influence is compromised and arteries constrict, inducing the hypertension and impaired perfusion of maternal organs, including kidney, liver, heart and brain, characteristic of preeclampsia [11,12]. As such, the second stage of preeclampsia can be considered, in principal, a disease of the maternal vascular endothelium [13].

Understandably, current therapies for preeclampsia are largely

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focussed on controlling maternal blood pressure [1,14]. The use of antihypertensives in preeclampsia has been responsible for reducing maternal stroke risk and maternal mortality [1,15]. However, the underlying endothelial dysfunction continues largely unabated by antihypertensives, such that the hypertension and end-organ damage eventually become refractory to therapy, and delivery of the baby becomes inevitable [1].

Ideally, the management of preeclampsia would include an approach that improves maternal vascular endothelial health, reduces oxidative stress and promotes vasorelaxation [2,14]. The naturally occurring isothiocyanate antioxidant sulforaphane may offer such an approach. **Sulforaphane is a safe and naturally occurring antioxidant found in cruciferous vegetables such as broccoli sprouts and may offer benefit to diseases characterised by oxidative stress.** An inducer of the antioxidant pathway nuclear factor erythroid 2 like-2 (NFE2L2) [16–19], sulforaphane shows promise in the treatment of cardiovascular disease outside of pregnancy [20–22]. Early *in vitro* studies also suggest that it may improve placental mitochondrial function [23], reducing placental oxidative stress that drives placental secretion of vasoactive compounds and the vascular dysfunction that jointly underlie preeclampsia [23]. Here, we aimed to investigate the effects of sulforaphane on human umbilical vein endothelial cell viability and vascular reactivity in rodent mesenteric and human omental arteries.

## 2. Materials and methods

### 2.1. Ethics

All animal experiments were approved by our institutional Animal Ethics Committee (MMCB2017/36) and conducted in accordance with the Australian Code of Practice and the National Health and Medical Research Council. Human placental and omental tissue collection was approved by the institutional Human Ethics Committee (HREC-01067B). **Human** tissue was collected after informed written consent from the donor.

### 2.2. Animals

Female adult wildtype C57Bl6J mice from the Animal Resource Centre (Perth, Australia) were used in this study, and were between 3 and 5 months of age. Mice were maintained on a 12 h light/dark cycle at 20 °C, with standard food pellets (Barastock, VIC, Australia) and water *ad libitum*.

### 2.3. Human umbilical vein endothelial cells

Primary human umbilical vein endothelial cells (HUVEC's) were collected from umbilical cords of healthy consenting women at the time of caesarean section (n = 8). Cells were isolated, as previously described [24–26]. Cells were grown on 0.2% gelatin in EBM™-2 endothelial cell growth media (Lonza) at 37 °C in a humidified atmosphere at 5% $CO_2$ and passaged to P2. Cells were plated at a seeding density of 2000 cells/well in a 96 well plate. Cells were incubated in the presence or absence of sulforaphane (1–50 μM) or placebo (DMSO, 1/565: equivalent to that in 50 μM sulforaphane) in quadruplicate. Cell viability was assessed using the CellTiter 96® $AQ_{ueous}$ One Solution Cell Proliferation Assay (MTS; Promega) following manufacturer's protocol after incubation for 24 or 48 h in normoxia (21% $O_2$) or hypoxia (1% $O_2$). Absorbance was measured at 490 nm in a 96 well plate reader (BioRad X-Mark microplate spectrophotometer).

### 2.4. Collection of human tissue

Placentae were collected from normotensive women undergoing elective caesarean >37 weeks gestation (n = 12) and from women diagnosed with early onset (<34 weeks) preeclampsia (n = 8). All placental samples were processed within 30 min of delivery. Additionally, omental tissue was collected from n = 8 normotensive women undergoing elective term caesarean section. Omental tissue was placed in cold in HEPES buffered physiological saline solution (PSS mmol/L: NaCl 120, KCl 5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, D-glucose 11.1, Na-HEPES 20 and $CaCl_2$ 2.5, and bubbled with carbogen (95% $O_2$ and 5% $CO_2$)) and stored at 4 °C until the time of assessment, usually within 2 h of collection. Inclusion criteria were: singleton pregnancy delivered at term without known fetal or maternal complications (thyroid disease, diabetes, hypertensive disorders of pregnancy). Exclusion criteria were multiple pregnancies, fetal and maternal complications, smoking, alcohol intake, drug use, medications (aspirin, antihypertensives, thyroxine, antipsychotics).

### 2.5. Trophoblast conditioned media

Primary cytotrophoblast cells were isolated from 12 term placentae of normotensive women having an elective caesarean section at term and cultured in Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS) and 1% streptomycin penicillin (Thermo Fisher Scientific, Auckland, NZ) for 24 h at 37 °C and 8% $O_2$, as previously described [24,26]. Cells were then incubated at $10^6$ cells/mL in 24 well plates for 72 h under hypoxic (1% $O_2$) conditions. On average, 8–12 mL of trophoblast conditioned media (TCM) was collected per term placenta. Conditioned media was collected and stored at −80 °C until later use. This type of media has previously been shown to cause endothelial dysfunction *ex vivo* in human umbilical vein endothelial cells [24,26,27] and in mouse mesenteric arteries [28].

### 2.6. Preeclamptic explant media (PEM)

Placentae of women with preeclampsia were collected at delivery. Placental cotelydons were excised, the basal and fetal surfaces removed and the placentae washed vigorously in cold Phosphate Buffered Solution (PBS) to remove all blood. Placental villi were dissected, as previously described [24,26], and a total weight of 100 g collected. Placental tissue was submerged in **6 mL** M199, 1% antibiotic-antimycotic, 1% L-glutamine (Thermo-Fisher) and incubated at 37°C and in 8% $O_2$. As explants were term samples they were cultured on the bottom of the well rather than in Matrigel [27]. After 24 h, conditioned media was collected and stored at −80 °C until later use. Preeclamptic media has been previously shown to have damaging effects on human vascular endothelium [28].

### 2.7. Isolation of mouse mesenteric arteries

Mice were euthanised by 2% isofluorane and cervical dislocation. The mesenteric arcade was isolated, as previously described[28], and immediately placed in ice-cold Krebs (physiological saline solution: PSS mmol/L: NaCl 120, KCl 5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25, D-glucose 11.1, and $CaCl_2$ 2.5, and bubbled with carbogen (95% $O_2$ and 5% $CO_2$)). Small mesenteric arteries (first and second order branches of the superior mesenteric artery) were isolated, cleared of fat and loose connective tissue and cut into rings 2 mm in length. After arteries were mounted on the myograph (model 610 M; Danish Myo Technology, Aarhus, Denmark), they were allowed to stabilise for 15 min at 37 °C before normalisation.

### 2.8. Reagents for vascular reactivity studies

All drugs were purchased from Sigma-Aldrich and dissolved in distilled water, except for sulforaphane (dissolved in dimethyl sulfoxide, DMSO; 5 mg/mL) and U46619 (ethanol). All subsequent dilutions were in distilled water.

### 2.9. Incubation of mesenteric arteries

Arteries from virgin mice were used, as this removed any influence of circulating pregnancy hormones. Each artery was incubated for 24 h at 37 °C and 5% $CO_2$ in a 16-well plate. In study 1, arteries were incubated in control medium (DMEM/F12, Life Technologies) with sulforaphane (1–20 μM) or DMSO (1/1400: equivalent to the dilution for 20 μM sulforaphane; n = 6–12 mice per treatment). These doses were selected as they are similar to those of our previous studies in human cells [24]. In study 2, arteries were incubated in control media (DMEM/F12) or trophoblast conditioned media (TCM; described below) to induce vascular dysfunction similar to that observed in preeclampsia [29] with or without sulforaphane (10 μM or 20 μM). After 24 h incubation, vascular reactivity was assessed, as described below. The incubation media was thoroughly washed out prior commencement of experimentation.

### 2.10. Assessment of vascular reactivity in mouse mesenteric arteries

To test tissue viability, all arteries were first exposed to high potassium PSS (KPSS; $K^+$ = 100 mmol/L, iso-osmotic replacement of $Na^+$ with $K^+$) and then washed out. Subsequently, the integrity of the endothelium was determined by submaximally pre-constricting arteries with PE (0.1–3 μmol/L) to 50–70% of KPSS contraction, then applying the endothelium-dependent vasodilator acetylcholine (ACh; 10 μmol/L). Arteries with >90% relaxation were deemed suitable for further analysis. The level of pre-contraction used when examining vasodilator function was kept constant across treatment groups. As the absolute contraction to KPSS varied significantly depending on treatment, contraction to KPSS and constrictor agonists were expressed as absolute force (mN) rather than as a percentage of KPSS.

Initially, cumulative concentration-response curves to the vasoconstrictors phenylephrine (PE; 1 nmol/L to 15 μmol/L) and endothelin-1 (ET-1; 0.1 nmol/L to 0.1 μmol/L) were constructed to assess whether sulforaphane pre-treatment influences agonist-mediated vasoconstriction. In other experiments, in un-incubated, pre-constricted mesenteric arteries, dose-response curves for sulforaphane (0.1 μmol/L to 15 μmol/L) were established to test whether sulforaphane induces relaxation.

As sulforaphane pre-incubation significantly reduced KPSS-mediated contraction in mesenteric arteries, we investigated if sulforaphane could inhibit $Ca^{2+}$ influx through voltage-gated $Ca^{2+}$ channels. In these experiments, arteries were incubated in $Ca^{2+}$-free EGTA (2 mM)-containing Krebs PSS for 40 min. This buffer was then replaced with $Ca^{2+}$-free KPSS and cumulative concentration-response curves to calcium chloride ($CaCl_2$; 10 μmol/L to 10 mmol/L) performed. This work was repeated, in separate mesenteric arteries that were incubated with TCM. After exposure to KPSS, cumulative concentration-response curves to PE (1 nmol/L to 15 μmol/L), angiotensin II (AngII; 0.1 nmol/L to 0.1 μmol/L) and endothelin-1 (0.1 nmol/L to 0.1 μmol/L) were performed to assess whether sulforaphane could still suppress vasoconstriction in the presence of TCM.

### 2.11. Assessment of reactivity in human omental arteries

To test the vasodilator actions of sulforaphane, cumulative concentration-response curves to sulforaphane (0.1 μmol/L to 15 μmol/L) were constructed in arteries (2 mm in length) submaximally constricted (to 50–70% of KPSS) using arginine vasopressin (AVP). Other arteries were first incubated for 3 h in PEM, which contains high levels of the anti-angiogenic factors sFlt-1 and soluble endoglin. After incubation, arteries were contracted with KPSS (100 mmol/L) to assess tissue viability, as detailed above. Then, arteries were submaximally pre-constricted with AVP, as mentioned above, and endothelial function tested by brief exposure to the endothelium-dependent vasodilator bradykinin (BK; 10 μmol/L), before quickly washing out. Cumulative concentration-response curves were constructed for BK (0.1 nmol/L to 10 μmol/L) to assess if PEM induced endothelial dysfunction. Maintaining reproducible and stable preconstriction of human omental arteries after incubation and exposure to BK was not possible. Therefore, we were unable to further investigate potential pathways disrupted by PEM by using pharmacological blockers.

In other experiments, arteries were incubated for 3 h in PEM and/or sulforaphane, as described above. After incubation, the arteries were exposed to the vasoconstrictors endothelin-1 (0.1 nmol/L to 0.1 μmol/L) or the thromboxane A2 mimetic U46619 (0.1 nmol/L to 0.1 μmol/L) to assess if PEM and sulforaphane could influence contraction.

### 2.12. Statistical analyses

All results are expressed as a mean ± SEM; 'n' represents the number of animals or human samples per group. Maximum contraction ($E_{max}$) to PE, AngII and endothelin-1 is expressed in mN. Maximum relaxation ($R_{max}$) to the endothelial agonists ACh and BK are expressed as a percentage of the level of pre-contraction to PE or **AVP**. Concentration-response curves were computer fitted to a sigmoidal curve using nonlinear regression (Prism version 7.0, GraphPad Software, San Diego, CA, USA) to calculate the sensitivity of each agonist ($pEC_{50}$). As maximal contraction induced by KPSS varied between treatment groups, for the calculation of $pEC_{50}$, contraction was expressed as a percentage of the $E_{max}$ in each artery, for each agonist. Group AUC, $pEC_{50}$, $R_{max}$ and $E_{max}$ values were compared within or between the single variable of treatment using one-way ANOVA with post-hoc analysis using Dunnett's test. $P < 0.05$ was considered statistically significant. Group AUC, $pEC_{50}$, $R_{max}$ and $E_{max}$ in human arteries were assessed using two-way ANOVA with Holm-Sidak post-hoc analysis and incubation medium (control or PEM) and treatment (sulforaphane) acting as the independent variables.

## 3. Results

### 3.1. Sulforaphane improves endothelial cell viability

We first sought to assess effects of sulforaphane on the viability of HUVECs under normoxic and hypoxic conditions. After 24 h of sulforaphane exposure in normoxia (21% $O_2$), HUVEC cell viability was unchanged, except at the highest doses of 20 μM and 50 μM which reduced cell viability (20 μM: P = 0.152, 50 μM: P < 0.0001, Fig. 1A). After 48 h in normoxia cell viability was unchanged at lower concentrations of sulforaphane, however, 10 μM, 20 μM and 50 μM decreased cell viability (10 μM: P = 0.039, 20 μM: P < 0.0001, 50 μM: P < 0.0001, Fig. 1B).

Under conditions of hypoxia (1% $O_2$), 1 μM and 5 μM sulforaphane improved HUVEC viability at both 24 and 48 h (Fig. 1C and D) (P < 0.01). Higher concentrations of sulforaphane either had no effect or reduced cell viability at 24 h (20 μM: P = 0.033; 50 μM: P = 0.01, Fig 1C) and 48 h (10 μM: P = 0.0003, 20 μM: P < 0.0001; 50 μM: P < 0.0001, Fig. 1D).

### 3.2. Sulforaphane reduces contraction and induces relaxation in small mesenteric arteries of mice

We next investigated effects of sulforaphane on the reactivity of mesenteric arteries of mice. After 24 h incubation, sulforaphane reduced arterial contraction to KPSS in a dose-dependent manner (Supp Fig 1A; P < 0.001). sulforaphane pre-incubation significantly affected phenylephrine-mediated contraction (Fig. 2A and B; $P_{TREAT}$0.0001). The highest dose of sulforaphane (20 μM) significantly decreased sensitivity (Supp Table 1; P < 0.001) to phenylephrine and reduced both maximal response (Supp Table 1; P < 0.0001) and AUC (Fig. 2B; Supp Table 1; P < 0.0001) compared to control. Similarly, 20 μM sulforaphane reduced mesenteric artery ET-1-mediated contraction (Fig. 2C and D; P < 0.0001), reducing sensitivity (Supp Table 1; P = 0.012), maximal response (Supp Table 1; P < 0.0001) and AUC (Fig. 2D; Supp Table 1; P

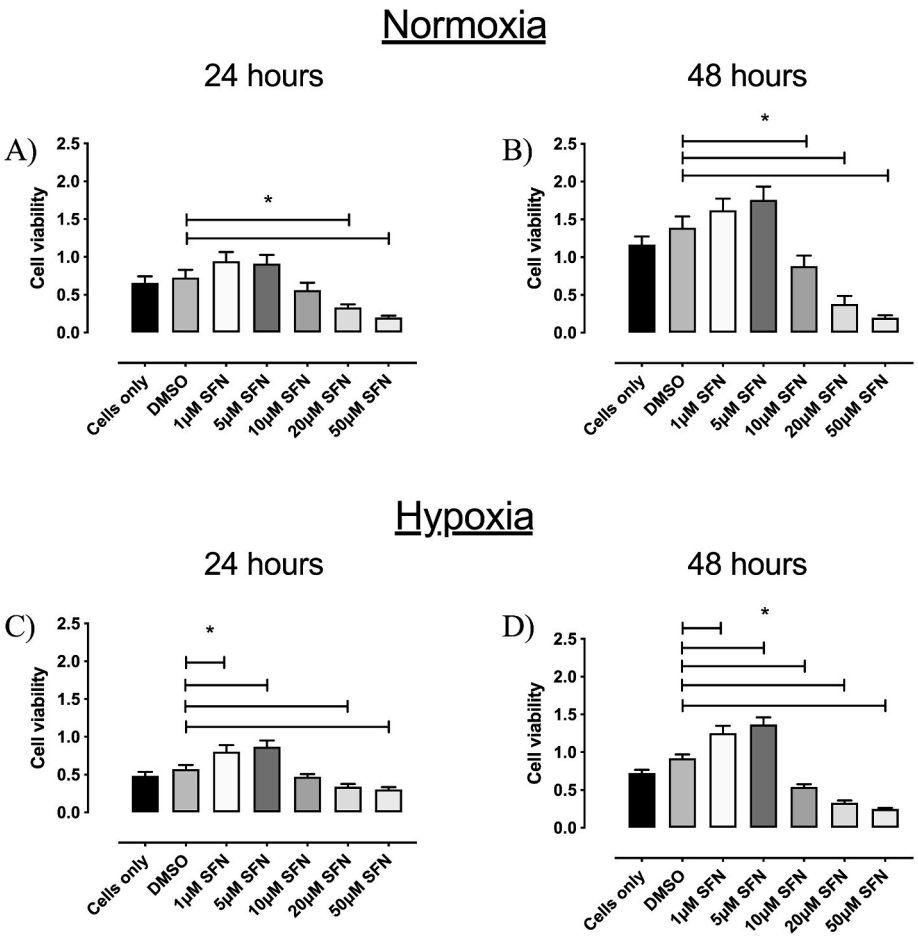


**Fig. 1.** Viability of HUVECs after exposure to DMSO (control) or SFN (1, 5, 10, 20 and 50 μM) after A) 24 h and B) 48 h in normoxia (21% $O_2$), and C) 24 h and D) 48 h in hypoxia (1% $O_2$) **(n = 6–8)**. Data are expressed as mean ± SEM. Analysis is by one-way ANOVA. Statistical significance ($P < 0.05$) on multiple comparison after post hoc correction (Dunnett) is shown with *.

< 0.0001).

Because the effects of sulforaphane on the mouse mesenteric artery appeared to be agonist-independent, we next investigated effects of sulforaphane on $Ca^{2+}$-induced contractions. In a dose-dependent manner, sulforaphane reduced mesenteric artery contraction to $Ca^{2+}$ (Fig. 2E and F), reducing sensitivity (Supp Table 1; 10 μM P = 0.04), maximal contraction (Supp Table 1; P < 0.0001 for 5, 10 and 20 μM sulforaphane) and AUC (Fig. 2F, P < 0.0001 for 5, 10 and 20 μM sulforaphane). At the highest concentration, 20 μM, sulforaphane almost completely prevented $Ca^{2+}$-induced contraction.

Next, we assessed whether sulforaphane could induce vasodilation in mice. Compared to the vehicle control (DMSO), sulforaphane induced concentration-dependent relaxation of mesenteric arteries (Fig. 2G). The highest sulforaphane concentration induced almost maximal relaxation (Fig. 2G; Supp Table 1; P < 0.0001 AUC, Fig. 2H, P < 0.0001).

We next sought to assess the effects of sulforaphane on mouse mesenteric artery contraction under *in vitro* conditions that mimic pre-eclampsia. To do this we exposed arteries to TCM alone, which increased contraction (AUC, Fig. 3A and B; P = 0.04) and sensitivity to phenylephrine (Supp Table 1; P = 0.0031). TCM did not alter responses to endothelin-1 (Fig. 3C and D, Supp Table 1) but did increase AUC to $Ca^{2+}$ (Fig. 3F, Supp Table 1; P = 0.04). TCM also increased sensitivity (Supp Table 1; P = 0.036), maximal response (Fig. 3G, Supp Table 1; P = 0.0002) and AUC (Fig 3H; P = 0.001) to AngII.

Relative to TCM treatment alone, co-incubation with sulforaphane reduced mouse mesenteric artery contraction to all vasoconstrictors. The highest dose of 20 μM sulforaphane reduced sensitivity (Supp Table 1; P = 0.003) and AUC (Fig. 3A and B; P = 0.007) to PE and endothelin-1 (Supp Table 1; P = 0.001; Fig. 3C and D; P < 0.0001). Sulforaphane exposure also reduced $Ca^{2+}$-induced contractions (Fig. 3E and F), with the highest dose of 20 μM reducing sensitivity (Supp Table 1; P = 0.01) and both 10 μM and 20 μM reducing maximal response (Supp Table 1; 10 μM P = 0.048, 20 μM P = 0.0013) and AUC (Fig 3F; 10 μM P = 0.008, 20 μM P < 0.0001) to $Ca^2$, relative to TCM treatment alone. Finally, sulforaphane treatment reduced maximal response (Supp Table 1; 10 μM P = 0.004, 20 μM P < 0.0001) and AUC (Fig. 3G and H; 10 μM P = 0.021, 20 μM P = 0.0006) to AngII, while 20 μM of sulforaphane significantly (Supp Table 1; P = 0.003) reduced sensitivity to AngII. After 24 h incubation in TCM, sulforaphane (10 & 20 μM) significantly reduced KPSS-mediated contraction in the mesenteric artery (Supp Fig 1B; P = 0.001).

### 3.3. Sulforaphane effects on human omental arteries

We next sought to assess the vasoactive properties of sulforaphane in human omental arteries taken from pregnant women. Compared with control (DMSO), sulforaphane induced dose-dependent relaxation of omental arteries such that the highest concentration produced almost complete relaxation (Fig. 4A and B; Supp Table 2; P < 0.0001; AUC, Fig 4B; P = 0.002).

As previously, we sought to mimic “preeclamptic-like” vessel injury *in vitro*. Here, we used media conditioned by preeclamptic placental explants (PEM). Three hours of incubation in PEM induced human omental artery endothelial dysfunction as evidenced by reduced

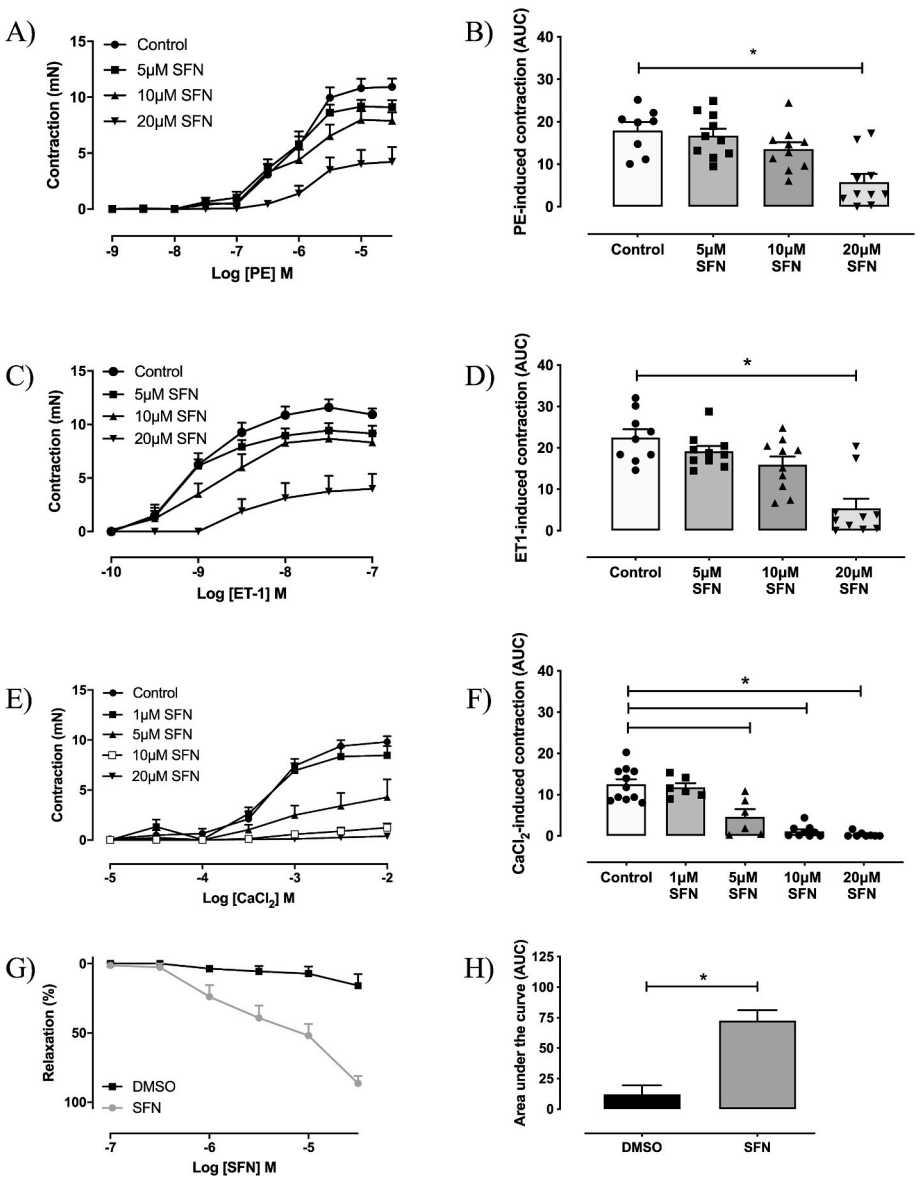


**Fig. 2.** Concentration-response curves and area under the curve (AUC) to (A & B) phenylephrine (PE), (C & D) endothelin-1 (ET-1) and (E & F) calcium chloride ($CaCl_2$) in the mesenteric artery of mice exposed to sulforaphane (SFN,1 μM, 5 μM, 10 μM and 20 μM) (n = 6–11). G) Concentration-response curve and H) AUC to control (DMSO) or SFN in the mesenteric artery of mice (n = 11). Data are shown as mean ± SEM and analysed using on-way ANOVA. Post hoc correction (Dunnett) or student t-test where relevant. *P < 0.05.

sensitivity (Fig. 4C, Supp Table 2; $P_{INT}$0.005, $P_{INJURY}$0.001), maximal relaxation (Supp Table 2; $P_{INT}$0.002, $P_{INJURY}$0.001) and AUC (Fig. 4D; $P_{INT}$0.0006, $P_{INJURY}$0.0002) to the endothelium-dependent vasodilator bradykinin. These effects were wholly prevented by co-incubation with 5 μM of sulforaphane (Fig. 4C and D; Supp Table 2; $P_{TREAT}$0.0001, $P_{TREAT}$0.002 and Fig. 4D; $P_{TREAT}$0.0002).

