J Physiol 596.23 (2018) pp 5593–5609

The Journal of Physiology

TOPICAL REVIEW

Antenatal prevention of cerebral palsy and childhood disability: is the impossible possible?

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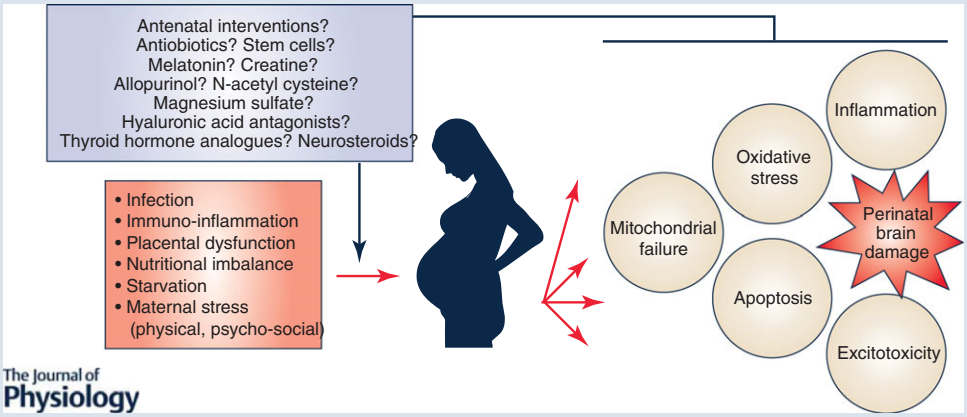
Edited by: Ole Petersen & David Wyllie



Abstract This review covers our current knowledge of the causes of perinatal brain injury leading to cerebral palsy-like outcomes, and argues that much of this brain damage is preventable. We review the experimental evidence that there are treatments that can be safely administered to women in late pregnancy that decrease the likelihood and extent of perinatal brain damage that occurs because of acute and severe hypoxia that arises during some births, and the additional impact of chronic fetal hypoxia, infection, inflammation, growth restriction and preterm birth. We discuss the types of interventions required to ameliorate or even prevent apoptotic and

Top row: **Stacey Ellery**, **Meredith Kelleher**, **Peta Grigsby**, **Irina Burd** and **Jan B Derks**. Bottom row: **Jon J Hirst**, **Suzie Miller**, **Larry S Sherman**, **Mary Tolcos** and **David W. Walker**. This review arose from papers presented by the authors at a minisymposium entitled 'Prevention of Cerebral Palsy and Childhood Disability – Is the Impossible Possible?', presented at the 5th International Conference on Cerebral Palsy and other Childhood-onset Disabilities in Stockholm, 1–4 June 2016. Considerable discussion took place from then until the date of publication to which all of the authors contributed. Rather than undertaking a formal systematic review of the existing literature in this important area of perinatal research, we have opted to present the ideas arising from our own biomedical research projects in order to emphasize the prospects for further development of these ideas into clinical practice. In this regard, all authors contributed equally to this work.

DOI: 10.1113/JP275595

necrotic cell death, and the vulnerability of all the major cell types in the brain (neurons, astrocytes, oligodendrocytes, microglia, cerebral vasculature) to hypoxia/ischaemia, and whether a pan-protective treatment given to the mother before birth is a realistic prospect.

(Received 21 November 2017; accepted after revision 15 May 2018; first published online 21 June 2018)
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Abstract figure legend Preventing perinatal brain damage.