PEM did not alter human omental artery sensitivity, maximal contraction or AUC to U46619 (Fig. 4E and F, Supp Table 2), but did increase maximal contraction and AUC to endothelin-1 (Fig. 4G and H, Supp Table 2). When arteries were co-incubated with PEM and sulforaphane, arteries demonstrated reduced contraction to U46619 (Fig. 4E, $P_{TREAT}$0.0024), as evidenced by reduced sensitivity (Supp Table 2, $P_{TREAT}$0.002), maximum contraction (Supp Table 2; $P_{TREAT}$0.0004) and AUC (Fig. 4F; $P_{TREAT}$<0.0001). Furthermore, sulforaphane reduced sensitivity (Supp Table 2; $P_{TREAT}$0.02), maximum contraction (Fig. 4F, Supp Table 2; $P_{TREAT}$<0.0001) and AUC (Fig. 4G; $P_{TREAT}$0.0001) to endothelin-1 relative to PEM treatment alone.

Incubation in PEM did not alter human omental smooth muscle contraction to KPSS (Supp Fig 1C; $P_{INT}$0.67, $P_{INJURY}$0.89). However, as in mesenteric arteries of mice, sulforaphane significantly reduced KPSS-mediated contraction relative to control (Supp Fig 1C; $P_{TREAT}$0.005).

## 4. Discussion

Here we have identified that a naturally occurring plant-derived antioxidant, sulforaphane, has vasoprotective actions in both mouse and human *ex vivo* models of 'preeclamptic-like' vascular dysfunction. Importantly, sulforaphane is able to protect against the enhanced vascular constriction and reduced sensitivity to endothelium-dependent vasodilation that occurs after exposure to the anti-angiogenic compounds known to contribute to the maternal vascular dysfunction that underlies preeclampsia. Sulforaphane is also capable of acting as an acute vasodilator and influences endothelial cell viability under conditions of hypoxia. These vasoprotective actions suggest that sulforaphane merits further assessment as a potential adjuvant therapy in preeclampsia.

Sulforaphane has received much attention for its ability to improve the health and function of the vasculature in hypertension and peripheral vascular disease outside of pregnancy, principally through reducing oxidative stress [30,31]. Sulforaphane activates the nuclear factor erythroid 2 like-2 (NFE2L2, commonly known as Nrf2)- antioxidant response element pathway to increase transcription and production of ROS scavenging enzymes, such as haem-oxygenase-1 (HO-1) [19]

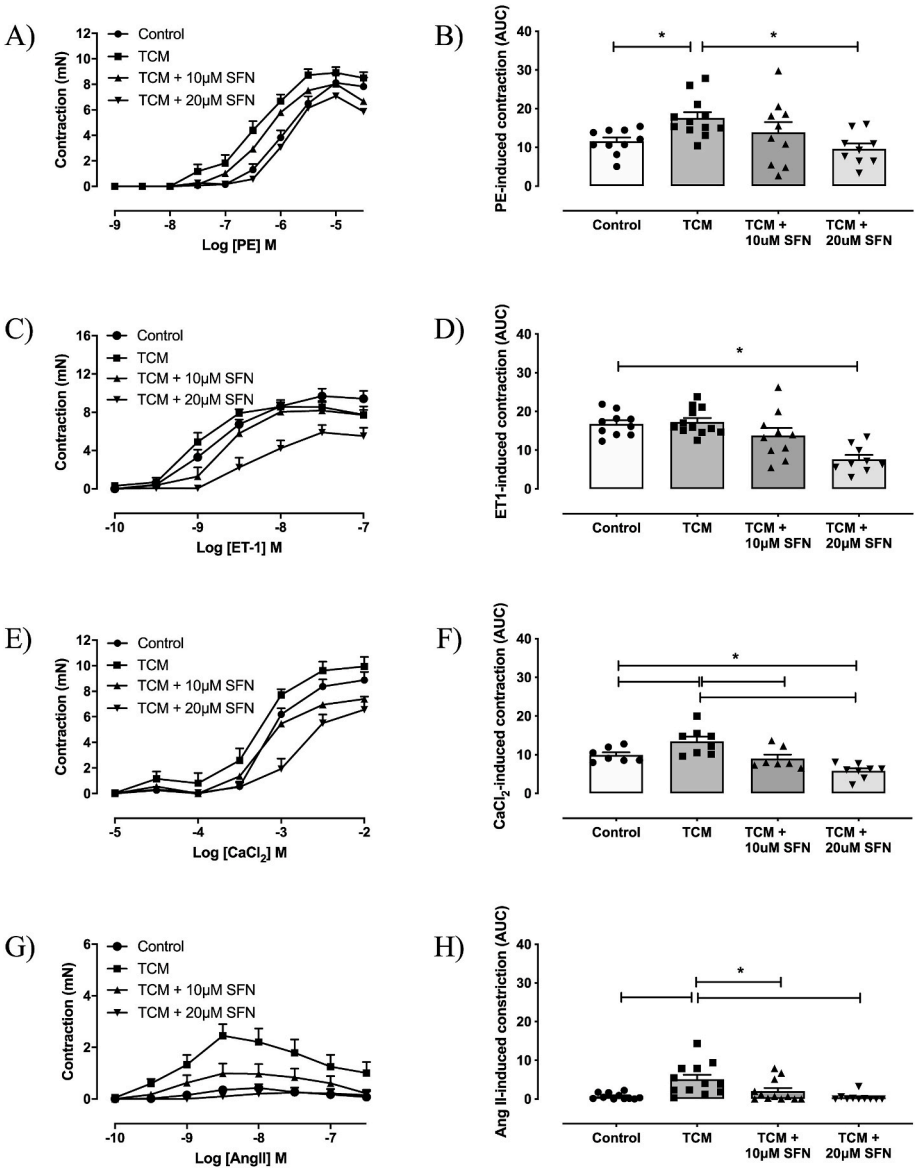


**Fig. 3.** Concentration-response curves and area under the curve (AUC) to (A & B) phenylephrine (PE), (C & D) endothelin-1 (ET-1), (E & F) calcium chloride ($CaCl_2$) and (G & H) angiotensin II (AngII) in mouse mesenteric arteries exposed to trophoblast conditioned media (TCM) with or without SFN (10 μM and 20 μM) (n = 6–11). Data are shown as mean ± SEM and analysed using on-way ANOVA with post hoc correction (Dunnett). *P < 0.05.

catalase, glutathione S-transferase, glutathione peroxidase and NAD(P)H:quinone oxidoreductase 1 [32,33]. Sulforaphane's ability to ameliorate endothelial dysfunction is attributed in part to the upregulation of Nrf2 [34]. In our work, 'preeclamptic-like' injury induced by PEM resulted in markedly impaired endothelial vasodilator function in human omental arteries, reducing the sensitivity to bradykinin by 25-fold. This endothelial dysfunction was completely prevented by acute exposure to sulforaphane. Endothelium-dependent relaxation in human omental arteries is mediated by nitric oxide, prostacyclin and endothelium-derived hyperpolarization (EDH). The restoration of endothelial function by treatment with sulforaphane likely reflects the recoupling of endothelial NOS and improved NO bioavailability [35], and EDH-mediated relaxation. Indeed, acute sulforaphane treatment was associated with a reduction in superoxide generation and improvement of NO and EDH-dependent relaxation in aged or diseased rat and human arteries [34]. EDH-mediated relaxation originates from the opening of small (S)- and intermediate (I)-conductance calcium-activated potassium ($K_{Ca}$) channels in endothelial cells [36]. Although Nrf2 activation upregulates large (B)-conductance $K_{Ca}$ channel activity in vascular smooth muscle [37], its role in the regulation of the endothelial $SK_{Ca}$ and $IK_{Ca}$ channels responsible for the classical EDH-mediated response is unknown and remains to be explored in future studies.

Enhanced contraction of the maternal vasculature is a key feature of preeclampsia. The ability of sulforaphane to restore endothelial vasodilator function will, in itself, facilitate the suppression of contraction [38]. In addition, in preeclampsia there is upregulation of ROS generation that enhances vessel responsiveness to vasoconstrictors. Normalisation of ROS generation by sulforaphane may be one mechanism by which sulforaphane ameliorates enhanced constriction in the setting of disease.

More recently, the capacity of isothiocyanates, such as sulforaphane, to reduce vascular contraction has been recognised. Others have shown sulforaphane can reduce enhanced vascular contraction in hypertensive pregnant rats [39], cerebral arterial vasospasm [40] and stroke prone rats [33]. These beneficial changes were associated with increased Nrf2 and expression of antioxidant enzymes [33]. Consistent with findings reported by others, in our study, sulforaphane ameliorated the enhanced

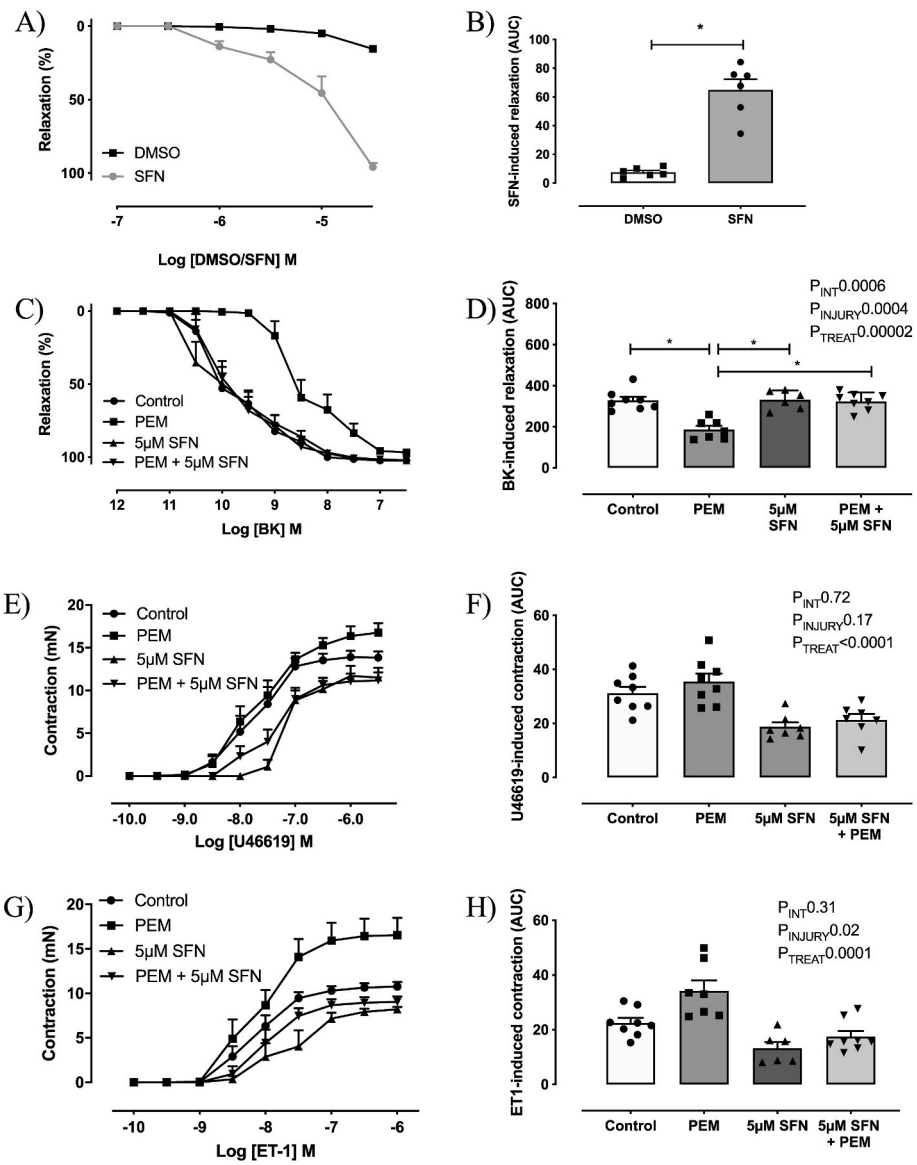


**Fig. 4.** A) Concentration-response curve and B) area under the curve (AUC) to DMSO (control) and SFN in omental arteries of healthy women undergoing elective caesarean delivered at term (n = 6–8). Concentration-response curves and area under the curve (AUC) to (C & D) bradykinin (BK), (E & F) U46619 and (G & H) endothelin-1 (ET-1) in omental arteries after exposure to placental explant media (PEM), SFN (5 μM) or PEM and SFN (5 μM) (n = 6–8). Data are shown as mean ± SEM and analysed using student t-test or two-way ANOVA with treatment (SFN) and injury (PEM) acting as the two independent variables. Significant interaction ($P < 0.05$) after post hoc correction is delineated by *.

contraction to a variety of constrictors evoked by 'pre-eclamptic-like' injury in mouse mesenteric and human omental arteries. The normalisation of ROS generation in the setting of disease and increased Nrf2 signalling are mechanisms which likely underpin the beneficial effects of sulforaphane treatment.

In our study, responsiveness to AngII in mesenteric arteries was significantly augmented following treatment with TCM. Enhanced responsiveness of the maternal vasculature to AngII is a feature of pre-eclampsia [41], probably via increased ROS generation. Sulforaphane treatment of mesenteric arteries in our study suppressed this enhanced responsiveness to AngII. Others have noted similar findings in other vascular beds, with an associated Nrf2 upregulation [33]. Thus, the activation of the Nrf2 pathway by sulforaphane in the setting of pre-eclampsia may reduce the extent of vascular complications. In addition, sulforaphane mitigates the damaging effects of sFlt-1 and oxygen free radicals, increasing NO and VEGF availability [24].

Aside from its ability to reverse enhanced contraction in disease, we also found that sulforaphane dose-dependently suppressed contraction to both receptor-dependent and receptor-independent agents in healthy arteries. Sulforaphane inhibited contraction evoked by depolarisation of the smooth muscle with high $K^+$ solution, suggesting that sulforaphane may modulate the activity of voltage-dependent calcium channels. To further explore this, we examined responses evoked by raising extracellular $Ca^{2+}$ concentrations in arteries already depolarised with high $K^+$ solution. Reintroduction of $Ca^{2+}$ to the bathing solution evoked concentration-dependent contraction, and concentrations of sulforaphane higher than 1 μM were found to inhibit contraction. These findings may reflect a reduction in $Ca^{2+}$ influx into vascular smooth muscle through inhibition of voltage operated calcium channels activity. The inhibition of agonist-mediated contraction by sulforaphane is likely multifactorial. As Nrf2 is able to upregulate smooth muscle $BK_{Ca}$ activity [37] it is possible that sulforaphane suppresses vascular contraction through enhanced $K^+$ channel activity and membrane repolarisation.

Beyond mitigating vascular dysfunction from 'preeclamptic-like' injury from explant and trophoblast conditioned media, we investigated the vasodilator effects of sulforaphane. In both pre-constricted human omental and mouse mesenteric arteries sulforaphane evoked dose-dependent relaxation, with almost complete relaxation at the highest dose. Others have demonstrated relaxation of the cerebral vasculature of pigs after administration of sulforaphane. The relaxation in the cerebral vasculature results from endogenously generated sulphide ($H_2S$) acting on smooth muscle $BK_{Ca}$ and ATP-sensitive K channels ($K_{ATP}$) [42]. We have since confirmed that sulforaphane was able to induce relaxation independently of the endothelium, suggesting that the vasodilator

actions of sulforaphane are dependent on the smooth muscle cells and unlikely to operate by enhancing endothelial production gaseous vasodilators. While $H_2S$, like NO, is endogenously produced by the endothelium [43], the sulforaphane metabolite L-Cysteine is enzymatically converted into $H_2S$ by cystathionineβ-synthase [44,45] bypassing the endothelium. The mechanism by which sulforaphane induces vascular relaxation likely involves membrane potential-dependent ($K^+$ channel activation and voltage-operated $Ca^{2+}$ channel inhibition) and -independent mechanisms (e.g. desensitisation of the contractile apparatus to $Ca^{2+}$).

The effects of sulforaphane treatment on vascular function make it likely that the beneficial effects extend to the regulation of blood pressure. Others have shown sulforaphane can reduce blood pressure and improve renal morphology in rats, with associated reductions in oxidative stress [31,46]. Given that preeclampsia is a condition defined by enhanced responsiveness to vasoconstrictors and worsening endothelial dysfunction, sulforaphane may similarly offer benefit in stabilising the vascular response of women with preeclampsia [14,47,48]. Furthermore, the actions of sulforaphane offers some promise for both the prevention of preeclampsia in women with pre-existing vascular dysfunction, who are at higher risk of developing preeclampsia. Low dose sulforaphane has been shown to promote endothelial cell viability in culture [24], via Nrf2 activation and improve placental cell mitochondrial function [23]. We also report that low doses of sulforaphane promoted endothelial cell viability under normoxic and hypoxic conditions. Higher concentrations of sulforaphane reduced viability, an observation consistent with the established hormetic properties of Nrf2 activators [49].

Based on our bioavailability data, doses of sulforaphane used to induce vasodilation in this study are considerably higher than those achievable through consumption of broccoli extract. This is an important consideration to ensure that consumption of a broccoli extract does not result in sudden and profound maternal hypotension that may limit placental and fetal perfusion and oxygenation. Doses used to improve human omental artery function in the face of injury are ~5x the level of bioavailable sulforaphane from broccoli extract [50], however our results offer proof of concept that sulforaphane can have protective effects on the maternal vascular endothelium. Evidence from *in vitro* work suggests that sulforaphane has a more profound effect on placental and endothelial function than another, similar Nrf2 activator, melatonin [25]. In this study we used a once off dose of sulforaphane for artery pre-treatment, and the effects of *in vivo* chronic exposure to lower levels of sulforaphane will become apparent from future large clinical efficacy trials.

### 4.1. Strengths and limitations

A strength of this work is the use of both mouse arteries and human arteries from pregnant women to investigate vascular reactivity. However, our work has a number of limitations. The doses of sulforaphane required to induce relaxation of arteries were supraphysiological. Our observation that at high concentrations sulforaphane compromises endothelial cell viability suggests a cautious approach to determining safe clinical dosing will be required. Nonetheless, our findings do provide proof-of-principle evidence for the antihypertensive potential of sulforaphane. Similarly, though we hypothesise that sulforaphane modulates calcium-mediated constriction, future studies are required to explore this mechanism in detail. Finally, after incubation in PEM, human omental arteries did not provide reproducible responses to certain drugs. Therefore, our initial mechanistic approach was performed in mouse arteries as a proof-of-concept investigation aimed at narrowing down potential vascular pathways for further investigation in human omental arteries. Our future vascular studies will now further this research on omental arteries from women with early-onset preeclampsia, characterised by vascular dysfunction.

## 5. Conclusions

Our findings suggest that sulforaphane may have exciting potential therapeutic effects as an adjuvant treatment for preeclampsia by protecting the vasculature against the damaging effects of preeclamptic anti-angiogenic compounds. We have shown that the naturally occurring antioxidant sulforaphane not only protects the vasculature of pregnant women from preeclamptic placental-derived anti-angiogenic factors but also has acute vasodilator effects on maternal vasculature. Our *in vitro* findings suggest that clinical investigation of sulforaphane as an adjuvant for preeclampsia is merited.

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## Declarations of interest

The authors have no conflicts of interest to declare.

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## Declaration of competing interest

The authors report no conflicts of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2020.09.001.

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# Chapter six

## *Measuring sulforaphane and its metabolites in human plasma: a high throughput method*

As detailed in the *Preamble*, the overarching goal of this thesis was to move to clinical evaluation of sulforaphane as an adjuvant therapy for preeclampsia. Given the lack of evidence for sulforaphane in this setting it was necessary to undertake the experimental studies described in previous chapters. However, while conducting those *in vitro* studies the planning and designing of the clinical elements of this research was already underway. Such planning was not without challenge as the purified sulforaphane used in the pre-clinical studies was not suitable for clinical administration. Instead, an appropriate broccoli extract for clinical evaluation needed to be identified.

In selecting suitable commercial formulations of sulforaphane, it is important to consider that the process of myrosinase activation is essential in the generation of bioactive sulforaphane following consumption of broccoli or a broccoli extract (Figure 3). Plant based sources of sulforaphane, such as broccoli extract, contain the precursor molecule glucoraphanin. Glucoraphanin requires activation by an enzyme located in the plant cell wall, mysrosinase, usually released by chewing of the plant material[71]. Through a process of myrosinase-catalysed hydrolysis, glucoraphanin is then converted into the antioxidant sulforaphane which is further degraded into a number of active metabolites *in vivo*[103,104]. Conjugation of sulforaphane begins immediately on entry of sulforaphane to a mammalian cell and initially sulforaphane glutathione (SFN-GSH) forms[71]. A series of conversion reactions then facilitates the formation of sulforaphane cysteine (SFN-CYS) and then sulforaphane cysteine-glycine (SFN-CYS-GLY)[71]. The final metabolite is sulforaphane N-Acetyl-Cysteine (SFN-NAC)[71]. Sulforaphane and metabolites act as gene transcription activators by releasing NFE2L2 from where it is

bound in the cytosol of the cell and inducing translocation to the nucleus[56,60,105]. Here, NFE2L2 increases transcription of cellular "safeguarding" genes in the antioxidant response element of cellular DNA, resulting in translation of a number of antioxidant enzymes that then undergo redox reactions to reduce damaging oxygen free radicals[58].

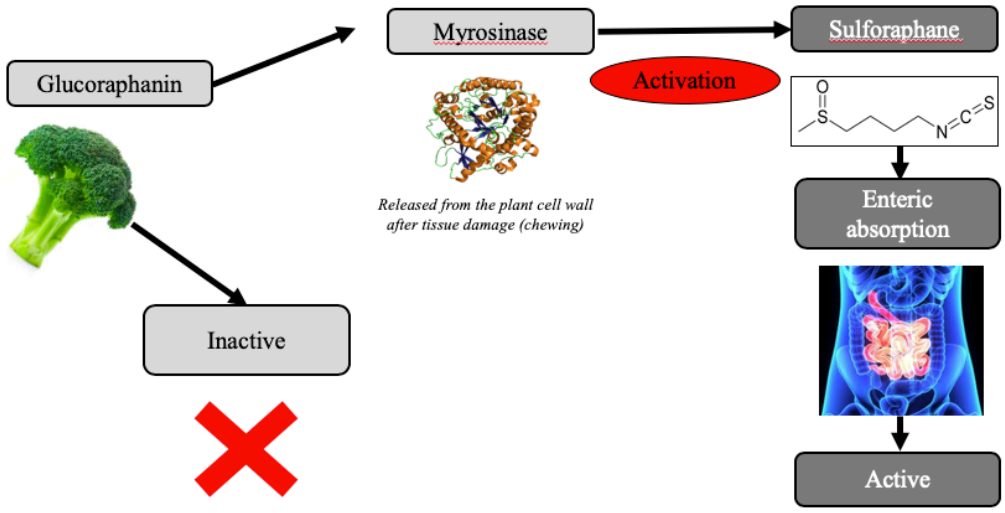


*Figure 3. Myrosinase activation of glucoraphanin into active sulforaphane.*

Figure 3. The plant cell wall of cruciferous vegetables contains glucoraphanin, an inert form of sulforaphane with limited bioavailability that requires activation by the enzyme myrosinase to become the metabolically active compound sulforaphane.

Determining an appropriate dose and select the extract formulation meant a method to measure and quantify levels of unconjugated sulforaphane and the four active metabolites (SFN-GSH, SFN-CYS, SFN-NAC and SFN-CYS-GLY) in plasma after consumption of the formulation was required. With the potential for a future clinical study in mind and the need to measure sulforaphane in clinical samples, a high throughput method that would allow rapid analysis of plasma levels of sulforaphane and metabolites was established. Ensuring the method was sufficiently sensitive but also efficient and capable of high throughput analysis was essential

so that it could be used to analyse a large number of clinical samples from future clinical trial. Unfortunately, no such method existed.

High performance liquid chromatography has been used to quantify sulforaphane but has limited reproducibility and sensitivity, particularly when concentrations are relatively low[106]. More recently, a sensitive and well validated method to quantify sulforaphane using liquid chromatography – mass spectrometry (LCMS) was developed to measure even low levels of sulforaphane metabolites in plasma and urine[107,108]. However, the elaborate plasma clean-up preparations required for this method limited the speed, and therefore sample number, with which analysis can be conducted. Given the relative instability of plasma samples (~12 hours) and problems associated with repeated freeze thaw cycles in plasma clean up, these studies needed a a faster, high throughput method that did not require lengthy sample preparation. In *Chapter five* the development and use of a rapid and high-throughput method using LCMS to accurately and sensitively quantify sulforaphane and metabolites in plasma is reported.

***Aim***

1. To develop a high throughput, sensitive method of measuring sulforaphane and metabolites in human plasma using liquid chromatography-mass spectrometry

My role in this study focused on design and conducting the clinical investigation. I designed the study protocol and conducted the clinical studies. Collaborators from Monash Institute of Pharmacological Investigation oversighted the LCMS. I conducted all the data analysis and manuscript preparation.

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# Measuring Sulforaphane and Its Metabolites in Human Plasma: A High Throughput Method

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**Abstract:** (1) Background: There is increasing understanding of the potential health benefits of cruciferous vegetables. In particular sulforaphane (SFN), found in broccoli, and its metabolites sulforaphane-glutathione (SFN-GSH), sulforaphane-cysteine (SFN-Cys), sulforaphane cysteine-glycine (SFN-CG) and sulforaphane-N-acetyl-cysteine (SFN-NAC) have potent antioxidant effects that may offer therapeutic value. Clinical investigation of sulforaphane as a therapeutic antioxidant requires a sensitive and high throughput process for quantification of sulforaphane and metabolites; (2) Methods: We collected plasma samples from healthy human volunteers before and for eight hours after consumption of a commercial broccoli extract supplement rich in sulforaphane. A rapid and sensitive method for quantification of sulforaphane and its metabolites in human plasma using Liquid Chromatography–Mass Spectrometry (LC–MS) has been developed; (3) Results: The LC–MS analytical method was validated at concentrations ranging between 3.9 nM and 1000 nM for SFN-GSH, SFN-CG, SFN-Cys and SFN-NAC and between 7.8 nM and 1000 nM in human plasma for SFN. The method displayed good accuracy (1.85%–14.8% bias) and reproducibility (below 9.53 %RSD) including low concentrations 3.9 nM and 7.8 nM. Four SFN metabolites quantitation was achieved using external standard calibration and in SFN quantitation, SFN-$d_8$ internal standardization was used. The reported method can accurately quantify sulforaphane and its metabolites at low concentrations in plasma; (4) Conclusions: We have established a time- and cost-efficient method of measuring sulforaphane and its metabolites in human plasma suitable for high throughput application to clinical trials.

**Keywords:** sulforaphane; Liquid Chromatography–Mass Spectrometry; pharmacokinetic

## 1. Introduction

The past decade has seen increasing interest in the potential health and medicinal benefits of naturally occurring antioxidants. Specifically, the health benefits of cruciferous vegetables have been identified by epidemiological studies [1–3], stimulating the identification of the likely active antioxidant components of these food groups. For example, resveratrol [4–6], vitamins C [7] and E [8], and selenium [9] have all been studied for their antioxidant therapeutic potential. Sulforaphane is another such plant-derived antioxidant that offers promise as a safe and clinically effective agent [10–15].

Glucoraphanin is an isothiocyanate found in cruciferous vegetables, particularly young broccoli (sprout and seeds) [16]. Glucoraphanin is converted into the active antioxidant, sulforaphane, in the gastrointestinal tract by myrosinase-catalyzed hydrolysis [16,17]. Sulforaphane is further metabolized into a number of other active metabolites (Figure 1). Sulforaphane and its metabolites are

gene transcription activators that release bound nuclear factor erythroid factor 2 (NFE2L2) in the cell cytosol, allowing its nuclear translocation [13,18–21]. In the nucleus, NFE2L2 induces transcription of a cassette of cellular "safeguarding" genes in the antioxidant response element (ARE) of cellular DNA [22]. This leads to the translation of several antioxidant enzymes that then undergo redox reactions to reduce damaging oxygen free radicals [23].

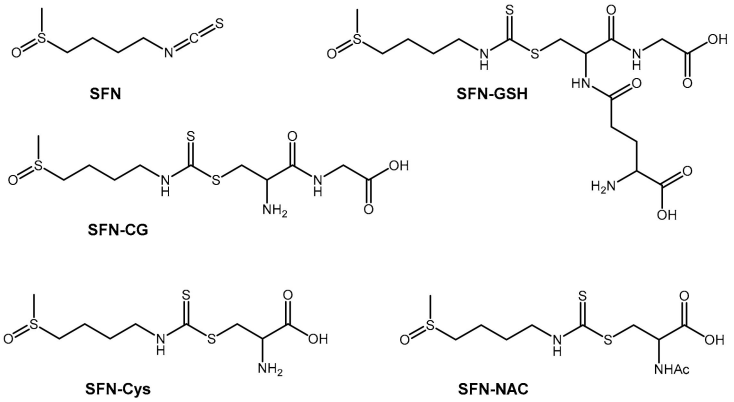


**Figure 1.** Molecular structures of sulforaphane and its metabolites: Sulforaphane (SFN) sulforaphane-glutathione (SFN-GSH), sulforaphane-cysteine (SFN-Cys), sulforaphane cysteine-glycine (SFN-CG) and sulforaphane-N-acetyl-cysteine (SFN-NAC).

Initial approaches to the measurement of sulforaphane and its metabolites in biological samples were limited in sensitivity and were unable to accurately quantify levels of metabolites in humans [24,25]. More recently, an approach offering increased sensitivity was described using liquid chromatography–mass spectrometry (LC–MS) [21]. This allowed quantification of sulforaphane and its metabolites at lower concentrations in both plasma and urine [26]. However, it also required extensive plasma clean-up during sample preparation, limiting the number of samples that could be handled at any given time. Given the relative fast thermal degradation of sulforaphane and its conjugates in human plasma at room temperature, with a reported half-life at 24 °C of between 6.16 and 0.49 h [27–29] and problems associated with sample degradation during lengthy plasma clean-up, a more rapid sample preparation methodology ahead of LC–MS would both allow processing of high numbers of samples, as would be required in large scale clinical trials, and may improve measurement accuracy. To that end, here we present a modified, cheaper, and high throughput method for the measurement of sulforaphane and its metabolites (Figure 1) using LC–MS.

## 2. Results

### *2.1. Method Development*

Method development was based on chromatographic separation and peak shape using pre-consumption (blank) plasma spiked with standards SFN-Cys, SFN-GSH, SFN, SFN-NAC and SFN-CG. Analyte-specific transitions settings (precursor *m*/*z*, Q1 pre-rod bias voltage, product m/z, collision energy and Q3 pre-rod bias voltage) were optimized by a LabSolutions software (Shimadzu, Kyoto, Japan, 2019) automated protocol from flow injection analysis of mixed pure standards at 1 µM concentration. HESI source parameters were optimized manually using flow injection mode.

Baseline peak separation of all analytes was achieved using aqueous reverse phase chromatography. As shown in Figure 2, in-source fragmentation of SFN conjugates was observed which resulted in SFN peaks (transition 177.8 > 113.9 *m*/*z*) being produced at the retention times of SFN-Cys (1.47 min), SFN-CG (1.59 min), SFN-NAC (2.02 min) and SFN-GSH (1.79 min). Similarly, SFN-GSH breakdown in source produced an additional SFN-CG peak at 1.79 min. Thus, good peak separation was important to avoid signal contamination. In-source fragmentation was observed only in plasma samples especially in higher spike concentrations and not in standards prepared in water, suggesting that fragmentation

is induced by matrix components of plasma. Changing ESI source parameters, such as voltage or interface temperature, reduced some, but not all, in-source fragmentation. Therefore, a calibration curve for quantitation was constructed in matched matrix–extracted plasma. The observed in-source fragmentation was reproducible and did not impede method accuracy, linearity or precision.

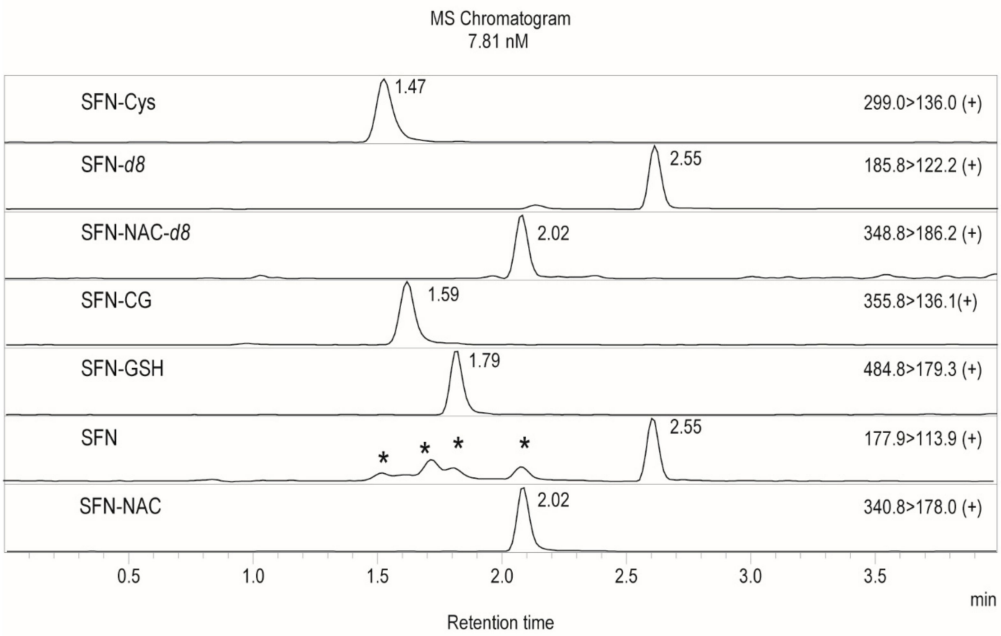


**Figure 2.** Extracted ion chromatograms of 7.8 nM spiked extracted plasma. SFN and SFN-CG peaks were produced due to in-source fragmentation are marked with arrows. Transitions from the top to the bottom: SFN-Cys, SFN-$d_8$, SFN-NAC-$d_8$, SFN-CG, SFN-GSH, SFN, and SFN-NAC. Peaks produced by in source ionization marked with " * ".

## 2.2. Sample Preparation

Previous LC–MS techniques require plasma sample preparation involving protein precipitation using cold methanol ethyl acetate [30] or trifluoroacetic acid [26,29,31,32]. Further sample clean-up using solid phase extraction techniques (SPE) is often employed [26,30,32,33]. As organic solvents lack extensive buffering capacity and given the sensitive nature of SFN and SFN conjugates, we aimed to eliminate the SPE step to prevent room temperature exposure and unnecessary sample handling, thereby minimizing sample degradation. We used methanolic precipitation of proteins as previously described [34,35] but modified the procedure to accommodate a smaller volume of plasma (25 µL). After extraction methanol was evaporated and sample resolubilized in the same volume of 0.1% formic acid solution to accommodate reversed phase chromatography gradient starting conditions. Attempts to inject methanolic extract resulted in poor peak shape of early eluting peaks and could not be improved by dilution or changing the gradient. Samples, standards, solvents, microcentrifuge tubes and vials were kept on ice throughout the process to minimize degradation of target compounds. Reduction of required plasma amount for analysis is desired in clinical setting, as collected plasma can be aliquoted and used for other tests or diagnostics.

During method validation it has become evident that SFN undergo degradation if exposed to higher than 4 °C temperature even for short periods of time. Also, loss of internal standard SFN-$d_8$ signal in time upon each injection was observed in couple of instances suggesting that perhaps in some batches of plasma SFN degradation is faster. High degree of degradation results in low SFN measurement accuracy. SFN is known to undergo thermal degradation at temperatures above −20 °C [36]. Short-term solution stability of SFN can be increased below pH 3–4. However, exposure to temperatures warmer than 4 °C will accelerate decomposition at acidic conditions. We were able to validate the method and show that if SFN stability is sufficient, the method is accurate and sensitive.

However, SFN accuracy will depend on how the samples are handled. It is crucial to keep all samples and standards on ice, use chilled solvents, tubes and vials throughout sample preparation and ensure autosampler temperature has reached 4 °C before samples are loaded. If SFN stability is not ensured, spiking SFN-$d_8$ internal standard will not be able to account for large losses and low accuracy will be obtained.

### 2.3. Accuracy and Linearity

As shown in Table 1, −11.8%–14.8% % bias was observed within linear range 3.9–1000 nM for SFN-GSH, SFN-Cys, SFN-NAC and SFN-CG and within 7.8 nM–1000 nM for SFN. Good accuracy for SFN was achieved by spiking 60 nM SFN-$d_8$ internal standard into extraction solvent (corresponding to 300 nM plasma concentration) and using area ratio in calibration curve generation of SFN. Linear fit with $1/A^2$ weighting factor was used for all target compounds. Good fit to this model was observed as represented by correlation coefficient ($R^2 > 0.99$).

**Table 1.** Summary of accuracy and linear range for sulforaphane and metabolites.

| Target Compound | Accuracy (% bias) | | | | | LOQ (nM) | Linear Range (nM) | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| | 3.9 nM | 7.8 nM | 11.7 nM | 200 nM | 1000 nM | | | |
| SFN | - | −2.70 | - | −11.8 | 12.2 | 7.8 | 7.8–1000 | 0.9947 |
| SFN-GSH | −5.70 | 11.3 | 2.65 | 2.6 | 0.1 | 3.9 | 3.9–1000 | 0.9944 |
| SFN-CG | −2.30 | 8.4 | −3.15 | 3.4 | −0.60 | 3.9 | 3.9–1000 | 0.9991 |
| SFN-Cys | 3.55 | 10.3 | 2.65 | 14.8 | 7.90 | 3.9 | 3.9–1000 | 0.9962 |
| SFN-NAC | 1.85 | −4.60 | −1.85 | 11.2 | 3.20 | 3.9 | 3.9–5000 | 0.9981 |

### 2.4. Limit of Quantification

For SFN-GSH, SFN-Cys, SFN-NAC and SFN-CG LOQ was 3.9 nM and for SFN–7.8 nM (Table 2).

**Table 2.** Monitored transitions and retention times of all analytes.

| Compound | *m/z*, Transition | Collision Energy | Retention Time (min) |
|---|---|---|---|
| SFN-Cys | 299.00 > 136.00 | −11 | 1.47 |
| SFN-GSH | 484.80 > 179.30 | −25 | 1.79 |
| SFN | 177.90 > 113.90 | −12 | 2.55 |
| SFN-NAC | 340.80 > 178.00 | −14 | 2.02 |
| SFN-$d_8$ | 185.80 > 122.20 | −10 | 2.55 |
| SFN-CG | 355.80 > 136.10 | −12 | 1.59 |

### 2.5. Precision

Repeatability was below 2% RSD for all target compounds at 200 nM QC level (n = 6) and below 8 %RSD at low concentrations: 3.9 nM and 11.7 nM levels (n = 4) for SFN-GSH, SFN-CG, SFN-Cys and SFN-NAC. For SFN repeatability was 8.61% at 7.8 nM (n = 4). Intermediate precision determined at 200 nM level on 3 different days was below 5.73% RSD for all analytes and 11.9% RSD for SFN (Table 3).

**Table 3.** Summary of precision and recovery for sulforaphane and metabolites.

| Target Compound | Repeatability ($n$ = 6) %RSD | | | | Intermediate Precision ($n$ = 3) %RSD | Recovery (% Difference) | | |
|---|---|---|---|---|---|---|---|---|
| | 3.9 nM | 7.8 nM | 11.7 nM | 200 nM | 200 nM | 40 nM | 200 nM | 1000 nM |
| SFN | - | 8.61 | - | 0.554 | 11.9 | 10.57 | 14.1 | −1.64 |
| SFN-GSH | 9.53 | 8.18 | 7.76 | 1.96 | 5.73 | 9.81 | 19.61 | −6.15 |
| SFN-CG | 3.68 | 4.28 | 3.95 | 1.19 | 5.47 | 15.2 | −10.1 | 0.846 |
| SFN-Cys | 4.01 | 5.95 | 1.03 | 1.72 | 1.85 | −5.63 | −1.97 | −0.26 |
| SFN-NAC | 6.66 | 3.38 | 3.12 | 1.25 | 0.656 | −8.92 | −4.67 | −3.12 |

### 2.6. Recovery

High recoveries of all analytes were observed with % difference below 19.61% (Table 3).

### 2.7. Sample Stability in Autosampler

Samples were stable for at least 12 h when kept at 4 °C in the autosampler, as determined from repeated injection of calibration curve dilutions after 12 h. The two calibration curves were nearly superimposable.

### 2.8. Matrix Effects

SFN-GSH, SFN-CG, SFN-Cys and SFN-NAC displayed acceptable matrix effects (between 2.63% and 29.1% difference in peak areas) and did not affect measurement accuracy. SFN suffered from ion suppression, with an 81%–86% decrease in peak area compared to the samples in water. Such large signal suppression resulted in reduced accuracy even when using a matched matrix to prepare calibration curve solutions. Attempts to chromatographically separate the interfering plasma components from the SFN peak were unsuccessful. We have shown that matrix effects in individual plasma samples for SFN were similar (% difference between −78.5% and −88.4% comparing plasma to water), but the accuracy between individual plasma samples was more varied between −23 and 50 %bias purely due to matrix effects. To account for signal suppression and improve accuracy of SFN, 60 nM SFN-$d_8$ was spiked into extraction solvent. Signal ratio SFN/SFN-$d_8$ was used to construct calibration curve which greatly improved the accuracy and method could be validated.

### 2.9. Application of Study Method to Human Samples

Pharmacokinetic profiles of each metabolite are outlined in Figure 3 for participant one (dotted line) and participant two (solid line) for participant two. As outlined in Table 4, the two participants had similar AUC values for SFN (P1: 424.9 and P2: 520.8), SFN-Cys-Gly (P1: 1264 and P2: 1007) but not for SFN-Cys (P1: 401 and P2: 245.5), SFN GSH (P1: 400 and P2: 530.3), SFN NAC (P1: 385.6 and P2: 172.5) and combined value. (P1: 2876 and P2: 2476). Mean peak value were largely similar; SFN (P1: 183.5 and 206.5) SFN-Cys (P1: 113.8 and P2: 112.2) SFN-GSH (P1: 150.1 and P2: 240.8), SFN-Cys-Gly (P1: 408 and P2: 419.2), SFN NAC (P1: 74.3 and P2: 35.6) and the combined value (P1: 906.2 and P2: 1014).

**Table 4.** Area under the curve (AUC) and mean peak of Participants one and two.

| Metabolite | Participant One | | Participant Two | |
|---|---|---|---|---|
| | **AUC** | **Mean Peak** | **AUC** | **Mean Peak** |
| SFN | 424.9 | 183.5 | 520.8 | 206.5 |
| SFN Cys | 401 | 113.8 | 245.5 | 112.2 |
| SFN-GSH | 400 | 150.1 | 530.3 | 240.8 |
| SFN-Cys-Gly | 1264 | 408 | 1007 | 419.2 |
| SFN-NAC | 385.6 | 74.3 | 172.5 | 35.6 |
| Total all metabolites | 2876 | 906.2 | 2476 | 1014 |

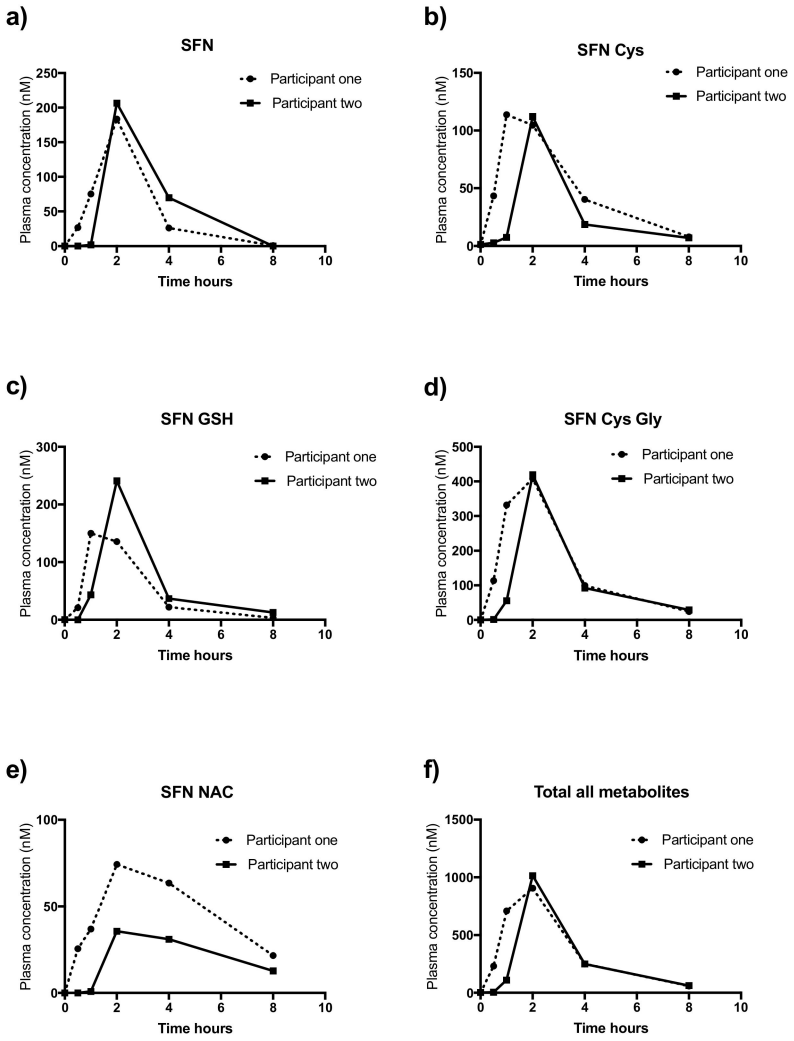


**Figure 3.** Pharmacokinetic profiles of SFN and metabolites in plasma taken from participant one (dotted line) and participant two (solid line) over 8 h. Metabolites are: (**a**) SFN, (**b**) SFN-Cys, (**c**) SFN-GSH, (**d**) SFN-CG, (**e**) SFN-NAC and (**f**) Total all metabolites (combined value of all metabolites). Y-axis represents measured concentration in ng/ML.

## 3. Discussion

A simplified methodology to allow high-throughput LC–MS analysis of plasma samples for the measurement of sulforaphane and its metabolites is described. Analysis time is greatly reduced by employing fast chromatography and simple plasma extraction procedure. These methodological simplifications better allow the use of LC–MS to process the large number of samples that are likely to be required in large clinical trials.

A number of sensitive LC–MS methodologies for quantification of SFN and metabolites in various biological samples have been reported [26,29–35,37,38]. However, few of these methods are suitable for use in human plasma [26,31,34]. All reported methods use triple quadrupole mass spectrometry coupled to High Performance Liquid Chromatography (HPLC) and stable-isotope-labelled internal standard (SIL IS) quantification, SFN-$d_8$ and SFN-NAC-$d_8$ and, with the exception of the method described by Janobi et al. [31], used Butyl-NAC as an internal standard. Early quantification of sulforaphane and its metabolites in human samples was established through cyclocondensation of all metabolites into a single compound rather than independent assessments of each metabolite [25,39,40]. The accuracy of this approach was limited by variation in the efficiency of the cyclocondensation reaction in combining all metabolites ahead of analysis [41–43]. To resolve this limitation, methods

were established that allowed the quantification of each metabolite with isotope-dilution tandem mass spectrometry [44]. However, even this approach had some limitations. First, reproducibility was limited with inter-assay coefficients of variation as high as 10%, compromising its application to clinical pharmacokinetic studies [44]. The sensitivity of the method was also insufficient to detect the low levels of some of the metabolites in plasma [45]. Finally, reported methodologies included lengthy sample preparation prior to analysis exposing already unstable metabolites to freeze–thaw cycles [26].

Careful selection of SIL IS and optimizing standard concentrations both reduced inter-assay variation and improved sensitivity [26,34,46]. However, these techniques still relied on multiple expensive internal standards introducing costs that would compromise feasibility in the setting of large clinical trials where frequent analysis of multiple samples must be undertaken. Even in the absence of financial concern, not all SIL standards are commercially available; SFN-CysGly, SFN-SGH and SFN-Cys, and so must be synthesized from SFN-$d_8$ in house [34]. Use of an SIL IS method can limit the sensitivity of the analysis by suppressing analyte signal at low concentration, thereby increasing the limit of detection [37].

In our hands, deuterated internal standards SFN-$d_8$ and SFN-NAC-$d_8$ produced remarkably lower signal intensities when spiked at the same concentrations as their non-deuterated versions. Low signals introduced inaccuracies and caused SFN-$d_8$ and SFN-NAC-$d_8$ to fail in the method validation step. We attempted to develop a sensitive and fast SIL-free LC–MS method. SFN conjugates could be quantified successfully using external standard quantification. However, for SFN, this approach proved challenging due to observed matrix effects and possible SFN degradation which resulted in reduced accuracy. This limitation was overcome by spiking extraction solvent with freshly prepared SFN-$d_8$. We also optimized methods for quantifying SFN metabolites by simplifying plasma preparation. We shortened the run time to eight minutes and used external standard calibration to quantify metabolites. We then confirmed this quantification method in human plasma after consumption of commercial sulforaphane preparation.

The mean peak of combined metabolites from our study (0.9 and 1 μM) using 120 mg of broccoli seed extract (~32 mg of SFN) was similar to work by Fahey et al. who investigated the pharmacokinetics of 350 mg of purified broccoli seed powder (mean 1.3 μM ± 0.5 μM) [47], though our dose was almost three-times less. The pharmacokinetic profiles of our study mirrored those of Fahey et al. in that excretion was complete 8 hrs after consumption. Our intervention peaked slightly later (~2hrs), than that of Fahey (~1 hr), likely due to our use of a capsule rather than liquid [47].

This simplified yet sensitive methodology allows high-throughput LC–MS analysis of plasma samples for the measurement of sulforaphane and its metabolites. The preliminary results confirmed method suitability to study sulforaphane supplementation in patients. Our methodological adaptations better allow the use of LC–MS to process the large number of samples that are likely to be required in future dose-finding studies and large clinical trials [48].

## 4. Materials and Methods

### *4.1. Materials*

LC–MS-grade acetonitrile was from Burdick and Jackson (Muskegon, MI, USA). LC–MS-grade formic acid (Optima™) was from Fisher Chemical (Thermo Fisher Scientific, Waltham, MA, USA). Reverse osmosis purified MilliQ water used in LC–MS analysis was from Millipore water purification system (Merck, Darmstadt, Germany). Analytical standards SFN, SFN-$d_8$, SFN-NAC-$d_8$, SFN-GSH, SFN-NAC, SFN-Cys were from Toronto Research Chemicals (Toronto Research Chemicals, Toronto, ON, Canada). CysGly (>85%) and pyridine were purchased from Sigma Aldrich (St. Louis, MI, USA). HF Bond Elute™ SPE cartridges C18 (6 mL tube, 500 mg bed) were purchased from Agilent Technologies (Colorado Springs, CO, USA). Myrosinase-activated broccoli sprout extract capsules, Broccomax™, were sourced from Jarrow Formulas (Los Angeles, CA, USA).

### 4.2. Application of Study Methods

A pharmacokinetic study was approved by the Monash Health Ethics Committee (HREC: 17-0000-169A) and conducted in accordance with the National Statement on Ethical Conduct [49]. The two healthy volunteer participants were identified within the community and approached for recruitment. Both participants provided written informed consent before they participated in this study. This research was conducted in accordance with the Declaration of Helsinki and the protocol was approved by an ethics committee (HREC 17-0000-169A). Inclusion criteria were non-pregnant, nulliparous women age 18–35. Exclusion criteria included current use of broccoli sprout extract, pre-existing medical condition (thyroid dysfunction, hepatic disease, renal disease, chronic inflammatory disease, polycystic ovarian syndrome), gastrointestinal disturbance, current infection, smoking, or any current medication (excepting the oral contraceptive pill). The characteristics of the participants are outlined in Table 5.

**Table 5.** Summary of participant demographics.

| Demographics | Participant One | Participant Two |
|---|---|---|
| Age (yrs) | 23 | 20 |
| BMI (m/kg$^2$) | 24 | 26 |
| Dietary restrictions | Nil | Nil |
| Medication | OCP[1] | Nil |
| Co-morbidities | Nil | Nil |

[1] Oral contraceptive pill.

The two participants fasted from midnight the day prior to commencing the study. Both were admitted to a clinical trial research center and an intravenous cannula placed in their non-dominant arm. Baseline venous blood (5 mL) was collected into ethylenediaminetetraacetic acid (EDTA) tubes immediately placed at 4 °C for 20 min before centrifugation for 20 min at 1200× *g* and 4 °C. Plasma was collected and aliquots immediately stored at −80 °C until analysis.

The participants were then observed consuming four Broccomax™ capsules, each containing 30 mg of broccoli seed extract and a dose of 8 mg of sulforaphane, as per manufacturer certificate of analysis, resulting in a total dose of 32 mg of sulforaphane (120 mg of broccoli seed extract). Otherwise they remained fasted for eight hours following. Further blood samples were collected into EDTA tubes at 30 min, one hour, two hours, four hours and eight hours after ingestion of the capsules and processed as above. For the eight-hour period the participants were monitored for potential side effects, reported or observed.

### 4.3. Chemical Synthesis of SFN-CG

*DL*-SFN-CG was synthesized using a modification of the methods described by Kassahun et al. [38] and Hauder et al. [26]. In our modified method NaOH solution pH 8.0 was replaced with pyridine, a Lewis base. 7.24 mg of CysGly (4 eq, 0.04044 mmol) dissolved in 100 µL of 50% EtOH and 1.8 mg of SFN (1 eq, 0.01015 mmol) dissolved in 100 µL of EtOH were mixed together in an Eppendorf tube. Three drops of pyridine were added using a syringe. Pyridine was used in excess and thus the exact amount was not measured. The reaction mixture was stirred for 5 h in the dark. Reaction progress was monitored qualitatively using LC–MS to check if levels of SFN changed. After 5 h the reaction was stopped using 10 µL 1M HCl. The reaction mixture was then diluted with MilliQ water to a volume of 600 µL. Two subsequent SPE clean-up steps were performed. C18 SPE cartridges were washed with 6 mL 0.1% formic acid in acetonitrile and conditioned with 6 mL of 0.1% formic acid in water. The reaction mixture was loaded and washed with 2 mL 0.1% formic acid in water. The reaction product was eluted using 2 mL 10% acetonitrile 0.1% formic acid solution, collecting 0.5 mL fractions. Two subsequent SPE purifications were used to purify SFN-CG. The first SPE purification filtered out pyridine but did not remove excess of CysGly due to the ethanol content in the crude reaction mixture.

After the first SPE purification, fractions 1–5 were collected and concentrated under nitrogen stream at 20 °C. After the second purification the pure product was obtained from fractions 2–5 after drying under vacuum.

In total, 2.1 mg (58% yield) of white solid; HRAM ESI-MS(+): *m/z* 356.07617 (100; [M + H], $C_{11}H_{22}O_4N_3S_3$, delta ppm −1.474); $^1$H NMR (600 MHz, DMSO-$d_6$), δ 1.67 (m, 4 H, CH), 2.52 (s, 3 H, CH3), 2.66 and 2.77 (m, 2 H, CH), 3.34 (dd, 1 H, CH), 3.50 (dd, 1 H, CH), 3.60 (t, 3 H, $CH_2$ and CH), 3.76 (s, 2 H, $CH_2$).)

### *4.4. Spectroscopic Data of Standard*

SFN-CG structure and purity were confirmed using high resolution accurate mass (HRAM) and $^1$H NMR analyses. HRAM was performed on QExactive HF mass spectrometer (Thermo Scientific, Waltham, MA, USA). Synthesized powdered SFN-CG was dissolved in water and directly infused using a syringe pump into mass spectrometer at 3 μL/min flowrate. The spectra recorded in positive ion mode. $^1$H NMR spectrum was recorded on Bruker Avance 600 MHz spectrometer (Bruker, Billerica, MA, USA).

### *4.5. Preparation of Stock Solutions*

In total, 100 mM stock solutions of SFN-GSH, SFN-Cys, SFN-CG and SFN-NAC were prepared in water with formic 0.1% and stored at −80 °C. SFN solution was prepared as 100 mM in EtOH with 0.1% formic acid. Individual stock solutions were mixed to prepare a 1 mM working solution. This solution was serially diluted with 0.1% formic acid solution to prepare 100 μM, 10 μM and 1 μM and 100 nM working solutions that were then used to spike QC samples and allow generation of calibration curve dilutions. All working solutions were prepared daily and discarded after use.

### *4.6. Preparation of Plasma Samples for LC–MS*

Single thawed plasma aliquots were used during method development and analysis. 25 μL of plasma was transferred to Eppendorf tubes with 100 μL of pre-chilled 0.1% formic acid in methanol spiked with 60 nM of SFN-d8 (corresponding to 300 nM plasma concentration), all kept on ice. Samples were quickly vortexed and shaken at 4 °C for 4 min. Samples were centrifuged at 1480× *g*, 4 °C for 10 min. Then, 100 μL of supernatant was transferred to clean Eppendorf tubes and the solvent evaporated at 20 °C under nitrogen stream for 30 min. Samples were resolubilized in 100 μL of 0.1% formic acid in water, quickly vortexed and sonicated in a water bath for 30 min with water bath temperature maintained below 25 °C. The samples were centrifuged at 1480× *g* at 4 °C for 10 min and the supernatant transferred into LC–MS vials. The samples were placed into LC–MS compartment (4 °C) and analyzed without delay.

### *4.7. Liquid Chromatography–Mass Spectrometry*

Liquid chromatography–mass spectrometry measurements were performed using triple-quadruple mass spectrometer Shimadzu LC–MS8050 (Shimadzu, Kyoto Japan) coupled with UHPLC system Nexera X2. Electrospray ionization in positive acquisition mode and multiple reaction monitoring mode (MRM) was used. Electrospray source parameters were set as follows: interface voltage 4.0 kV, interface temperature 300 °C, desolvation temperature 250 °C, heat block temperature 300 °C, nebulizing gas, heating gas and drying gas flow 3.0, 8.0 and 10.0 L/min respectively, CID gas pressure 270 kPa. Chromatographic separation was performed on an Hypersil Gold C18aq column (1.9 μm particles, 150 × 2.1 mm, Thermo Scientific) equipped with a guard column ($C_{18}$, 4.0 × 2.0 mm). The mobile phase consisted of 0.1% formic acid in water (A) and in acetonitrile (B). Injection volume was 2 μL. The gradient started at 10% B at time 0 and increased to 99% B by 3.5 min, kept at 99% B until 4.2 min and then returned to 10% B at 4.3 min and kept at 10% B until 8 min.

### *4.8. Method Validation*

Method validation criteria were adopted from FDA recommendations on chromatographic bioanalytical method validation [50]. The method was validated using QC samples prepared by spiking pooled pre-consumption time point plasma (blank plasma) from multiple patients at LLOQ, low, medium and high concentrations 3.9 nM or 7.8 nM, 11.7 nM, 200 nM and 1000 nM. Calibration curve solutions were prepared in the matched matrix keeping plasma components concentration the same in all dilutions. The calibration curve covered the range 3.9 nM–1000 nM and was generated by serial dilution of extracted spiked sample (1000 nM spike of all standards) with extracted unspiked sample. Accuracy is the closeness of the measurement to the true value. Accuracy was determined by measuring QC samples at four concentration levels 3.9 nM or 7.8 nM, 11.7 nM, 200 nM and 1000 nM and is expressed as % bias. Recovery is extraction efficiency of sample preparation procedure and is expressed as % of the nominal concentration value. Recovery was determined comparing samples spiked before and after extraction at concentrations levels 40 nM, 200 nM and 1000 nM. Spiking 80% of the nominal concentration values after extraction accounts for 20% of sample volume loss during supernatant transfer step (100 µL is transferred from total 125 µL of sample). Matrix effects, signal suppression or enhancement from coeluting plasma components were assessed by analysing spiked extracted plasma and spiked water. % difference was calculated between measured values for each analyte at three different concentration levels 40 nM, 200 nM and 1000 nM and between extracted individual blank plasma at 200 nM spike. Autosampler stability was assessed by reanalyzing the same calibration curve samples after 12 h in the autosampler. The limit of quantification (LOQ) is the lowest point of calibration curve with accuracy between 80% and 120%. Intraday precision (repeatability) determined from 6 or 4 consecutive injections of four QC levels −3.9 nM or 7.8 nM (LOQ), 11.7 nM (low QC), 200 nM (medium QC) and 1000 nM (high QC). Intermediate precision was inferred from 200 nM QC sample analysis on three different days.

### *4.9. Statistical Analysis*

Area under the curve (AUC) and mean peak were determined using GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA). These values were not compared statistically due to low numbers (n = 2).

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**Sample Availability:** Samples are not available from authors.

# Chapter seven

## *Establishing sulforaphane bioavailability and effects on blood pressure in women with pregnancy hypertension*

Having developed and established the analytical methods to accurately measure the level of sulforaphane and its metabolites in human plasma, two clinical investigations were conducted. First, to identify whether the formulation of broccoli extract – myrosinase activated or inactivated – altered the bioavailability of sulforaphane. Myrosinase is a naturally occurring enzyme located within a plant cell wall. It is released on tissue damage, through chewing, but is also present, to a much lesser extent, within the microbiome of the gastrointestinal tract[71,104]. Optimal myrosinase activation transforming inactive glucoraphanin into sulforaphane occurs when broccoli sprouts are heated from 25-70ºC. Overheating inactivates the enzyme quaternary protein structure[103,104] and greatly reduces enzyme activity. However, efficacy is only apparent acutely at particularly high doses or with chronic exposure[72]. In fact, clinical investigations into the therapeutic potential of sulforaphane have met with some[61,72,109], though limited[110–112] success, possibly due to ineffective myrosinase activation. Feasibility issues exist in establishing a therapeutic using broccoli sprout extract; capsule and liquid[111,112] formulations lack crucial chewing processes and physical consumption of sufficient broccoli to reach therapeutic levels is not plausible.

An artificially activated myrosinase preparation was identified and an investigation established to determine whether this preparation would convey higher plasma levels than a non-activated counterpart. An open label, single arm crossover study to compare the bioavailability of the two preparations was conducted. Such information would facilitate further large-scale clinical investigations into the therapeutic potential of a broccoli extract.

Having confirmed which of the two available formulations afforded better bioavailability, an appropriate dose in what would be the future target population, women with hypertensive disorders of pregnancy, was sought. Importantly, clinical trials to evaluate the *in vivo* effects of sulforaphane have shown an apparent lack of side effects or safety concerns[61,113]. This dose escalation study was necessary given the significant changes to drug absorption and metabolism that occur during pregnancy[114,115]. Preeclampsia in particular is a condition of volume overload with further derangements to cardiovascular output and often abnormal liver function and renal clearance. While studies investigating antioxidants for preeclampsia have met with negative results, few of these had appropriate preceding dosing and pharmacokinetic studies[116]. Similarly, few studies have adequate pharmacovigilance and safety data despite the vulnerability of the pregnant population[115].

To identify the bioavailability of sulforaphane and metabolites in a preeclamptic cohort a dose escalation study in women with hypertensive disorders of pregnancy was conducted.

***Hypothesis***

An activated broccoli extract will confer higher plasma levels of sulforaphane and metabolites and that pregnant women will require different doses of broccoli extract than non-pregnant women.

***Aims***

1. To compare plasma concentrations of sulforaphane and metabolites after consumption of a myrosinase activated broccoli extract and a non-activated extract.
2. To conduct a bioavailability, dose escalation study of broccoli extract in women with hypertensive disorders of pregnancy.

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MATERNAL FETAL MEDICINE/BIOLOGY: ORIGINAL ARTICLE

# Sulforaphane Bioavailability and Effects on Blood Pressure in Women with Pregnancy Hypertension

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## Abstract

Sulforaphane, an isothiocyanate found in cruciferous vegetables such as broccoli, shows promise as an adjuvant therapy for preeclampsia. To inform future clinical trials, we set out to determine the bioavailability of sulforaphane in non-pregnant and preeclamptic women. In six healthy female volunteers, we performed a crossover trial to compare the bioavailability of sulforaphane and metabolites afforded by an activated and non-activated broccoli extract preparation. We then undertook a dose escalation study of the activated broccoli extract in 12 women with pregnancy hypertension. In non-pregnant women, an equivalent dose of activated broccoli extract gave higher levels of sulforaphane and metabolites than a non-activated extract ($p < 0.0001$) and greater area under the curve (AUC) (3559 nM vs. 2172 nM, $p = 0.03$). Compared to non-pregnant women, in women with preeclampsia, the same dose of activated extract gave lower levels of total metabolites ($p < 0.000$) and AUC (3559 nM vs. 1653 nM, $p = 0.007$). Doubling the dose of the activated extract in women with preeclampsia doubled levels of sulforaphane and metabolites ($p = 0.02$) and AUC (1653 nM vs. 3333 nM, $p = 0.02$). In women with preeclampsia, activated broccoli extract was associated with modest decreases in diastolic blood pressure ($p = 0.05$) and circulating levels of sFlt-1 ($p = 0.0002$). A myrosinase-activated sulforaphane formulation affords better sulforaphane bioavailability than a non-activated formulation. Higher doses of sulforaphane are required to achieve likely effective doses in pregnant women than in non-pregnant women. Sulforaphane may improve endothelial function and blood pressure in women with pregnancy hypertension.

**Keywords** Preeclampsia · Bioavailability · Antioxidant · Sulforaphane · Broccoli

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## Introduction

Over the past 20 years, there have been significant advances in the prediction [1, 2], prevention [3] and diagnosis [4, 5] of preeclampsia. There have been no such major improvements in the management of women with established disease. Controlling maternal hypertension to allow prolongation of pregnancy remains the mainstay of care, as it did 50 years ago [6]. However, insights into the pathophysiology of preeclampsia have offered opportunities to develop new and hopefully more effective therapies. In particular, sustained oxidative injury to the maternal vasculature, secondary to circulating antiangiogenic factors, appears central to the progression of the disease [7]. Understanding how those antiangiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1) and activin A, released from the injured preeclamptic placenta, drive progressive and sustained systemic oxidative stress and endothelial dysfunction [8–11] has identified some promising new therapeutic targets [6, 12–16].

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In particular, there has been growing interest in plant-sourced antioxidants, including pomegranate and beetroot juice [17, 18] and resveratrol [19, 20]. Most recently, sulforaphane, an isothiocyanate found in cruciferous vegetables such as broccoli [21, 22], has been proposed as a therapy for preeclampsia [23]. Sulforaphane is both a potent antioxidant and an anti-inflammatory, acting both via the antioxidant releasing nuclear factor erythroid factor 2 (NFE2L2) and by inhibiting nuclear factor κβ (Nf-κβ) and transforming growth factor β (TGFβ) pathways [21, 24].

However, a woman would need to consume about 2 kg broccoli per day to achieve therapeutic levels of sulforaphane. Conveniently, sulforaphane is available in capsule or tablet form as glucoraphanin, a derivative of the amino acid methionine [22]. Glucoraphanin is metabolically inert, requiring hydrolysis by myrosinase to form active sulforaphane [22, 25] and, by subsequent conjugation, into four active metabolites: sulforaphane cysteine (SFN-CYS), sulforaphane cysteine-glycine (SFN-Cys-Gly), sulforaphane-N-acetylcysteine (SFN-NAC) and sulforaphane glutathione (SFN-GSH) [22]. Sulforaphane and its metabolites are each gene transcription activators, releasing bound NFE2L2 in the cell cytosol allowing nuclear translocation [26]. In the nucleus, NFE2L2 increases transcription of cellular "safeguarding" genes in the antioxidant response element of cellular DNA, resulting in translation of a number of antioxidant enzymes that then undergo redox reactions to reduce damaging oxygen free radicals [27].

Commercially available preparations of sulforaphane are available either as glucoraphanin alone or as myrosinase-activated glucoraphanin. We set out to compare the bioavailability of sulforaphane and its metabolites afforded by each of these preparations in non-pregnant women and then optimise dosing in women with preeclampsia to inform the design of future efficacy studies [28]. This work is particularly important given the limited data available regarding pharmocokinetic drug distribution in pregnancy. Establishing an appropriate dose for the pregnant population before clinical taregt is an essential, though often omited, phase of study design.

## Materials and Methods

Patients were not involved in the design of the studies, establishing the research questions or development of recruitment procedures.

Two commercially available sulforaphane-containing nutritional supplements were assessed, a myrosinase-activated broccoli seed extract (BroccoMax®, Jarrow Formulas, Los Angeles, CA) and a non-activated broccoli sprout extract (Broccoli Sprouts Extract™, Source Naturals, Scotts Valley, CA).

### Bioavailability Studies

#### Comparing Two Supplements in Non-pregnant Women

To assess the bioavailability of sulforaphane and its metabolites afforded by each of the two supplements, we first undertook a crossover study. Six healthy volunteer women were recruited from the local community. Inclusion criteria were as follows: non-pregnant, age 18–35 years. Exclusion criteria were as follows: current use of broccoli extract, existing significant medical condition (hypertension, thyroid dysfunction, hepatic disease, renal disease, polycystic ovarian syndrome), gastrointestinal disturbance, chronic inflammatory disease, infection, smoking, alcohol abuse, or illicit drug use. Each participant gave written informed consent prior to screening and enrolment.

Each participant fasted from midnight the day prior to the study. On the morning of the first study day, each woman was admitted to the clinical trials research centre and an IV cannula inserted. A baseline blood sample was collected. Each participant then swallowed four capsules of the activated broccoli seed extract BroccoMax®, equivalent to 32 mg of sulforaphane, but otherwise remained fasted for 8 h during which time a further five blood samples (at 30 min, 1 h, 2 h, 4 h and 8 h after ingestion of the sulforaphane preparation) were collected. After a 2-week "wash out" period, the study protocol was repeated for each participant but administering Broccoli Sprouts Extract™, the non-activated broccoli sprout extract, at an equivalent sulforaphane dose.

#### Dose Finding Study in Women with Hypertensive Disorders of Pregnancy

Next, to determine dosing for women with preeclampsia, we undertook a limited dose escalation study in women with hypertensive disorders of pregnancy, using the myrosinase-activated BroccoMax® supplement. Of the two preparations, we used this one because it had afforded the better sulforaphane bioavailability in the comparative study. We recruited 12 women with a singleton pregnancy and a diagnosis of preeclampsia or gestational hypertension, as defined by national guidelines [29]. Inclusion criteria were as follows: ≥ 18 years of age, singleton pregnancy with a live fetus, normal mid trimester morphology scan, systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, gestation $> 24^{+0}$ weeks. Exclusion criteria were as folllows: eclampsia, current use of a broccoli extract, multiple pregnancy, known congenital abnormality, contraindication to continuing with pregnancy (maternal or fetal reason), inability to provide informed consent.

Following written, informed consent, women had an IV cannula inserted, a baseline blood sample collected and a baseline blood pressure recorded. Blood pressure

recordings were measured from the right arm with the same sphygmomanometer and by the same observer throughout. Prior to administration of BroccoMax®, a cardiotocograph (CTG) was performed and the study continued only if the fetal heart rate was normal for gestation. Six women each took four BroccoMax® capsules, and then, a further six women each took eight capsules. For each participant, the CTG remained in place for the first hour after BroccoMax® administration and removed if the fetal heart rate was normal. Blood pressure was taken half hourly for the first 2 h, hourly for the next 2 h, and then two-hourly for the final 2 h. In the event of a blood pressure reduction of more than 30 mmHg systolic and/or 15 mmHg diastolic from baseline, the CTG was to be restarted. Blood samples were collected at the same time points as for study 1. Women were not fasted for this study. However, they were all hospital inpatients and received the same ward diet.

### Sample Processing

For both studies, venous blood (5 mL) was collected into ethylenediaminetetraacetic acid (EDTA) tubes and immediately placed at 4 °C for 20 min before centrifugation for 20 min at 1200*g* at 4 °C. Plasma was collected, aliquoted and immediately stored at − 80 °C until analysis. Single thawed aliquots were used during all biochemical analysis. Samples were not subject to repeated freeze-thaw cycles.

### Sulforaphane and Other Analytes

Plasma samples were analysed for sulforaphane and its metabolites using liquid-chromatography mass-spectrometry (LC-MS), as previously described [30]. Briefly, LC-MS was performed using a triple-quadruple mass spectrometer (LCMS8050, Shimadzu, Kyoto, Japan) coupled with Ultra High Performance Liquid Chromatography system (Nexera X2, Shimadzu). Electrospray ionisation in positive acquisition mode and multiple reaction monitoring mode (MRM) was used. Electrospray source parameters were set as follows: interface voltage 4.0 kV, interface temperature 300 °C, desolvation temperature 250 °C, heat block temperature 300 °C, nebulizing gas, heating gas and drying gas flow 3.0, 8.0 and 10.0 L/min respectively, CID gas pressure 270 kPa. Chromatographic separation was performed on an Hypersil Gold C18 column (1.9 μm particles, 150 × 2.1 mm, Thermo Scientific, Waltham, MA, USA) equipped with a guard column ($C_{18}$, 4.0 × 2.0 mm). The mobile phase consisted of 0.1% formic acid in water (A) and in acetonitrile (B). Injection volume was 2 μL. The gradient started at 10% B at time 0 and arrived to 99% B by 3.5 min, kept at 99% B until 4.2 min and then returned to 10% B at 4.3 min and kept at 10% B until 8 min. Transitions monitored were as follows: SFN-Cys: 299.00 m/z > 136.00 m/z, − 11 eV, 1.47 min; SFN-GSH: 484.80 m/z > 179.30 m/z, − 25 eV, 1.79 min; SFN: 177.90 m/z > 113.90 m/z, − 12 eV, 2.55 min; SFN-NAC: 340.80 m/z > 178.00 m/z, − 14 eV, 2.02 min; SFN-CG: 355.80 m/z > 136.10 m/z, − 12 eV, 1.59 min.

Soluble fms-like tyrsoine kinase-1 (sFlt-1) and placental growth factor (PlGF) were quantified using the B.R.A.H.M.S. Kryptor Compact PLUS (Thermo Fisher Scientific, Henningsdorf, Germany) as previously described [31] and in accordance with the manufacturer's instructions. Activin A was measured using an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA), as previously described [23].

### Human Research Ethics

Both studies were approved by the Monash Health Research Ethics Committee (HREC: 17-0000-169A and HREC:18-0000-514A) and were conducted in accordance with the National Statement on Ethical Conduct [32]. All participants provided written informed consent and all recruited participants completed their respective studies with no study withdrawals or loss to follow-up.

### Statistical Analyses

To quantify sulforaphane and metabolites, we assessed the area under the curve (AUC) for each metabolite. Data were expressed as mean ± standard error of the mean (SEM). We compared the AUC and mean maximum concentration (CMax) between activated and non-activated formulations in six non-pregnant women, and between four and eight capsules of the activated formulation (BroccoMax®) in 12 pregnant women with hypertension. Circulating levels of the angiogenic markers (sFlt-1, PlGF and activin A) were expressed as fold change from control and were compared using a two-way ANOVA with the Holm-Sidak post hoc correction. Pharmacokinetic profiles and blood pressure were compared using a 2-way ANOVA with time ($P_{TIME}$) and either dose ($P_{DOSE}$) or formula ($P_{FORM}$) as the two independent variables. Interaction $p$ values are also reported ($P_{INT}$). Continuous maternal characteristics were assessed for normality using the Sharpiro-Wilk test and compared using $t$ test or the Mann Whitney $U$ with data presented as mean with SD or median and IQR as appropriate. Categorical variables were assessed using Pearson's chi$^2$. A value of $< 0.05$ is considered statistically significant. All analysis was performed using GraphPad Prism 7.0 (San Diego, CA).

## Results

### Participant Demographics

The mean (SD) age of the six healthy volunteers in the comparative study was 24.5 (4.03) years. Three (50%) of the women were taking the oral contraceptive pill. None was smokers. Table 1 summarises participant details for dosing finding study in pregnant women.

### Non-activated Versus Activated Broccoli Extract Formulations

The plasma levels of sulforaphane and its metabolites following ingestion of either a non-activated or activated broccoli formulation in the six non-pregnant women are shown in Fig. 1. Plasma levels of sulforaphane and all metabolites increased following administration of both formulations. There was a significant difference between formulations at 2 h for all metabolites (all $p < 0.05$), except for sulforaphane-N-acetylcysteine which was significantly different at 4 h ($p = 0.03$). The activated preparation resulted in a greater AUC for all metabolites (all $p < 0.05$), except unconjugated sulforaphane ($p = 0.2$). CMax of SFN-GSH (Fig. 1A) and SFN-Cys-Gly (Fig. 1E) were significantly higher following the activated than the non-activated preparation ($p = 0.0003$ and $p = 0.03$). There were no significant differences between formulation for the mean CMax for all other metabolites. A combined profile of all metabolites (Fig. 1K) shows that the activated formulation afforded higher levels of total metabolites over time ($p \leq 0.0001$), a greater AUC ($p = 0.03$) and a higher mean CMax ($p = 0.01$) than the inactivated formulation.

### Dose Escalation Study in Women with Preeclampsia

The bioavailability profiles of sulforaphane and its metabolites in women with pregnancy hypertension following administration of either four or eight capsules of the activated preparation are shown in Fig. 2. While there were differences between the profiles of each metabolite, in general the administration of eight capsules increased the peak serum concentrations about 2-fold compared to four capsules. These differences were seen at 4 h ($p = 0.01$) and 8 h ($p = 0.002$).

Eight capsules also approximately doubled the AUC for most metabolites, except SFN-Cys and SFN. Compared to four capsules, eight capsules increased the mean peak of SFN-GSH and SFN-NAC (Fig. 2A, $p = 0.01$; Fig. 2C, $p = 0.006$, respectively). A composite profile of all metabolites shows that, compared to four capsules, eight capsules approximately doubled AUC (1653 nM vs. 3333 nM, $p = 0.02$, Fig. 2L).

### Comparison of Preeclamptic and Non-pregnant Women

A comparison of bioavailability data following administration of BroccoMax® in non-pregnant and pregnant women with hypertension is shown in Fig. 3. We found significantly higher levels of all metabolites in the non-pregnant cohort, with peak levels attained sooner. Levels of all metabolites were significantly different at 2 h and SFN-Cys, SFN-GSH and SFN were also significant at 1 h (Fig. 3A). When we compared the total exposure, the AUC in the non-pregnant women was about double that seen in hypertensive pregnant women (3559 nM vs. 1653 nM, $p = 0.007$; Fig. 3B). The AUC in hypertensive

**Table 1** Demographic information of 12 pregnant women in dose escalation study

| | No. of BroccoMax capsules | | *p* value |
|---|---|---|---|
| | Four | Eight | |
| Number of women | 6 | 6 | - |
| Nulliparous | 4 (67%) | 5 (83%) | - |
| Median (IQR) maternal age (years) | 31 (27–36) | 28 (21.5–36) | 0.45 |
| Median (IQR) gestation (weeks$^{+days}$) | $37^{+6}$ ($32^{+5}$–$39^{+3}$) | $36^{+5}$ ($33^{+2}$–$39^{+1}$) | 0.95 |
| No. (%) Smoker | 1 (17%) | 1 (17%) | - |
| No. (%) gestational diabetes | 2 (33%) | 1 (17%) | - |
| No. (%) gestational hypertension | 1 (17%) | 3 (50%) | - |
| No. (%) preeclampsia | 5 (83%) | 3 (50%) | - |
| Antihypertensive medication | | | |
| Labetalol | 5 (83%) | 4 (67%) | |
| Nifedipine | 1 (17%) | 1 (17%) | |
| Mode of birth | | | |
| Caesarean section | 2 (33%) | 3 (50%) | |
| Vaginal birth (all inductions) | 4 (67%) | 3 (50%) | |

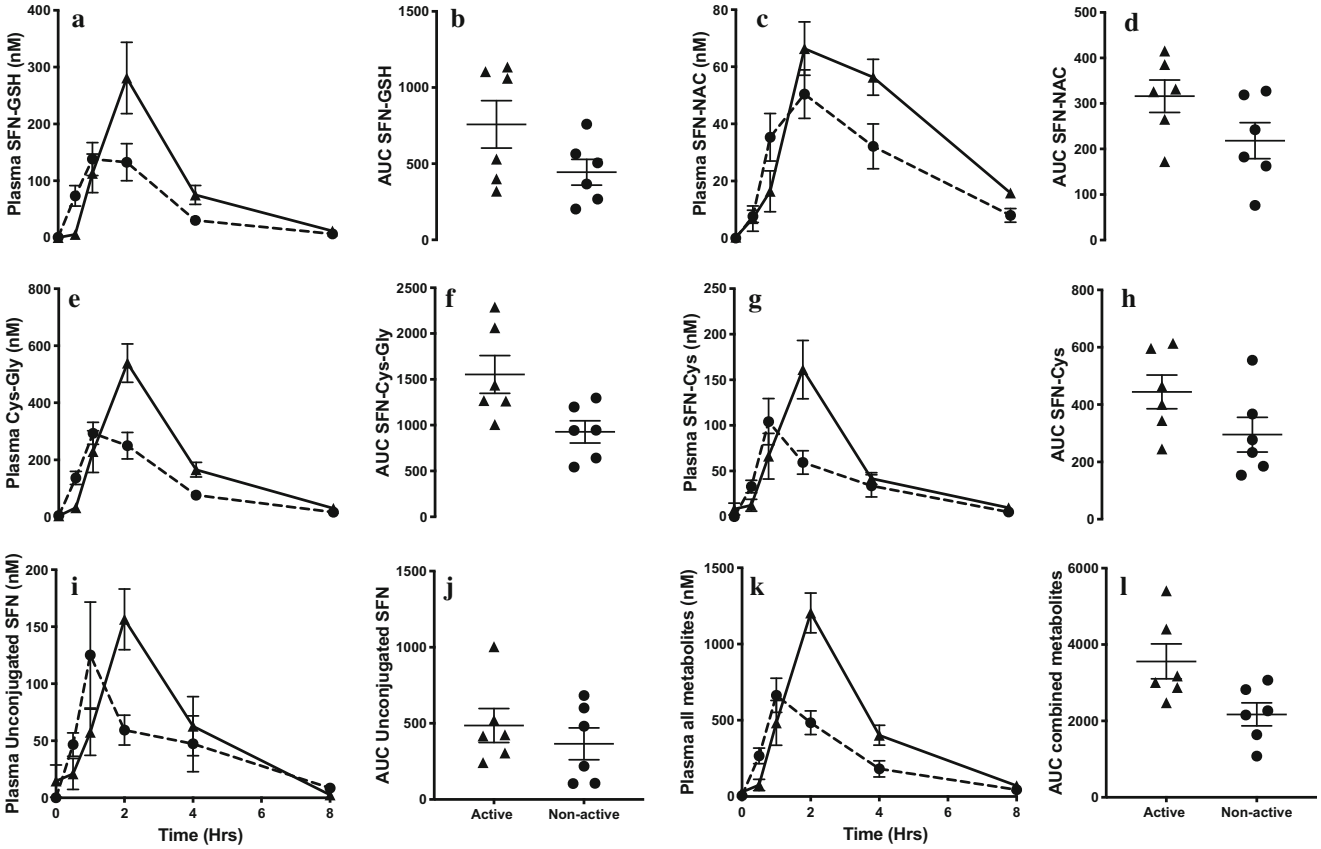


**Fig. 1** Pharmacokinetic profiles and area under the curve comparing active (▲) and non-active (●) groups for each metabolite and combined total of all metabolites: SFN-GSH ((A) $P_{INT} < 0.0007$, $P_{FORM}$ 0.40; (B) AUC 845.7 vs. 443.9, $p = 0.04$), SFN-NAC ((C) $P_{INT} < 0.002$, $P_{FORM}$ 0.38; (D) AUC 315.5 vs. 195.5, $p = 0.05$), SFN-Cys-Gly ((E) $P_{INT} < 0.0001$, $P_{FORM}$ 0.22; (F) AUC 1555 vs. 870.5, $p = 0.03$); SFN-CYS ((G) $P_{INT}$ 0.0008 $P_{FORM}$ 0.41; (H) AUC 444.3 vs. 217.9, $p = 0.008$), unconjugated SFN ((I) $P_{INT}$ 0.0045, $P_{FORM}$ 0.77; (J) AUC 485.8 vs. AUC 317, $p = 0.2$) and combined metabolites ((K) $P_{INT} < 0.0001$, $P_{FORM}$ 0.25; (L) AUC 3559 vs. 2172, $p = 0.03$). Statistical analysis was 2-way ANOVA with time and formula as variables for PK profiles and paired *t* test for AUC. $N = 6$. Significance is $p \leq 0.05$. Values are mean ± SEM

pregnant women taking eight capsules was similar to the AUC in non-pregnant women taking four capsules (3333 nM vs. 3559 nM, respectively; Fig. 3B).

### Blood Pressure and Biomarkers

Figure 4 shows the blood pressure profiles of the 12 pregnant women with hypertension immediately before and for 8 h after ingesting the BroccoMax® capsules. There was a possible trend towards a modest (~ 10%) reduction in diastolic but not systolic blood pressure over time (Fig. 4A; $p = 0.05$), irrespective of dose. Although the higher dose of BroccoMax® appeared to have a greater effect on maternal blood pressure than the lower dose, this was not significantly different. Plasma levels of sFlt-1 significantly decreased 2 h after BroccoMax® (Fig. 4C), irrespective of dose. There were no significant changes in PlGF (Fig. 4D), sFlt-1:PlGF ratio (Fig. 4E), or activin A (Fig 4F).

## Discussion

In this study, we have described the bioavailability of sulforaphane and its metabolites in both pregnant and non-pregnant women following administration of commercially available broccoli extract formulations. Such information is necessary to inform future clinical trials assessing sulforaphane as a potential adjuvant therapy for preeclampsia. We have also shown a possible reduction in blood pressure and circulating sFlt-1 levels in pregnant women with hypertension.

The key strength of this work is that it is the first report of the clinical use sulforaphane in pregnant women. While in vitro studies of sulforaphane as a future therapy for preeclampsia have been promising [22, 33], with evidence that it mitigates both trophoblast and endothelial injury, until now there have been no published reports of its use in pregnant women. The comparative dose finding in what would be the target population of future clinical trials—women with preeclampsia—is also a strength. Less than one in twenty

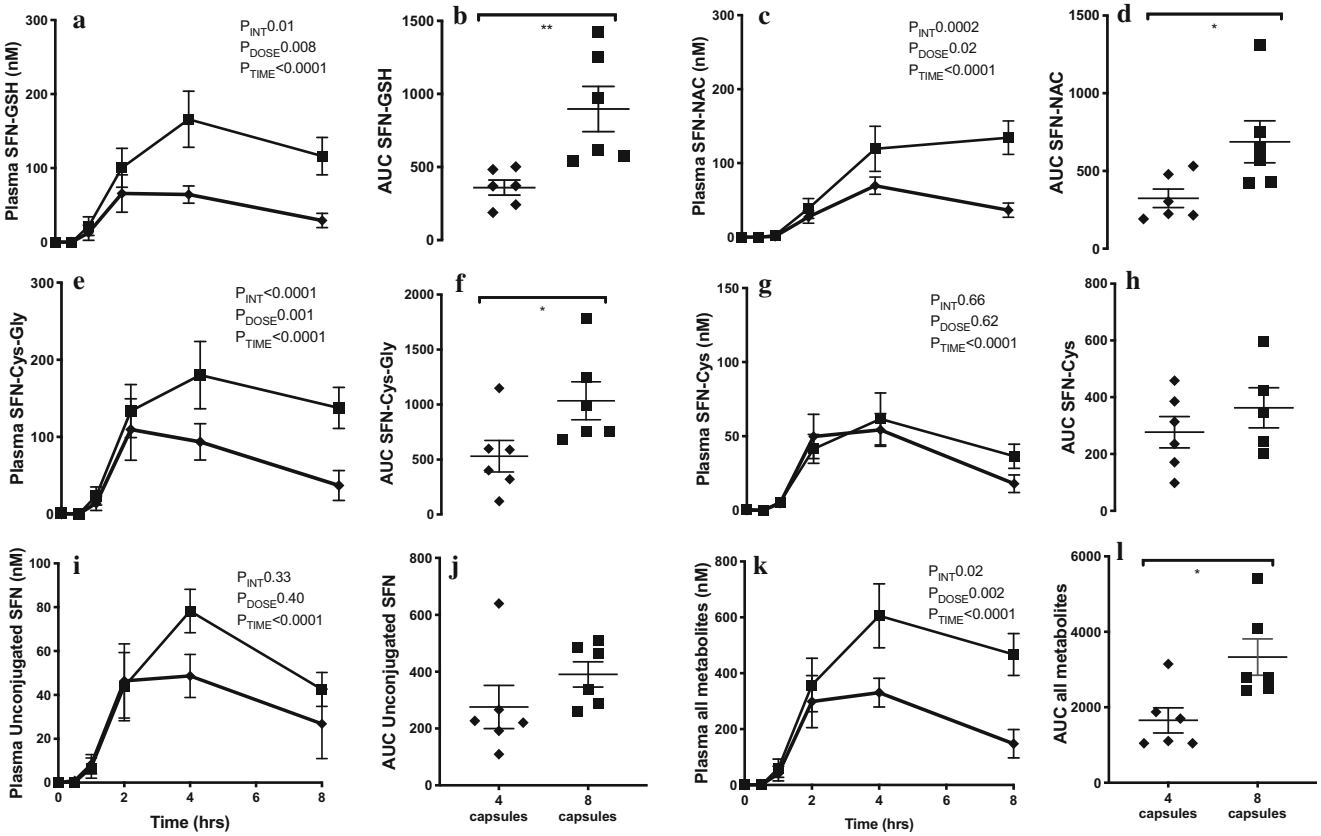


**Fig. 2** Pharmacokinetic profiles and area under the curve comparing four (♦) and eight capsules (■) in women with hypertensive disorders of pregnancy: SFN-GSH ((A) $P_{INT}$ 0.01, $P_{DOSE}$ 0.008; (B) AUC 359.5 vs. 896.8, $p = 0.01$); SFN-NAC ((C) $P_{INT}$ 0.0002, $P_{DOSE}$ 0.02; (D) AUC 345 vs. 687, $p = 0.03$); SFN-Cys-Gly ((E) $P_{INT} < 0.0001$, $P_{DOSE}$ 0.001; (F) AUC 1555 vs. 870.5, $p = 0.03$); SFN-Cys ((G) $P_{INT}$ 0.66, $P_{DOSE}$ 0.62; (H) AUC 277.1 vs. 362.8, $p = 0.77$); unconjugated SFN ((I) $P_{INT}$ 0.33, $P_{DOSE}$ 0.40; (J) AUC 275.9 vs. 390, $p = 0.220$); total ((K) $P_{INT}$ 0.02, $P_{DOSE}$ 0.002; (L) AUC 4: 1653 vs. AUC 8: 3333, $p = 0.02$). Statistical analysis was 2-way ANOVA with time and dose as independent variables for PK profiles and paired *t* test for AUC. $N = 12$. Significance is $p \leq 0.05$. Values are mean ± SEM

drugs used in pregnancy have been appropriately studied to define pharmacokinetics and ensure satisfactory therapeutic levels [34]. The key limitation is the relatively small number of women studied to date. However, for the purposes of assessing bioavailability and comparing formulations and doses, there was no requirement to study larger numbers of women.

Over recent years, sulforaphane has garnered increasing interest as a potential medicinal antioxidant with therapeutic applications in diseases as diverse as cardiovascular disorders

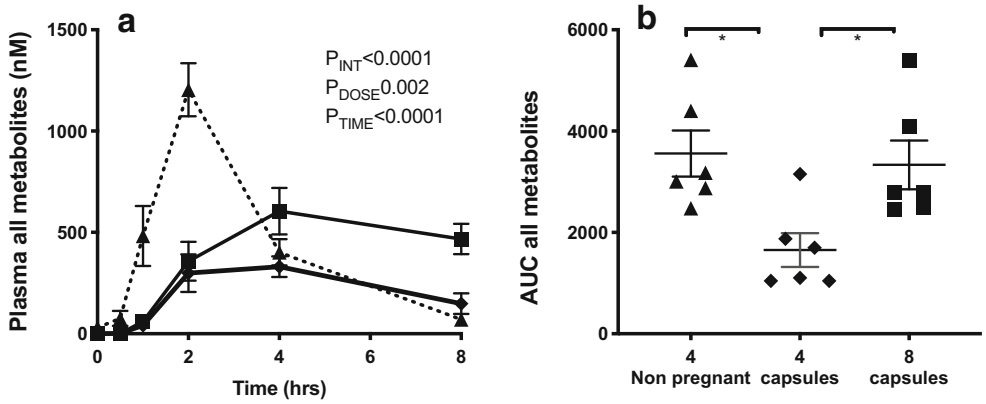


**Fig. 3** Comparison of combined metabolite levels for non-pregnant (▲) and four (♦) and eight capsules (■) in women with hypertensive disorders of pregnancy. (A) pharmacokinetic profile $P_{INT} < 0.0001$, $P_{DOSE}$ 0.002, $P_{TIME} < 0.0001$ and (B) area under the curve (non-pregnant vs. 4 capsules, $p = 0.01$; 4 capsules vs. 8 capsules, $p = 0.03$). Statistical analysis was 2-way ANOVA with time and dose as independent variables for PK profile and one-way ANOVA with group as the variable for AUC. Significance is $p \leq 0.05$. Values are mean ± SEM

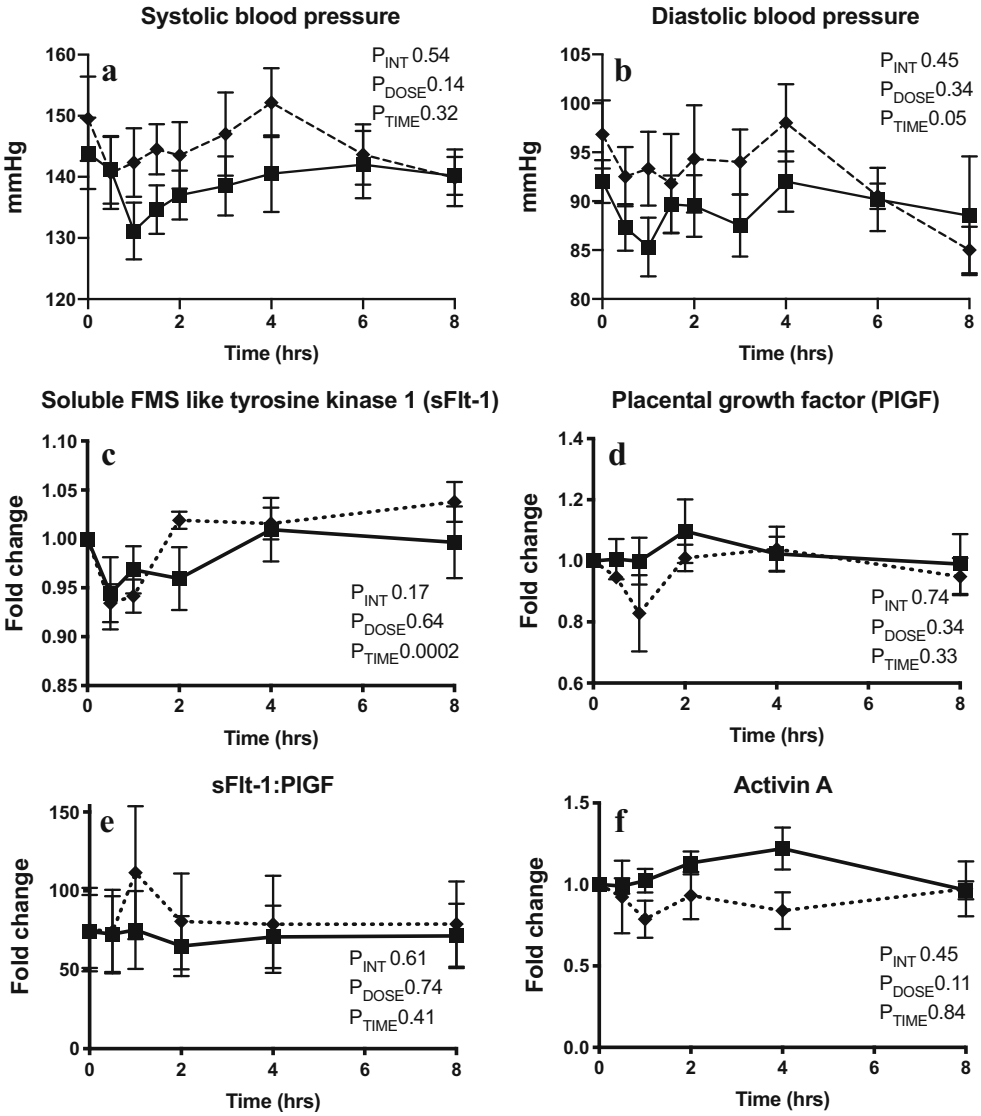


**Fig. 4** Blood pressure profiles and plasma concentration of biomarkers of women with hypertensive disorders of pregnancy immediately before and for 8 h after ingesting 4 (♦) and 8 (■) BroccoMax™ capsules. (A) Systolic blood pressure comparing 4 and 8 capsules over time $P_{INT}$ 0.54, $P_{DOSE}$ 0.14, $P_{TIME}$ 0.32; (B) diastolic blood pressure comparing 4 and 8 capsules over time $P_{INT}$ 0.45, $P_{DOSE}$ 0.34, $P_{TIME}$ 0.05; (C) soluble FMS-like tyrosine kinase-1 (sFLt-1) $P_{INT}$ 0.17, $P_{DOSE}$ 0.64, $P_{TIME}$ 0.0002; (D) placental growth factor (PlGF) $P_{INT}$ 0.74, $P_{DOSE}$ 0.34, $P_{TIME}$ 0.33; (E) sFlt-1:PlGF ratio $P_{INT}$ 0.61, $P_{DOSE}$ 0.74, $P_{TIME}$ 0.41; (F) activin A $P_{INT}$ 0.45, $P_{DOSE}$ 0.11, $P_{TIME}$ 0.84. Data are expressed as raw value (A and B) fold change from baseline (C–E) or ratio (F). Statistical analysis was 2-way ANOVA with time and dose as independent variables. $N = 12$. Significance is $p \leq 0.05$. Values are mean ± SEM

[35], cancer [36, 37], environmental respiratory diseases [38, 39], and neurological disorders such as autism [40] and schizophrenia [41]. Key to the success or failure of the application of sulforaphane in any of these clinical settings has been consideration of the need for sulforaphane to be activated by myrosinase. Myrosinase is an enzyme found in plant cell walls. It is released during plant cellular damage, as occurs during chewing [22], allowing hydrolysis of the inactive glucoraphanin to sulforaphane. This is the typical pathway of sulforaphane generation from broccoli that is eaten during a meal. However, ingestion of sulforaphane as a broccoli sprout or seed extract in a capsule bypasses the myrosinase step [22, 42] and the small amount of glucoraphanin hydrolysis undertaken by gastrointestinal bacteria has been thought to be insufficient to afford therapeutic levels of sulforaphane [22, 42]. Our observation that consumption of four capsules of an activated formulation of broccoli seed extract afforded higher plasma levels of sulforaphane and its metabolites than a similar dose of a non-activated formulation confirms the benefits of myrosinase activation. However, that reasonable levels of sulforaphane and its metabolites were also attained after consumption of the non-activation preparation suggests that bacterial hydrolysis may contribute more than previously thought [22].

The pharmacokinetic profile of sulforaphane and its metabolites afforded by the activated formulation in non-pregnant women was very similar to that previously reported for a reconstituted purified sulforaphane powder derived from broccoli seed [42]. In particular, in both studies, clearance was essentially complete 8 h after administration. The CMax in our study was slightly later, at 2 h, than the 1 h reported for the reconstituted powder. This is likely because we used an encapsulated formulation rather than a liquid [42] that would be more readily absorbed. Taken together, these studies suggest that, to maintain therapeutic levels, multiple daily dosing will likely be required.

Being mindful of the changes in drug distribution, metabolism and clearance that occur in pregnancy [34, 43], we undertook a limited dose escalation study to explore what doses of the activated formulation would be required by women with preeclampsia to achieve comparable blood concentrations to non-pregnant women. To achieve comparable levels of sulforaphane and its metabolites, women with preeclampsia require double the dose of BroccoMax® compared to non-pregnant women, an important insight. In non-pregnant women, consumption of four capsules of BroccoMax® resulted in a CMax of 1.2 μM—a level that in vitro improves endothelial and placental function [22, 33]. To achieve similar plasma levels in women with preeclampsia requires a dose eight capsules.

We monitored blood pressure during the dose escalation study as a safety measure. While we observed a small fall in blood pressure, it is not appropriate to claim here that BroccoMax® has any sustained antihypertensive effect in pregnant women with high blood pressure. Assessing efficacy was not the intent of our study. Nonetheless, an antihypertensive effect is consistent with a previous report outside of pregnancy [44]. Sulforaphane has both endothelial-dependent and endothelial-independent vasodilatory effects, the former most likely via antioxidant pathways [23] and the latter via vascular smooth muscle relaxation [45]. Future efficacy studies of sulforaphane as an adjuvant therapy in preeclampsia will certainly require careful monitoring of blood pressure following commencement of therapy.

Similarly, the modest reduction in sFlt-1 following BroccoMax® supports in vitro observations [23]. It is likely that any decrease in sFlt-1 results from reduced placental oxidative stress [23] and improved trophoblast mitochondrial function [33]. Oxidative stress increases mitochondrial activity [33], an effect that increases sFlt-1 production [46] and reversible with resveratrol [46]. However, as promising as this observation is, it is nothing more than that at this stage. This study was not designed to assess effects of BroccoMax® on antiangiogenic factors. Nonetheless, that there was any effect at all merits follow-up in future efficacy studies.

## Conclusion

We have provided bioavailability data to inform the design of a future efficacy trial of sulforaphane in women with preeclampsia [28] and have reminded us of the importance of dose finding studies in pregnant women prior to progressing to clinical trial.

**Code Availability** Not applicable

**Authors' Contributions** AGC, SM, KP and EMW designed the study protocols and undertook the clinical studies. AGC, DA and DJC performed the LC-MS. AGC wrote the first draft of the manuscript. All authors contributed to manuscript revisions, and read and approved the final manuscript.

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**Data Availability** Data are available on request from the corresponding author.

## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no competing interests.

**Human Research Ethics Statement** Both studies were approved by the Monash Health Research Ethics Committee: RES17-0000-169A, approved 7 April 2017, and RES18-0000-514A, approved 10 September 2018.

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# Chapter eight

## *Prolong: a double-blind placebo, randomised controlled trial of broccoli sprout extract in women with early onset preeclampsia. A clinical trial protocol.*

The culmination of this research was to be a clinical trial. This chapter describes the detailed design of that trial, including required sample size and main outcomes. Given that delaying delivery of women with preeclampsia is to improve perinatal outcome, any efficacy trial of a new therapy for preeclampsia should have meaningful child health measures, such as neurodevelopment at 2 years of age, as the primary outcome. However, such a trial would likely require many hundreds, if not thousands, of participants, depending on the gestation eligibility and anticipated size effect. Before the costs of such a trial could be justified it was necessary to undertake a proof-of-principle trial confirming, or not, that a broccoli extract could prolong pregnancy in women with early-onset preeclampsia. Given that melatonin is able to prolong pregnancy by almost a week in such women[55] and that sulforaphane has more impressive *in vitro* effects than melatonin[55,63], it is likely that BroccoMax will be able to prolong pregnancy too. However, this needs formal testing before a large multicentre randomised controlled trial with child health outcomes as the primary outcomes can be established.

While the early *in vitro* studies were underway and prior to the dose finding studies, design of a clinical trial began, with diagnosis-to-delivery interval as the primary outcomes. A proof-of-principle trial. The dose of broccoli extract was based on the existing literature[104]. However, it became apparent from *Chapter seven* that the bioavailability of sulforaphane in pregnant women with hypertension was lower than expected, requiring higher doses than initially anticipated and that was included in the study design protocol. For this reason, doses in the clinical trial registry reflect the dose of BroccoMax that will be used in the clinical trial, rather

than the does outlined in the published protocol. Subsequent amendments to study design are now updated in the clinical trials registry. A logistical issue facing the development of this trial is the relatively low number women who will be eligible to participate in this trial, and indeed the naturally smaller number who will consent. Increasing the catchment area from one hospital to nationwide, and possibly trans-Tasmin, may reduce the time taken to evaluate this potential therapeutic for preeclampsia. The decision expand recruiting centres will, of course, rest with those who move forward with the clinical trial.

***Hypothesis***

Sulforaphane will safely increase the length of pregnancy, recorded as time between diagnosis and delivery of the baby (diagnosis to delivery interval) in women with early onset preeclampsia.

***Aims***

To design a trial that would:

1. Determine whether broccoli extract can safely prolong the interval between enrolment and delivery (recorded in days) in women with early onset (<34 weeks) preeclampsia.
2. Investigate the effects of a broccoli supplement on production of maternal circulating biomarkers of placental and endothelial health in women with early onset (<34 weeks) preeclampsia.
3. Assess the effects of a broccoli extract on maternal and perinatal outcomes (safety and tolerance) in women with early onset (<34 weeks) preeclampsia.

BMJ Open

# Prolong: a double-blind randomised placebo-controlled trial of broccoli sprout extract in women with early onset preeclampsia. A clinical trial protocol

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## ABSTRACT

**Introduction** Preeclampsia is a leading cause of maternal and perinatal morbidity and mortality. There is a need for adjuvant, targeted therapies to improve outcomes. Broccoli sprout extract, rich in the antioxidant sulforaphane, reduces oxidative stress and placental secretion of the antiangiogenic factors that contribute to vascular dysfunction in preeclampsia. We propose a phase III trial investigating broccoli sprout extract. We will assess broccoli sprout extract in women with early onset (<34 weeks) preeclampsia, investigating (1) the interval between enrolment and delivery (days), (2) biomarkers of placental and endothelial function and (3) maternal and fetal outcomes.

**Methods** A double-blind, placebo-controlled randomised trial will be conducted at Monash Health, Melbourne, Australia. One hundred and eighty women (45 each arm of each stratum) with early onset preeclampsia (defined as per Society for Obstetric Medicine of Australia and New Zealand guidelines) will be recruited. Consenting women will be randomised to receive an oral dose of either broccoli sprout extract (24 mg of activated sulforaphane) or identical placebo, twice daily until delivery. Maternal blood will be collected antenatally for measurement of biomarkers of preeclampsia, including soluble fms-like tyrosine kinase 1 (sFlt-1), placental growth factor (PlGF), soluble endoglin (sEng) and activin A, as well as circulating sulforaphane metabolites. Maternal and perinatal outcomes will be monitored throughout. All clinical care decisions, including the timing of delivery, will be made by the treating team, blinded to treatment allocation. Participation in this trial will not affect routine care. At delivery, maternal and cord blood and placentae will be collected to measure sulforaphane metabolites and sFlt-1, PlGF, sEng and activin A.

**Ethics and dissemination** Approval to conduct the trial has been granted by Monash Health Human Research and Ethics Committee (RES-18-0000-109A). Deidentified data will be published in peer-reviewed journals and presented at learnt society conferences, both nationally and internationally. This study has not yet commenced and is pre-results.

**Trial registration number**
ACTRN12618000216213

## Strengths and limitations of this study

- Study design is a double-blind, randomised, placebo-controlled trial.
- Intervention is a naturally occurring nutritional supplement with an excellent safety profile.
- Sample size not adequate for secondary outcomes.
- Study participants restricted to women with early onset preeclampsia.

## INTRODUCTION

Preeclampsia is defined as new onset hypertension after 20 weeks' gestation with associated maternal organ dysfunction and/or fetal growth restriction.[1] It complicates 5%–8% of pregnancies and is a leading cause of maternal and perinatal morbidity and mortality worldwide.[1] Even in high-resource settings the risk of neonatal mortality is fivefold greater in those born to a mother with preeclampsia compared with those born to a normotensive mother. This increased mortality is largely due to associated fetal growth restriction and the need for premature delivery. Indeed, preeclampsia is the leading cause of iatrogenic premature delivery, implicated in 20% of all premature births.[1] Unfortunately, the incidence of preeclampsia has not changed over the last century and, beyond controlling maternal blood pressure, we continue to lack effective targeted therapies for this serious disorder.[1 2]

Though much remains unknown about the pathological progression of preeclampsia, it is broadly accepted that a placenta, chronically injured by ischaemic-reperfusion insult, releases excessive vasoactive and inflammatory factors into the maternal circulation. In turn, these factors induce systemic maternal endothelial dysfunction.[3] The resulting

vasoconstriction and increased vessel permeability cause hypertension, oedema, renal endotheliosis and secondary organ ischaemic injury. For the last 50 years, the pharmacological management of preeclampsia has aimed solely to correct the maternal hypertension, allowing safer continuation of the pregnancy in the interests of improving fetal maturity. While the focus on controlling hypertension has improved maternal and perinatal outcomes it has neglected the underlying pathological processes of the disease and limited the potential gains in mitigating fetal risk, particularly in the setting of early onset disease.[1] Seeking to prolong the pregnancy further by targeting the oxidative stress-induced endothelial dysfunction is an additional approach worth exploring.

In particular, inducers of the nuclear factor E2-like related factor 2 (Nrf2) antioxidant pathway offer an attractive approach. Inducing Nrf2 would be expected to have anti-inflammatory and antioxidant effects in both the placenta and in the maternal vasculature. Nuclear factor E2-related factor 2 is an endogenous inducer of cellular antioxidants.[4 5] Under physiological conditions, bioavailable levels of Nrf2 are regulated by cytosolic binding to kelch-like ECH-associated protein 1 (KEAP-1), preventing rapid proteasome degradation.[5] Exposure to oxidative stress induces cysteine modifications to KEAP-1, loss of binding to Nrf2 and translocation of Nrf2 to the nucleus.[4] Within the nucleus, by combining with small maf-proteins in the promoter region of antioxidant 'safeguarding' genes, Nrf2 stimulates antioxidant response elements resulting in the transcription of mRNA for a number of cellular antioxidants and phase II enzymes.[4] Numerous studies have shown therapeutic benefits from Nrf2 stimulation both in maintaining endothelial health and in treating vasculopathies.[6]

The Nrf2 inducer sulforaphane is a naturally occurring organosulphur abundant in broccoli sprout extract[7–9] that has attracted attention in cardiovascular and cancer medicine.[7 8] It stabilises Nrf2 by impairing ubiquitination and increasing Nrf2 phosphorylation, thereby preventing proteasomal degradation and causing cytosolic accumulation.[5] Sulforaphane also induces cytosolic transcription and nuclear translocation of Nrf2. As such, sulforaphane uses the Nrf2 pathways to enhance production of phase II and antioxidant enzymes, improving cellular resilience to oxidative stress.[4 10]

## Rationale

Preeclampsia remains a leading cause of maternal and perinatal morbidity and mortality worldwide.[1] While the introduction of antihypertensives 60 years ago represented a major advance in the care of women with preeclampsia, further progress has all but stalled. Future benefits in maternal and/or perinatal outcomes are likely to come from improved screening and prevention[11] or from more effective treatment, beyond simply managing maternal hypertension.[12 13] In particular, therapies that target the maternal endothelial dysfunction that underlies the hypertension offer promise in further improving maternal and perinatal outcomes. The antioxidant and anti-inflammatory sulforaphane may be one such therapy. Preliminary data from our group support a role for sulforaphane in reducing placental production of the antiangiogenic factors soluble fms-like tyrosine kinas 1 (sFlt-1) and activin A. We have further shown that sulforaphane improves endothelial cell health and function after activation with tumour necrosis factor alpha and serum from pre-eclamptic women. Whether sulforaphane has beneficial in vivo effects on placental and/or endothelial function in women with early onset preeclampsia remains unexplored. We aim to examine this possibility in our clinical trial, *Prolong*.

### Aims and hypothesis

We hypothesise that administration of Broccomax will significantly increase duration of pregnancy, specifically the interval between diagnosis of preeclampsia and delivery.

The overarching aim of this trial is to assess the utility of a commercial broccoli sprout extract (BroccoMax) as an adjuvant therapy in the management of women with early onset (<34 weeks) preeclampsia.

*Aim 1.* To assess whether broccoli sprout extract can safely prolong the interval between enrolment and delivery (recorded in days) in women with early onset (<34 weeks) preeclampsia.

*Aim 2.* To assess the effects of a broccoli sprout supplement on production of maternal circulating biomarkers of placental and endothelial health in women with early onset (<34 weeks) preeclampsia.

*Aim 3.* To assess effects of a broccoli sprout extract on maternal and perinatal outcomes (safety and tolerance) in women with early onset (<34 weeks) preeclampsia.

## Methods and analysis

### Study design

Double-blind, randomised, placebo-controlled superiority trial (figure 1).

### Sample size

The size effect on the primary outcome was based on the results of a trial of melatonin as an adjuvant therapy in women with early onset preeclampsia.[14 15] In that trial, melatonin prolonged the enrolment-to-delivery interval by 6 days, from a mean (SD) of 10.4 (8.3) to 16.4 (11).[15] Using these data, we calculated that 42 women in each treatment group (1:1 ratio) would be sufficient to detect a 6-day difference in mean (two-sided comparison) enrolment-to-delivery interval with 80% power. To allow for a 5% attrition rate, we elected a sample size of 45 in each arm, equating to a total of 90 participants. Randomisation for this study will be stratified within two gestation brackets: $24^{+0}$–$30^{+0}$, $30^{+0}$–$33^{+6}$. Because the power analysis was performed based on a study with a single stratum, we elected to have 90 participants in each stratum, requiring a total of 180 participants. This study is powered on the

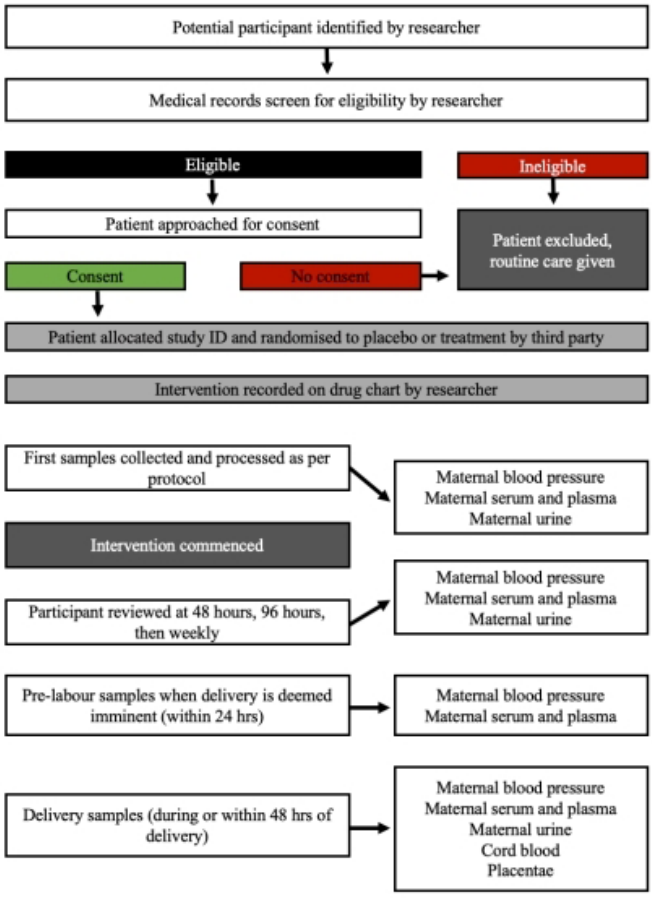


**Figure 1** Flowchart indicating participant recruitment, enrolment and sample collection. Potential participants will be identified from the labour ward and clinic and will be screened for eligibility by the research team. Eligible women will be approached for consent to participate. Where a woman is not eligible or declines to participate, no change will be made to her routine care and she will not be approached again. Consenting participants will be randomised to receive either broccoli sprout extract or placebo which will be written on the participant drug chart and given as per hospital protocol. Samples will be collected throughout the participant stay in hospital. Initial samples will include maternal blood pressure, maternal bloods (10 mL for serum and plasma) and maternal urine (50 mL). At 48, 96 hours, then weekly until delivery, maternal bloods and urine will be collected and blood pressure recorded. Immediately prior to labour maternal blood will be collected. After delivery, placentae will be collected along with cord blood (5 mL). Maternal urine sample will also be collected.

primary outcome of interval between enrolment and delivery, rather than secondary outcomes.

## Trial sites

Women will be recruited from Monash Medical Centre and Jessie McPherson Private Hospital, Clayton, Victoria, Australia. Both sites are level 6 maternity services, as per Victorian government Maternity Capability Framework.[16]

### Participant inclusion criteria

A woman will be eligible for inclusion in the trial only if the following criteria are met:

1. Aged 18–45 years.
2. Singleton pregnancy.
3. Diagnosis of preeclampsia, as defined by the Society for Obstetric Medicine of Australia and New Zealand guidelines.[17]
4. Gestation between $24^{+0}$ and $33^{+6}$ weeks.
5. Live fetus.
6. Able to safely continue pregnancy for at least 48 hours, as determined by the treating obstetrician.
7. No known significant fetal anomaly.
8. Able to give written, informed consent.

### Participant exclusion criteria

A woman will not be eligible for inclusion in this trial if any of the following criteria apply:

1. Eclampsia.
2. Current use of broccoli sprout extract supplement.
3. Contraindications to use of broccoli sprout extract supplement (eg, intolerance of broccoli sprout).
4. Unknown gestation.
5. Unwillingness or inability to follow the procedures outlined in the participant information and consent form.
6. Mentally, cognitively or legally incapacitated or ineligible to provide informed consent.
7. Corecruitment/participation in another clinical trial where there is a pharmaceutical, herbal or nutritional intervention (such trial interventions would also include complementary and alternative medicines).

## Participant recruitment

Potential participants will be identified by the research team from the antenatal clinic, pregnancy assessment unit, in-patient wards and birth suite at Monash Medical Centre. Following discussion with the attending clinical team caring for the woman, eligible women will be approached by a member of the research team who has no involvement in the provision of patient care and provided with the participant information and consent form for the trial. The research team member will provide a verbal explanation of the trial, including a description of the trial processes, the voluntary nature of the trial, and that a decision to participate, or not, will not affect normal clinical care. No trial-related procedures will be performed on any individual without their prior written, informed consent.

Women who provide written and informed consent to participate will be randomised to receive either broccoli sprout extract (BroccoMax, Jarrow Formulas, Los Angeles, California, USA) or an identical placebo (Jarrow Formulas). Allocation will be determined by a computer-generated sequence. After recruitment, each participant will be provided with a unique code so as to maintain participant confidentiality.

## Randomisation

A randomisation sequence will be generated by a perinatal statistician not involved in the clinical trial, using a computer-generated code. Because the gestation at

diagnosis of preeclampsia may influence the duration of the interval between diagnosis and delivery, randomisation will be stratified within two gestation brackets: $24^{+0}$–$30^{+0}$, $30^{+0}$–$33^{+6}$. Randomisation will be done through block sequence to ensure equivalent sample sizes are allocated to each treatment group (BroccoMax or placebo).[18]

The randomisation sequence will be provided to the pharmacist who will allocate capsules (BroccoMax or placebo) to each participant and will dispense the allocated intervention into bottles accordingly. The pharmacist will maintain a record of participant trial identification number and treatment group.

## Intervention

Each participant will take three Broccomax capsules, each containing 8 mg of activated sulforaphane (total of 24 mg), twice daily (BD) or three identical placebo capsules twice daily (BD). Both participants and the research team will be blinded to group allocation. Capsules (BroccoMax or placebo) will be dispensed by the pharmacy in individualised bottles containing sufficient capsules for 5 days, with additional capsules (amount known only by the research team), and provided to the midwives in charge of ward care. Dosing will be recorded on the patient drug chart and administered as per hospital protocol.

Where participants are discharged home they will record taking the capsules in a patient self administration diary and return the capsule bottle, including any residual capsules, after 5 days, or sooner if delivered earlier. After delivery, residual capsules will be collected and discarded; they will not be reissued to a participant.

## Outcomes

### Primary outcome

The interval between enrolment and delivery, recorded in days.

### Secondary outcomes

The secondary outcomes will be collected principally as measures of safety and tolerability.

1. Preeclampsia severity, as assessed by: escalation of antihypertensive therapy, systolic and diastolic blood pressures, severe renal involvement (serum or plasma creatinine >90 µmol/L, oliguria <80 mL/4 hour), haematological involvement (haemolysis (schistocytesor red cell fragments on blood film, raised bilirubin, raised lactate dehydrogenase >600 IU/L, decreased haptoglobin), platelets $<10^4$/µL, disseminated intravascular coagulation) liver transanimases >500 IU.
2. Indication for delivery (maternal or fetal compromise).
3. Mode of labour and birth (prelabour caesarean section, intrapartum caesarean section, induced or spontaneous labour, spontaneous vaginal birth, assisted vaginal birth).
4. Composite maternal outcome including maternal death, eclampsia, HELLP (haemolysis, elevated liver enzymes, low platelets) syndrome (haemolysis (lactate dehydrogenase ≥600 u/L, platelet count $<100\times10^9$/L, aspartate aminotransferase >60 /L, haemolysis on peripheral blood smear or a raised haptoglobin level), pulmonary oedema (clinical signs and symptoms warranting treatment in the presence of oxygen saturations <90%), thromboembolic event (significant deep vein thrombosis or pulmonary embolus), placental abruption (retroplacental clot of >15% of maternal surface), major postpartum haemorrhage (>1000 mL of blood loss), severe renal impairment (creatinine >125 umol/L or need for dialysis), liver haematoma or rupture.
5. Intrauterine fetal death (stillbirth).
6. Changes in fetal surveillance (fetal Doppler studies—umbilical or middle cerebral artery pulsitility index, or abnormal ductus venosus—amniotic fluid volume <5 cm, abnormal fetal heart rate on CTG (cardiotocography).
7. Birth weight <5th percentile.
8. Gestation at birth.
9. Composite neonatal outcomes, including neonatal death before hospital discharge, 5 min Apgar score <7, umbilical lactate >5.0 at birth, admission to the neonatal intensive care unit (NICU), diagnosis of respiratory distress syndrome, bronchopulmonary dysplasia (need for oxygen after 28 days of life), sepsis, necrotising enterocolitis, intraventricular haemorrhage (grade III or IV), stage 4 or 5 retinopathy of prematurity, as determined by the treating clinician.
10. Duration of NICU care (days).
11. Maternal serum and placental angiogenic markers sFlt-1, soluble endoglin, placental growth factor (PlGF) and activin A.
12. Maternal TSH (thyroid stimulating hormone) and free and total T3/T4 (measured at baseline and after delivery).

Maternal demographics will be sourced from patient medical records. These will include maternal BMI (body mass index), smoking status, drug and alcohol use, age, parity, maternal comorbidities (thyroid dysfunction, diabetes (gestational type I or type II)) and maternal medications.

Additional covariates will include baseline sulforaphane and circulating sFlt-1 and PlGF levels. Adjustment will be made in statistical modelling for any significant difference in these covariates between treatment arms.

## Sample collection and storage

Samples will be collected at a number of time points (figure 2). All blood (10 mL for serum and plasma and 5 mL of cord blood) and urine samples (50 mL) will be centrifuged at 4°C and stored on-site at −80°C. Placental cotyledons will be removed, washed free of blood and either fixed in 10% buffered formalin or frozen in RNAlater (Sigma-Aldrich) until analysis. All biomarker investigations will be performed using ELISA and run in triplicates. Sulforaphane and its metabolites will be

| | Enrolment | Intervention | Antenatal | | | | |
|---|---|---|---|---|---|---|---|
| TIMEPOINT | -24 hours | 0 | 48 hrs | 96 hrs | Weekly | Before delivery | After delivery |
| | ENROLMENT | | | | | | |
| Eligibility screen | X | | | | | | |
| Informed consent | X | | | | | | |
| Allocation | X | | | | | | |
| | INTERVENTION | | | | | | |
| Placebo | | | | | | | |
| Broccoli Sprout extract | | | | | | | |
| | ASSESSMENT | | | | | | |
| Blood pressure | | X | X | X | X | X | X |
| Blood sample | | X | X | X | X | X | X |
| Urine sample | | X | X | X | X | | X |
| Placental sample | | | | | | | X |
| Umbilical cord blood | | | | | | | X |

**Figure 2** Timeline for sample collection. After eligibility screening by the research team, eligible participants will be consented within 24 hours. Consenting participants will be randomised to receive either broccoli sprout extract or placebo which will be written on the participant drug chart and given as per hospital protocol. This will be classified as time point 0. Samples will be collected throughout the participant stay in hospital at the beginning of treatment, 48 and 96 hours later and then weekly until and including delivery.

measured in plasma by liquid chromatography mass spectrometry using an established in-house methodology.

Information regarding participant demographics, blood pressure, fetal biometry and results from routine investigations will be collected from patient records. All information will be deidentified and stored on password-protected devices within the institution. Only the research team will have access to the dataset.

## Proposed analysis

This is a superiority trial. Participant data will be analysed using intention to treat. All continuous measures will be assessed for normality of distribution. Differences in the primary outcome, time from enrolment to delivery in days, and secondary outcomes (safety data) will be compared between the two treatment groups. Continuous variables will be compared with a t-test (normally distributed variables) or Mann-Whitney U (non-normally distributed data). Categorical data will be assessed using a $\chi^2$. If possibly, non-parametric data will be transformed to allow parametric comparisons. The interaction between gestation at diagnosis and treatment group will also be assessed and regression approaches (using either an interaction term or gestation as a covariate) will be used to assess the relationship between treatment arm and time to delivery after assessing assumptions. Survival analysis will also be performed (after assessing assumptions) to account for censoring and survival/failure will be graphed with Kaplan-Meier curves. Linear mixed models regression will be used to compare differences in maternal angiogenic markers, TSH and T3/T4 over time between the two treatment groups. If there is a non-constant interaction between time and the outcome of interest, we will include this parameter in the model and investigate biochemical samples at specific pregnancy time points.

In the initial analysis, correction will only be made for baseline characteristics. Where appropriate, adjustment will be made using regression using a multivariate model.

A p value <0.05 (two-tailed) will be considered statistically significant.

## Adverse events

While not expected, there may be unexpected adverse reactions associated with broccoli sprout supplements when used in pregnancy. To date, clinical studies have not demonstrated any serious adverse reactions to broccoli sprout supplements. However, metabolic changes during pregnancy may alter the pharmacological properties in unanticipated ways. A senior obstetrician on the treating team will monitor participants for the duration of their inpatient admission. The investigator will be contactable by phone at all times. Adverse event (AE) assessment and reporting will be undertaken in line with the requirements of the Sponsor, Monash Health and the National Health Medical Research Council.[19] All observed or volunteered AE and serious AE (SAE) will be recorded and reported in detail in participant medical records, to the Monash Health Human Ethics Committee and the Sponsor, Monash Health within 24 hours.

Written summaries of the trial status will be submitted to the sponsor, annually or more frequently, if requested. All participant information and trial records will be securely stored to allow retrieval for audit or review purposes.

## Data safety monitoring board reporting

A data safety monitoring board (DSMB) has been established to ensure the safe continuation of this trial by reviewing data on the following:

1. Maternal admission to intensive care unit or coronary care unit.
2. Apgar score <7 at 5 min of age requiring active resuscitation (±subsequent admission to the NICU).
3. Fetal surveillance outcomes (Doppler studies, CTG, biophysical profile).
4. Maternal or perinatal death.
5. All SAE/AEs submitted to the Sponsor, Monash Health.

The DSMB may request unbinding and will advocate for cessation or re-evaluation of the trial if either arm has a statistically significant or a 50% above baseline increase in any of these outcomes.

## Trial discontinuation or modification

The trial will prematurely, permanently or temporarily cease recruitment if the investigator, or the sponsor believes that there are important issues pertaining to

maternal and/or fetal welfare. Given the progressive nature of preeclampsia, worsening disease will not be considered an indication for discontinuation.

The trial will conclude when:

1. All participants (n=180) have been studied, delivered and discharged from Monash Health.
2. Data collection and entry is complete and database lock has occurred.
3. All data analysis has been performed.
4. All necessary reporting has been completed.

There will be no allowance for modification of the trial intervention or protocol after recruitment has commenced unless directed by the DSMB or the HREC (Human Research Ethics Committee) .

### Unblinding

Unblinding in the trial may occur in the following circumstances:

1. To make clinical treatment decisions or when an unexpected SAE occurs and the intervention must be made known. This is called emergency unblinding.
2. During an unmasked analysis in accordance with the trial analysis plan.
3. At the request of the DSMB.
4. At the conclusion of the trial to determine the effect of the intervention.

When all participants (n=180) have completed the trial, all data entry and processing are complete and the database has been locked, the CPI (clinical principal investigator) will contact the Clinical Trials Pharmacy and request that unblinding take place, prior to statistical analysis.

### Ethics and dissemination

This trial will be conducted in compliance with all stipulations of this protocol, the conditions of Monash Health HREC approval, and all other relevant local national and international guidelines. Any amendments to the trial conduct, except those necessary to remove an apparent, immediate hazard to the participant, will be submitted, in writing to the Monash Health HREC, for their review and approval, before they are implemented

Data will be published in peer-reviewed journals and presented at conferences, both nationally and internationally. All patient information will be deidentified for the purpose of publication.

### Patients and public involvement

Patients were not involved in the design of this trial, establishing the research question or development of recruitment procedures. Participants will be provided with the opportunity to receive the study findings ahead of publication or presentation at learnt meetings.

## DISCUSSION

Prolong is a pragmatic superiority trial designed to increase the interval between enrolment and delivery for women with preeclampsia. Here, we propose the use of a novel antioxidant to target the oxidative stress underlying preeclampsia. Through this trial, we aim to add to the collective knowledge about novel therapeutics for preeclampsia and, if successful, ultimately establish a new medical intervention that improves outcomes for women with preeclampsia and their babies.

If effective, we believe that adjuvant use of a broccoli sprout extract, or a similar sulforaphane source, will significantly reduce the serious disease burden attributed to preeclampsia. Cheaply and simply reducing the morbidity and mortality associated with disease for both mother and child will have application in both high-resource and low-resource settings. However, sample size limitations are inevitable in a phase III trial and we acknowledge that there is a risk of under power and type II error. Therefore, this study was designed to power for only our primary outcome. Future investigation with larger populations and further assessment of short-term and long-term infant outcomes will be necessary. Similarly, the single centre nature of this trial and subsequent issues in population bias are a limitation of this study that will be addressed in future investigations. Larger trials of the efficacy and clinical application of broccoli sprout extract will be necessary if prolong produces positive results. We hope that this initial trial will provide sufficient evidence to support and inform future such trials.

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**Contributors** The original concept for this study came from EW. The trial design was established through discussions between EW and AGC, with considerable input from SAM and KRP. The manuscript was written by AGC with drafting from SAM, KRP and EW. All authors have read and approved the final manuscript.

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**Patient consent for publication** Not required.

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# Chapter nine

## *Discussion and future directions*

As detailed in the *Preamble* (page 11), from the outset the intent of this thesis was to develop a novel adjuvant therapy for preeclampsia based on the broad hypothesis that mitigating the placental and endothelial oxidative stress, particularly the latter, would be effective. Sulforaphane was chosen as the candidate therapy because of its proven effects in other disciplines[72,117–119] and its safety profile[71,74]. The intent was to complete the first efficacy trial of sulforaphane in this thesis. This was not achieved. "The best laid schemes o' mice an' (wo)men; Gang aft a-gley" and all that!

Instead, this thesis focused on developing *in vitro* data to better understand the mechanisms underlying sulforaphane mediated placental cell protection and conducting the bioavailability studies necessary to properly inform clinical trial design. Both the bioavailability study and efficacy trial could not be completed within the confines of this PhD. However, the trial design was part of these studies. It will be for others to show whether sulforaphane is an effective adjuvant therapy or not.

This last chapter of my thesis provides insights into some of the limitations of the experiments and studies that enabled fulfilment of this PhD. Submitting a thesis by publication did not afford an opportunity to consider those limitations in detail within the chapters due to restrictions on word limits placed on peer reviewed papers. This final chapter also reflects on the learnings from this research and likely future directions.

As one might expect, across these PhD studies there were numerous limitations that, in hindsight, would have been overcome through different experimental design. In the endothelial

and trophoblast experiments (*Chapter three)* sulforaphane improved placental and endothelial function under conditions of hypoxic and oxidative stress. Given current understanding of sulforaphane as a NRFE2 inducer, a NFE2L2 gene knockdown technique was used to confirm this pathway. However, sulforaphane also protected blood vessel cells against inflammation by activating NFE2L2[76,120,121]. Certainly, the findings in this chapter of an increase in NFE2L2 nuclear translocation and cellular expression in vascular cells support this mechanism in endothelial cells. However, in dispersed placental cytotrophoblasts, silencing NFE2L2 did not prevent the protection awarded from sulforaphane treatment. Subsequent validations of the NFE2L2 knockdown (unpublished) showed that this occurred despite a successful knockdown.

The interconnected nature of cell signalling pathways means that sulforaphane likely exerts multifactorial antioxidant effects to protect cells against ROS injury. It is known that sulforaphane can directly inhibit the pro-inflammatory pathways triggered by IL-1β[122], NF-κβ[123–125] and TGF-β[76,126], inhibiting production of proinflammatory cytokines. Sulforaphane may also work via nuclear factor erythroid-2-related factor 3 (NFE2L3). NFE2L3 is a basic Lucene zipper nuclear transcription activator that is more abundant in trophoblasts than any other cell type. NFE2L3 is the most recently discovered member of the cap 'n' collar family and possesses a similar heterodimer structure to NFE2L2, however is relatively poorly characterised[127,128]. This is due, in part, to the specific expression of NFE2L3 in only a few tissue types; placental tissue, breast carcinoma and, to a lesser extent, hepatic cells, express high levels of NFE2L3[129]. Like NFE2L2, NFE2L3 is believed to activate the ARE of cellular safeguarding genes, though to a lesser extent[130,131]. However, such experiments have only been conducted in non-placental cell lines and have never been quantified respective to the relative abundance of NFE2L3[132]. NFE2L3 has a similar heterodimer structure to NFE2L2 and is also released from KEAP-1 binding sites following cysteine modifications, through protein ubiquitination, to KEAP-1[128]. Sulforaphane likely also activates NFE2L3, which may confer

the NFE2L2-independent placental cell protection from sulforaphane observed in my trophoblast experiments (*Chapter one*). Investigating the antioxidant potential of NFE2L3 in both preeclamptic and normotensive placental cells is a worthwhile endeavour, though was not possible within this thesis.

Sulforaphane may improve mitochondrial respiration, the major source of cellular ROS particularly in the face of hypoxic-reperfusion injury[85]. Already we know that sulforaphane modulates mitochondrial fission and fusion dynamics independently of NFE2L2, though the role of sulforaphane in modulating respiration has not been assessed[95]. For this reason, mitochondrial investigations (*Chapter four*) were undertaken.

While these studies were important in showing that sulforaphane is able to protect placental mitochondria, the precise mechanism(s) underlying this were not able to be defined. How sulforaphane so profoundly protects the placental mitochondria in the face of acute injury could not be conclusively identified. Such experiments would be possible using an oxygraph[133], but cannot be undertaken with the Seahorse whole cell respiration model that was used. This is an unfortunate reality of a cell type with low respiration and that fuses into a syncytial structure that is destroyed during the mitochondrial isolation. Nevertheless, these studies highlighted important and relevant findings that sulforaphane can protect mitochondria against a hypoxic reperfusion model of injury. While it may be possible to use the oxygraph to assess placental tissue, or isolated cytotrophoblasts, I chose to assess the syncytiotrophoblast as the *in vivo* cellular entity responsible for antiangiogenic factors in preeclampsia. Gene data was used for assessing the underlaying mechanism. This showed that sulforaphane may promote mitochondrial biogenesis and fusion while reducing fission in acute hypoxia which may explain the improved respiration[134],[135]. Interestingly, the mitochondrial experiments (*Chapter four)* saw an acute interaction between hypoxia and SFN, with a 60-fold increase in NFE2L2

after 4 hrs of hypoxia. This normalised by 24 hours. It may be that the timeframes studied were wrong and that acute 4hr assessments in my endothelial and trophoblast experiments (*Chapter three)* may have seen an effect from NFE2L2 silencing. This would be worthwhile for others to consider. It would also be interesting to explore the effects of sulforaphane after inflammatory injury, such as with TNF-a in protecting mitochondrial function however was not possible within the constraints of this experiment which focused on a hypoxic-reperfusion model of injury.

Difficulty was encountered when inducing hypoxic injury in dispersed trophoblasts during the *in vitro* experiments of *Chapter three.* Secretion of sFlt-1 and sEng increased in hypoxia in explant tissue but not trophoblasts. Possibly, the gestation (>38 weeks) of the placentae used in these experiments was an issue. The presence of sFlt-1 under control conditions may be due to placental aging and stress from the isolation process. Interestingly, many consider hypoxia as the driver of protein cleavage to form sFlt-1 (post translational cleavage) and sEng(membrane bound proteolytic cleavage) [27,30], studies using hypoxia alone in trophoblasts found that sFlt-1 and sEng were no different to control[26] or did not report on a normoxic control[136]. Rather, others have found success with direct administration of a HIF-1alpha inducer[137,138]. Possibly syncytial proliferation is limited when a finite number of cells are plated. Being the source of sFlt-1 and sEng, the syncytial size may be a rate limiter for the amount of secreted sFlt-1 and sEng in hypoxia. It may also be that a chronic 24-hour insult allows sufficient time for the placental syncytiotrophoblast to adapt to a lower oxygen tension. A 24 hour injury, though commonplace in trophoblast studies[53,55,139–141], may not accurately reflect the repeated hypoxic reperfusion injury faced by the preeclamptic placenta, Consequently, different lengths of hypoxic insult were explored in future experiments. A modest reduction in sFlt-1 was observed only eight hours post ingestion of a broccoli extract

in my bioavailability study (*Chapter seven)*, suggesting that adaptations to sFlt-1 secretion likely begin before 24hrs.

Similarly, in the endothelial and trophoblast studies (*Chapter three)*, the same changes in 8-isoprostane (8-IP) were not observed, a marker of lipid peroxidation, as were with the pro-inflammatory cytokine activin A. This may be explained by the immediate nature of superoxide generation and lipid peroxidation from xanthine-xanthine oxidase and the fact that while sulforaphane may prevent lipid peroxidation it is unlikely to reverse the process. Being an unstable molecule, 8-isoprostane may have degraded in the 24-hour time period between induction of oxidative stress and media collection. To better understand the role of 8-IP and the timing of a rise, or not, in 8IP, in future sampling and measuring 8-IP in media at multiple timepoints, both shortly after induction of oxidative stress and after 48 hrs would be advisable. Indeed, it was with this added insight, in the mitochondrial experiments (*Chapter four*), an hypoxic and reperfusion (superoxide) model were included with two different lengths of injury: acute (4hrs) and chronic (24hrs).

Hypoxic and reperfusion injury elicited a very different response to the mitochondrial electron transport chain. Not surprisingly, acute oxygen deficiency (4 hrs, 1% $O_2$) profoundly impaired respiration likely due to inadequate oxygen molecules available to act as the necessary electron accepter at complex IV. Interestingly, by 24 hours, hypoxic mitochondrial respiration had normalised, though membrane health was impaired. Others have identified similar responses to 24 hours of injury with an oxygen chelator[142]. This adaptation may account for the lack of increased sFlt-1 and sEng observed in the early *in vitro* studies of *Chapter three*.

Similarly, the acute superoxide injury altered respiration while by 24 hrs of superoxide injury the mitochondria had largely recovered. Contrary to expectation superoxide injury did not mimic that of hypoxic injury. Instead, maximal and spare respiratory capacity increased in

superoxide injury. Possibly, superoxides increased production of uncoupling protein 1 (UCP1)[143,144]. UCP1 facilitates leak of protons from the electron transport chain, reducing the electrochemical gradient and diverting energy away from oxidative phosphorylation of ADP into ATP[143,144], which may explain this increased respiration, or at least, oxygen consumption. Though it could not be known at the time of analysis, measuring UCP1 would be an important addition to gene and/or protein analysis in future studies. Importantly, this study showed that acute hypoxic and reperfusion injury have different, but profound effects on respiration, supporting a role for mitochondrial derangement as a driving force behind cellular dysfunction after the repeat hypoxic reperfusion injury of preeclampsia.

To investigate how this adaptation may occur gene analysis was conducted in mitochondrial experiments *(Chapter four)* to identify possible upregulations of fission and fusion pathways. The balance of mitochondrial fission and fusion is necessary for cellular homeostasis[88–90] and for the maintenance of a stable mitochondrial matrix membrane that houses the five complexes of the electron transport chain[89]. Low oxygen tensions skew this dynamic process towards excessive fission as a rescue attempt to increase mitochondrial density and therefore ATP production thus enabling continued cellular respiration. However, this adaptive process reduces the efficiency of the electron transport chain by damaging the mitochondrial membrane, increasing permeabilization and eventually triggering mitophagy and intrinsic apoptosis[84]. The hypothesis of this study was that the recovery of basal ATP seen in my chronic cells resulted from increased fission, at the expense of membrane health[91]. Surprisingly, there was no difference in fission and fusion gene expression, indicating that this chronic recovery mechanism occurs independently of skewed fission and fusion dynamics. It may be that there is an adaptive response within the placenta to maintain mitochondrial function at chronically low oxygen tensions. These studies were unable to identify the exact pathways by which the chronically injured placenta adapts to hypoxic and superoxide environments. As with all

cellular entities, this process is likely multifactorial and there was insufficient time to conclusively identify the pathways involved within this PhD.

A limitation of the endothelial and trophoblast studies (*Chapter three)* was the absence of preeclamptic samples. Investigating the effect of sulforaphane on preeclamptic tissue would overcome the issues faced in ensuring hypoxic insult induced sufficient injury. Rather than one insular injury, preeclamptic tissue faces repeated hypoxic reperfusion injury[24]. It would be useful to see the effect of sulforaphane on placental cells that have endured *in vivo* stress. Time constraints and small cell numbers limited my ability to include preeclamptic cells in the investigation. Most published trophoblast investigations lack preeclamptic samples[139,141], largely due to feasibility challenges in acquiring samples and in obtaining sufficient cell counts. Additionally, gestational and phenotypic variation makes it difficult to account for confounders or have consistency in experiments of preeclamptic tissue. Nonetheless, it is an important component of preeclamptic research and so preeclamptic samples were included in the mitochondrial investigations (*Chapter four)*.

Though, importantly, sulforaphane increased respiration in preeclamptic placental samples, the small size of severely preeclamptic placenta meant insufficient cell numbers were isolated to enable gene expression analysis in preeclamptic cells. Additionally, these samples lacked did not have gestation matched normotensive controls to compare with preeclamptic cells due to an absence of placentae delivered before 34 weeks without confounding comorbidities, such as chorioamnionitis or growth restriction. As such the observation of higher respiration in preeclamptic placentae may be a true representative of adaptive processes, as has been noted by others[21], or may simply be a function of gestation. Completing mitochondrial assessments of placentae from the participants in *Prolong* would allow us to understand how *in vivo* sulforaphane modulates mitochondrial function, with a placebo control for comparison.

The doses of sulforaphane used in the experiments on trophoblast and endothelial cell function (*Chapter three)* were greater than in subsequent studies. Having shown that sulforaphane confers benefits *in vitro*, the lowest effective concentration was sought, so as to translate this into an oral dose, based on other literature. Consequently, before commencing the mitochondrial studies (*Chapter four*) dose-response tests were conducted. Two smaller doses were used to better replicate the dosing regimen of clinical investigations. Given the timing of injury, this necessitated that sulforaphane be administered prior to the induction of injury. The preventative approach of treating cells with sulforaphane before injury, in contrast to previous experiments, is a limitation of the study design. However, it does allow time for sulforaphane to activate transcription of antioxidant enzymes[63], prior to initiation of insult.

Much lower doses of sulforaphane (1uM) were more effective than the dose used in the first study (20uM). Higher doses completely supressed mitochondrial respiration, irrespective of injury. While only *in vitro*, this finding raised potential safety concerns for high doses of sulforaphane; an important implication for future clinical studies. The sensitivity of cellular response to different doses illustrated that, unlike melatonin for which high concentrations (100uM) do not induce mitochondrial toxicity[64], there would be a need for a clinical bioavailability and dose finding study of sulforaphane before progressing to clinical trial. The dose for bioavailability studies was selected based on the intent to achieve a circulating level of 1uM, albeit mindful that circulating levels are not tissue levels. It was pleasing that a cMax of 1.2uM was observed in non-pregnant women after consumption of four capsules of broccoli extract. These PK dosing studies also showed that *in vivo* levels were well below the 20uM dose that suppressed mitochondrial respiration.

Similarly, dose-response investigations were conducted in my wire myography experiments (*Chapter five)*. In vessels, 5uM of sulforaphane offered the greatest benefit with similar results

from 1uM. Interestingly, unlike in the mitochondrial studies (*Chapter four)*, higher doses of sulforaphane (10 uM) did not damage human tissue; they simply did not confer additional benefit. Higher doses of 20uM were not assessed. Unfortunately, repeat dosing could not be incorporated into this study. The isolated human blood vessels were not able to tolerate more than 3 hrs of *ex vivo* incubation before smooth muscle damage was such that they were unable to respond to constrictive stimuli. Longer incubation periods with multiple dosing of sulforaphane would certainly be a more valid way to assess the effects of clinical use of sulforaphane on vascular function in women with preeclampsia however *ex vivo* methodologies do not currently allow this. Nevertheless, the experiments of chapter five offered valuable insights into potential clinical utility of sulforaphane as a vascular therapeutic. While the cMax doses of hypertensive women consuming eight capsules did not reach the 5uM used in my vascular experiments (*Chapter five)*, consecutive doses of broccoli extract may result in cumulative plasma levels. The AUC neared the 5uM dose and importantly 1uM in the optimisation studies showed similar benefit to 5uM. Data on circulating levels will be available from *Prolong* where consecutive blood samples will be analysed for plasma levels of sulforaphane and metabolites. Similarly, the effect of chronic exposure to sulforaphane and metabolites will become apparent when vascular assessments are done on vessels collected at caesarean from the participants of *Prolong.*

Given the damaging effect of high levels of sulforaphane on mitochondrial respiration, in my vascular experiments (*Chapter five)* it was important to establish whether high doses of sulforaphane impaired vascular function. Additionally, the capacity of sulforaphane to act as a vasodilator was investigated. From these studies it was evident that sulforaphane can protect maternal and mouse vasculature against the damaging effects of placental-derived toxins associated with preeclampsia. Promisingly, sulforaphane also acted as a direct vasodilator at high doses without damaging smooth muscle or the endothelium, as shown by the maintained

potassium response. This exciting result certainly deserves future investigation to fully understand the possible therapeutic benefits of sulforaphane as a modulator of vascular function.

While preeclamptic placental explant conditioned media (PEM) significantly impaired omental vessel function, increasing sensitivity to constrictors and impairing relaxation, except in the presence of sulforaphane, the mechanisms behind this remained unclear. Nonetheless, the enhanced vascular response to constrictors, particularly endothelin-1, and consequential worsening endothelial dysfunction, characteristic of preeclampsia makes these vaso-protective effects of sulforaphane particularly exciting[12,145–147]. Unfortunately, the fragility of human vasculature limited mechanistic investigations into the effect of injury and sulforaphane on human vasculature. Instead, murine vessels were used to conduct mechanistic investigations. Ideally, in future studies, continued optimisation of experimental conditions for human tissue would allow further studies using a series of blockers to determine the pathways by which sulforaphane protects the maternal vasculature.

Similarly, it was not possible to assess the pathways underlying sulforaphane's ability to act as a direct vasodilator in human tissue. The vessels exposed to sulforaphane were subsequently so refractory to vasoconstrictors that further experiments were impossible. As the potassium response was preserved this effect was not the result of smooth muscle damage. Others have identified similar vasodilatory effects from SFN and have linked these to an increase in hydrogen sulphide ($H_2S$) production[148]. While $H_2S$, much like NO, is endogenously endothelial derived[149], L-Cysteine (SFN-Cys) from SFN metabolism is enzymatically converted into $H_2S$ by cystathionineβ-synthase[150,151]. $H_2S$ has negative ionotropic effects, blocking calcium channels and extracellular calcium influx[152,153]. It also directly induces smooth muscle relaxation by activating K(ATP channels)[148,154,155]. $H_2S$ has antioxidant and mitochondrial

effects, particularly at complex III. So, increased $H_2S$ may also explain the NFE2L2-independent and profound mitochondrial rescue effects observed in the early *in vitro* experiments of this thesis (*Chapters three and four)*[156].

In the bioavailability study (*Chapter seven)*, a modest reduction in maternal diastolic blood pressure after consumption of broccoli extract was observed. This is not the first study to note more significant changes to diastolic than systolic blood pressure with cruciferous vegetable interventions[157]. This finding may reflect peripheral vascular relaxation without changes to cardiac contractility[158]. The observed reduction in diastolic blood pressure took place across the first two hours, when SFN-Cys peaked. As discussed, SFN-Cys converts to $H_2S$ *in vivo* with consequential vasodilatory effects on vascular smooth muscle. Investigating the role of each individual metabolite on cardiac and vascular contractility would be an interesting series of experiments in future investigations, though is not necessary before progressing to clinical trial. $H_2S$ was not measured in these or my wire myography experiments. As such, even at the conclusion of this investigation the mechanism behind sulforaphane's effects on the vasculature remain to be defined. Future mechanistic investigation would no doubt be beneficial in shedding light on how sulforaphane exerts an effect and whether purified sulforaphane may be useful to control hypertensive crises.

The vaso-protective and vasodilatory effects of sulforaphane prompted clinical translation of a broccoli seed extract, rich in sulforaphane. Initially a dose was chosen based on experimental work in conjunction with previous literature. However, purified sulforaphane cannot be administered to humans so direct translation of the dosing from my laboratory work into dose selection was not appropriate without a better understanding of bioavailability. The challenge was to identify a dose of broccoli extract that would achieve therapeutic levels of sulforaphane in pregnant women such that it could be used in clinical trial. To do so, an accurate means of

determining how much *in vivo* sulforaphane, and active metabolites, are achieved after consumption of a broccoli extract was required.

Current published methods for measuring sulforaphane are time consuming, with multiple freeze-thaw cycles that enable compound degradation, or cannot delineate concentrations of individual metabolites[107,159,160]. Primarily, the aim of this study was to develop a method that does not require plasma clean-up, a lengthy process that would limit the high-throughput and clinical trial utility of the method, while maintaining accuracy. The method optimisation experiments that make up *Chapter six* required a sensitive, high throughput method using liquid chromatography-mass spectrometry to quantify sulforaphane and metabolites in human plasma. This method was necessary to complete the bioavailability experiments (*Chapter seven)*.

The next step was to determine bioavailability of sulforaphane and metabolites after consumption of a broccoli extract. Clinically useful preparations of broccoli extract have historically been limited in their utility due to insufficient myrosinase activation of glucoraphanin into sulforaphane[71,161]. To achieve sufficient circulating sulforaphane, a concentrated formula of broccoli extract was required; this is not feasible through consumption of fresh produce. However, myrosinase is only released from plant cell walls on tissue damage such as chewing [71,103,111] and homogenised juice based preparations of broccoli extract have issues with unpalatable flavour, bloating and compliance[72,104]. Theoretically an artificially "myrosinase activated" formula should confer more bioavailability and hence have greater clinical utility. This investigation was conducted in non-pregnant women. More timepoints would be an important addition to future studies so as to allow the calculation of a half-life, which was not possible with only six samples per participant, though certainly collecting more than six samples may limit recruitment. The activated formula certainly had greater

bioavailability however the non-activated preparation also provided reasonable levels of active compounds, likely due to the small, but present, myrosinase activity in the enteric microbiome[71].

The cMax of combined metabolites from our study (1.2uM) using 120mg of broccoli seed extract (~30mg of SFN) was similar to work by Fahey et al. who investigated the pharmacokinetics of 350mg of purified SFN powder, also from broccoli seed, (mean 1.3uM) [162], though our dose was almost three-times less. The pharmacokinetic profiles of the commercial preparation used in non-pregnant women broadly mirrored those of Fahey et al. In this study, cMax was attained in non-pregnant women slightly later (~2hrs), than that of Fahey (~1hr), likely due to the use of a capsule rather than liquid [162]. Both the non-pregnant cohort of this study and Fahey et al. had extraction completed by 8 hrs; strikingly different to pregnant women, who maintained relatively high plasma levels of sulforaphane and metabolites even after 8 hrs. The number of samples and timing of this study was based on the first investigation in non-pregnant women, and that of Fahey. In pregnant women however, further blood samples would have been useful to better understand the excretion and pharmacokinetics of broccoli extract in pregnancy. This was, however, limited by cannula patency and patient recruitment so collecting more samples may have been challenging.

The cMax of the activated formula was 1uM; the same dose that showed experimental promise in the mitochondrial studies. The initial intent was to use this information to identify the dose for clinical trial. However, after considering the pregnancy related changes to drug metabolism, cardiac output and volume distribution[114,163], a bioavailability study was undertaken in women with pregnancy induced hypertension, to better ensure an appropriate dose was selected for the target population. Though the past decade has seen an increase in the hunt for novel therapies for preeclampsia, the majority of studies lack appropriate dose-finding investigations[164]. In fact,

only 4.4% of drugs used in pregnancy have appropriate pharmacokinetic analysis for the pregnant woman[115].

This pharmacokinetic study was not designed as an efficacy trial. As such the numbers of women in this study were too small to identify dose related differences in blood pressure and antiangiogenic compounds and these data cannot be used to inform effectiveness. The small size and short duration meant that blood pressure and biochemical improvements were modest with a small, though statistically significant, drop in diastolic pressure and sFlt-1. Variations in disease severity and patient gestation may have also limited our ability to obtain tight efficacy data. Ideally this study would have multiple groups clustered by gestation to identify gestation related differences in drug bioavailability. While it would have been interesting to increase the sample size, with a larger cohort of participants and increase the length of blood pressure and angiogenic monitoring, it was deemed not necessary for the purpose of this dose finding study. It would also have been useful to have included a cohort of women consuming the intervention for three-five days. This would have offered better insights into circulating levels attained with sustained use, and into possible haemodynamic and biochemical changes over time. Ultimately the results from this study are important both to inform dosing for the clinical trial *Prolong,* but also as a reflection on the importance of dose finding before clinical trial for the scientific community at large. Similarly, that some changes to antiangiogenic level and blood pressure were observed is an important finding and supports ongoing clinical investigation into the clinical application of sulforaphane.

Efficacy data will be obtained from the clinical trial *Prolong*. Continuous dosing with the broccoli extract and longer monitoring will allow an observation of improvements in angiogenic balance and blood pressure over days, rather than hours. Future studies that may investigate sulforaphane preventatively across preconception and the first and second

trimesters will need to include bioavailability studies in early pregnancy. Additionally, given the multifaceted effects of sulforaphane, such studies would require extensive safety analysis. Rather than use efficacy outcomes for dose selection, plasma levels after ingestion of eight capsules were similar to concentrations used in my mitochondrial studies and used this as rationale for dose selection. Ideally, a more efficient means of delivering this dose would be identified prior to clinical trial, such as a highly concentrated capsule. Though recruitment in the bioavailability study was never limited by patient resistance to number of capsules, this certainly may be an issue in the clinical trial.

Though sulforaphane is safe, and possibly feto-protective in pregnant animal models,[74] it was pertinent to establish safety data when giving the broccoli extract to pregnant women with vulnerable babies. In particular, considering the profound vasodilatory effects of sulforaphane in *ex vivo* studies it was essential to ensure that the extract did not induce placental hypoperfusion and maternal hypotension. No CTG abnormalities were observed nor did maternal blood pressure drop by more than 20mmHg. Further data on fetal exposure to sulforaphane will come from *Prolong* where umbilical vein concentrations of sulforaphane and metabolites will be measured and numbers will be sufficient to overserve fetal outcomes. As a once off exposure, assessing fetal outcomes after this bioavailability study would not offer value.

The ultimate aim of this thesis was to clinically investigate a broccoli extract in women with preeclampsia. As such, while conducting the laboratory and clinical studies (*Chapters three to seven)*, a protocol for a large, double blind placebo-controlled trial of broccoli extract in women with preeclampsia was developed (*Chapter eight)*. Unfortunately, as this was published before the importance of dose escalation studies became apparent from early data, the dose in the protocol is too low and does not reflect the correct dose, as shown in the clinical trial registry.

*Conclusion*

This thesis includes a series of integrated and translational studies designed to explore the use of a broccoli extract as a possible therapy for preeclampsia. Starting with bench investigations, the proof of concept data to suggest biological plausibility for sulforaphane as an adjuvant to protect maternal placental and vascular function was first established. The mechanisms behind sulforaphanes actions were then explored, identifying direct mitochondrial and vascular modulation. In particular sulforaphane can act as a direct vasodilator with possible calcium-channel blocking effects. Next, a useful method for quantifying sulforaphane and metabolites in human plasma was established and ca pharmacokinetic study in non-pregnant women conducted. From this, it was apparent that pregnant women require twice the dose of sulforaphane of non-pregnant women, highlighting the importance of dose-finding in pregnancy investigations. A dose of broccoli extract to carry forward in clinical trial was identified, as were modest changes in diastolic blood pressure and sFlt-1 with acute sulforaphane treatment. Finally, this thesis includes a clinical trial protocol for a large, placebo controlled randomised trial investigating sulforaphane as a possible therapy for preeclampsia. Overall, the studies described in this doctoral thesis have sought to investigate sulforaphane as possible novel therapy to improve the health outcomes of pregnant women with preeclampsia and their babies.

*Future directions*

This thesis sets the ground work for experimentally sound clinical evaluation of a broccoli extract. If proved effective it would be a safe and cheap intervention that can be readily transported to third world countries where the maternal and perinatal burden of preeclampsia, particularly early onset disease, is overwhelming. Further mechanistic investigation into the possible utility of purified sulforaphane as an antihypertensive, for use in multiple medical

fields and to manage hypertensive crises may also be warranted. Similarly, pharmacological efforts to optimise sulforaphane concentration in a smaller number of capsules is advisable to aid in clinical investigation. From here the clear step forward is to conduct *Prolong*, a randomised controlled trial investigating sulforaphane as a possible therapeutic for preeclampsia. *Prolong* will offer insight into whether administration of a broccoli extract to women with preeclampsia can delay delivery in women with early onset preeclampsia. Beyond that, tissue samples collected from the participants of *Prolong* will allow exploration of the *in vivo* mechanisms behind sulforaphane in the placenta and the vasculature. Further studies including childhood follow up may offer additional insight into whether a broccoli extract has protective effects for babies born to women with preeclampsia. In this regard, our research group is currently examining the efficacy of maternally administered melatonin as an *in utero* neuroprotectant in growth restricted fetuses (ACTRN12617001515381). Broccoli extract may also offer benefit for women with placental diseases beyond the SOMANZ definition of preeclampsia, including normotensive but growth restricted pregnancies. Given the effects of sulforaphane on the placenta, it would also be useful to assess whether it was a useful preventative therapy, much in the way that low dose aspirin is used in women at high risk of preeclampsia[165]. In fact, given the safety data surrounding sulforaphane and the possible feto-protective effects, population based preventative studies giving broccoli extract, as an addition to pregnancy multivitamins, from preconception to delivery may be appropriate. In any event, future directions should now focus on clinical research so that broccoli extract can be evaluated as a possible safe and cheap intervention that can be used to improve the lives of pregnant women with preeclampsia and their babies.

# Research presentations

***Invited presentations***

1. Pursuing adjuvant therapies for preeclampsia

   *Royal Darwin Hospital Grand Rounds, Darwin, Australia, May 2021*

2. Pursuing adjuvant therapies for preeclampsia: could sulforaphane be the answer?

   *Mount Sinai Hospital, Toronto, Canada, February 2020*

3. Why do a BMedSc(hons)? - BMedSc(hons) seminar,

   *Monash University, Victoria, June 2020*

4. Can sulforaphane reverse mitochondrial dysfunction in the preeclamptic placenta?

   *Leiden University Medical Centre Research Group, Leiden, Netherlands*, *March, 2019.*

5. Should I do an honours year? – Biomedical and Science Student Seminar,

   *Monash University, Victoria, September, 2019*

6. Why do a BMedSc(hons)? - BMedSc(hons) seminar,

   *Monash University, Victoria, June, 2019*

7. Foundation Day Guest Speaker

   *Newhaven College, Foundation day, Phillip Island, Victoria, August, 2019*

8. Girls like me - inspiring young women to follow a career in STEMM

   *STEMM sisters programme, Inverloch, Victoria, Australia, September, 2019*

9. Preeclampsia; novel therapies and research

   *Bass Coast Obstetric Network Training Day, November, 2019*

10. Prolong: a randomised placebo-controlled trail of broccoli sprout extract for preeclampsia

    *Midwife Education, Monash Health, Clayton, Victoria, April, 2018*

11. Prolong: a double blind, randomised placebo-controlled trial investigating broccoli extract for early onset preeclampsia,

    *Australasian Trials (IMPACT) network, Auckland, New Zealand, March, 2018*

12. Investigating sulforaphane for preeclampsia,

    *Salamonsen Lecture, Hudson Institute of Medical Research, Clayton, Victoria, October, 2018*

13. Sulforaphane for Preeclampsia,

*Victorian Research Symposium, Melbourne, Victoria, June, 2018*

***Conference presentations***

1. **Langston-Cox A,** Marshall SA, Palmer KR, Wallace EM

   Developing a Broccoli Extract as a Therapy for Preeclampsia: Using Pharmacokinetics to Guide Dose Selection. *Perinatal society of Obstetrics and Gynaecology, Online,* **plenary session oral presentation** *April, 2021*

2. **Langston-Cox A,** Marshall SA, Palmer KR, Wallace EM

   Sulforaphane Improves Vascular Reactivity in Arteries After Preeclamptic-like Injury *Perinatal society of Obstetrics and Gynaecology, Online,* **oral presentation** *April, 2021*

3. **Langston-Cox A,** Marshall SA, Palmer KR, Wallace EM

   Sulforaphane Improves Vascular Reactivity in Arteries After Preeclamptic-like Injury *Society of Reproductive Investigation, Vancouver, Canada*, **oral presentation** (cancelled due to COVID) *March, 2020*

4. **Langston-Cox A,** Marshall SA, Palmer KR, Wallace EM

   Sulforaphane Protects Placental Mitochondrial Function Against Preeclamptic Injury In Vitro

   *Society of Reproductive Investigation, Vancouver, Canada*, **poster presentation** (cancelled due to COVID)*, March, 2020*

5. **Langston-Cox A,** Marshall SA, Palmer KR, Wallace EM.

   Developing a broccoli sprout extract as an adjuvant therapy for preeclampsia*. Royal Australia and New Zealand College of Obstetrics and Gynaecology Meeting*, **oral presentation,** *October, 2019*

6. **Langston-Cox A,** Muccini AM, Ellery S, Marshall SA, Palmer KR, Wallace EM. Sulforaphane prevents hypoxia induced placental mitochondrial dysfunction. *Perinatal Masterclass, Prato, Italy,* **oral presentation**, *April, 2019*
7. **Langston-Cox A,** Muccini AM, Ellery S, Marshall SA, Palmer KR, Wallace EM. Differing effects of hypoxia and hyperoxia in causing placental mitochondrial dysfunction. *Perinatal Masterclass, Prato, Italy*, **poster presentation,** *April, 2019*
8. **Langston-Cox A,** Muccini AM, Ellery S, Marshall SA, Palmer KR, Wallace EM. Sulforaphane Improves Placental Mitochondrial Function In Vitro. *Perinatal society of Obstetrics and Gynaecology, Brisbane,* **oral presentation,** *March, 2019*
9. **Langston-Cox A,** Fernando S, Malhotra A, Wallace E.M, Davies-Tuck M. Neonatal outcomes in late-preterm babies: Do they differ by maternal region of birth? *Perinatal Society of Obstetrics and Gynaecology, Auckland, New Zealand,* **oral presentation** *March, 2018*
10. **Langston-Cox A,** Marshall SA, Palmer KR**,** Wallace E.M. Sulforaphane improves endothelial function and reduces placental oxidative stress in vitro: a potential adjuvant therapy for preeclampsia. *Perinatal society of Obstetrics and Gynaecology, Auckland, New Zealand*, **oral presentation,** *March, 2018*
11. **Cox AG,** Marshall SA, Palmer KR, Wallace EM.

    Prolong: a randomised placebo-controlled trail of broccoli sprout extract for preeclampsia. *Interdisciplinary Maternal and Perinatal Australasian Trials (IMPACT) network, Auckland, New Zealand,* **oral presentation** *March, 2018*
12. **Cox AG,** Cooray S.D., Robinson PJ. Bahemia N, Davis SR, Bell RJ, Topliss DJ, Schneider HG, Sztal-Mazer S. Iodine: the forgotten mineral? Awareness and use of iodine amongst women attending a low risk pregnancy. *Society of Obstetric Medicine*

*of Australia and New Zealand Meeting, Canberra, Australia* **poster presentation**

*October, 2017*

***Manuscripts included in this thesis***

1. **Langston-Cox A**, Marshall SA, Palmer KP, Wallace EM. Current and emerging therapies in the emergency management of preeclampsia. *Expert opinion on pharmacotherapy* 20(6):701-12 doi:0.1080/14656566.2019.1570134
2. **Langston-Cox A**, Palmer KP, Marshall S.A, Wallace EM, Melatonin for the management of preeclampsia: a review. *Antioxidants* 2021;10(3):376 doi:10.3390/antiox10030376
3. **Cox A,** Gurunghe S, Rahaman R, Leaw B, Chan ST, Muljadi R, Sign H, Mockler JC, Murthi P, Lim R, Wallace EM. Sulforaphane improves endothelial function and reduces placental oxidative stress in vitro. *Pregnancy hypertension* 2019;16:1-10 doi:10.1016/j.preghy.2019.02.002
4. **Langston-Cox A,** Muccini AM, Marshall SA, Palmer KP, Wallace EM, Ellery SJ, Sulforaphane improves syncytiotrophoblast mitochondrial function after *in vitro* hypoxic and superoxide injury. *Placenta* 2020: doi:10.1016/j.placenta.2020.05.005
5. **Langston-Cox A**, Leo CH, Tare M, Wallace EM, Marshall SA. Sulforaphane improves reactivity in mouse and human arteries after "preeclamptic-like" injury *Placenta* 2020: doi: 10.1016/j.placenta.2020.09.001
6. **Langston-Cox A**, Dovile A, Creek DJ, Palmer KP, Wallace EM, Marshall SA. Measuring Sulforaphane and its Metabolites in Human Plasma: A High Throughput Method. *Molecules* 2020;25(4):829doi:10.3390/molecules25040829
7. **Langston-Cox A**, Dovile A, Creek DJ, Palmer KP, Wallace EM, Marshall SA. Sulforaphane bioavailability and effects on blood pressure in women with pregnancy hypertension. *Reproductive Sci* 2021 doi:10.1007/s43032-020-00439-5
8. **Langston-Cox A**, Marshall SA, Palmer KP, Wallace EM. Prolong: a double blind, randomized, placebo-controlled trial in women with preeclampsia. A protocol. *BMJ open* 2019;9(10):e027493 doi:10.1136/bmjopen-2018-027493.

***Other manuscripts***
*written during PhD studies but not part of the studies*

9. Cooray SD, **Langston-Cox A**, Robinson PJ, Bahemia N, Davis SR, Bell RJ, Topliss DJ, Schneider H, Sztal-Mazer S. Awareness and use of iodine supplementation among Australian women attending a low risk pregnancy clinic. Aust N Z J of Obstet Gynaecol 2018;58(5):e16-17 doi: 10.1111/ajo.12829
10. Gurusinghe S., **Cox A.G.,** Rahman R., Leaw B., Chan S.T., Muljadi R, Singh H., Mockler J.C., Murthi P., Lim R., Wallace E.M. Resveratrol mitigates trophoblast and endothelial dysfunction partly via activation of nuclear factor erythroid-2 related factor-2. *Placenta* 2017;60:74-85 doi:10.1016/j.placenta.2017.10.008

11. **Cox A,** Fernando S, Malhotra A, Wallace EM, Davies-Tuck M. The influence of maternal ethnicity on neonatal respiratory outcome. *Archives of Diseases in Childhood*, 2018;105(1) doi: 10.1136/archdischild-2018-316418
12. Marshall SA, **Langston-Cox A,** Parry LJ, Wallace EM. Targeting the vascular dysfunction: potential treatments for preeclampsia. *Microcirculation* doi: 10.1111/micc.12522

# Professional development

## *Awards*

***2021***

1.Nutrition award, Perinatal Society of Australia and New Zealand

***2020***

2. President's Award, Society of Reproductive Investigation

3. Veski Research Fellowship

4. Homeward Bound Women's Leadership Programme

***2019***

5. Young Scientist Award, Royal Society of Victoria

6. Ritchie Centre Travel Grant

7. Monash University Travel Grant

8. Research Programme Scholarship

9. School of Clinical Sciences 3-Minute Thesis 1st Place

***2018***

10. Best Oral Presentation in Obstetrics, Perinatal Society of Australia and New Zealand

11. Ritchie Centre 3-Minute Thesis 1st Place

12. Hudson Retreat People's Choice Award

13. Hudson Institute Values Award

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