Introduction

The incidence of brain damage arising from 'oxygen starvation' and infection at birth, or from preterm birth, is about 5-fold greater in low-income than in high-income countries (Liu *et al.* 2016). Even in 'first world' countries where women have access to the best medical care, the unpredictable nature of fetal hypoxia, fetal growth restriction (FGR), preterm birth and hypoxic/asphyxic events during fetal–neonatal transition presents a unique set of challenges to clinicians. There are few if any *in utero* sentinel events in fetal life that indicate either that damage to the brain is imminent, or that it might have already occurred and is becoming progressively worse, and which finally manifest as cerebral palsy (CP). However, there is much evidence to suggest that brain injury – especially with FGR – occurs *in utero* rather than after birth as shown by fetal MRI and the presence of established injury at birth (Dubois *et al.* 2008; Ramenghi *et al.* 2011; Businelli *et al.* 2015; Miller *et al.* 2016). Preterm birth is an additional and important risk factor for CP, often associated with the form of white matter damage known as periventricular leukomalacia (Back, 2006; Volpe, 2009). Prematurity leads to a spectrum of neurological deficits, including persistent neuromotor abnormalities (commonly spastic diplegia), cognitive and planning deficits, and problems of sensorimotor integration that lead to functional impairments in learning, academic difficulties and problems with social–emotional adjustment.

The challenge

The challenge for obstetricians and neonatologists is to devise strategies that protect the immature brain before birth, i.e. by finding an effective prophylactic treatment that can be used antenatally, or immediately after birth. We recognize that to deliver a treatment via the maternal compartment treats not only the mother but also the placenta, an organ with a high metabolic rate and a source of oxygen/nitrogen free radicals and cytotoxic metabolites that can compromise organ function in the fetus (Myatt & Cui, 2004; Burton *et al.* 2016). In this review, we discuss the proposal that antenatal and very early post-birth treatments are available that can dramatically decrease, and even eliminate, the brain damage that arises when the fetus becomes chronically deprived of oxygen due to placental insufficiency, is infected, or faces the serious physiological challenge of preterm birth. While it is expected such treatments would be delivered only to 'at risk' patients (i.e. where fetal compromise is already present, or considered highly likely to arise), an important consideration is whether the proposed treatments are also benign in the absence of the extreme conditions discussed above that cause perinatal brain damage. This is important, because it addresses the globally significant problem of intrapartum and postpartum brain damage present in underdeveloped and developing countries where clinical resources are limited, where pregnant women may receive little care, and where the global burden of CP is the greatest. Clearly, the approach we would like to recommend must be suitable for use in any birth setting and not just tertiary level hospital care. It should also be inexpensive and simple to use, and yet based on the emerging mechanistic physiology of the inflammation, oxidative stress and neurotoxicity widely agreed to induce altered brain development and damage in infants.

What approach should we take?

The recognition that head cooling (hypothermia) commenced within 6 h of birth is effective in reducing the severity of brain injury following hypoxic–ischaemic encephalopathy (HIE) in term infants (Edwards *et al.* 2010) shows that the pathogenic processes leading to injury can be interrupted, providing a strong impetus to examine further options targeting the pathways likely to be involved in injury to the young brain. However, a protective strategy must prevent or correct the pathophysiology that causes tissue injury, whether in the brain or elsewhere. This is particularly challenging in perinatal HIE because, as pointed out above, the pathways that lead to cellular degeneration in the immature brain are certainly multifactorial, and include glutamate-mediated excitotoxicity, oxidative stress, inflammation, inhibition of progenitor cell differentiation and maturation, demise of mitochondrial function, and induction of cell-death

signalling pathways (Ferriero, 2004). Magnesium sulphate has a rapid neuroprotective effect, and can be given to mothers shortly before delivery. The evidence is clear that this protects against gross motor dysfunction, reduces the risk of CP in infants born preterm, and is not associated with adverse long-term maternal or neonatal outcomes (Doyle *et al.* 2009; Crowther *et al.* 2017). Notwithstanding that there is now general agreement on the dose, timing and indications for using magnesium sulphate (see the Royal College of Obstetricians and Gynaecologists (RCOG) Scientific Advisory Committee Opinion Paper no. 29, 2011), its use only when labour is imminent or already present may create time-critical situations that conflict with other demands of obstetric care. Furthermore, the risk of adverse effects associated with this medication (albeit, low) mandates that its use will remain largely confined to tertiary level medical care settings. Thus, a requirement that we are keen to address is finding a prophylaxis that can be administered safely in a very wide range of settings. It is our opinion that current treatments do not address the problem of preventing, limiting or repairing pre-existing fetal brain injury.

Timely identification of the unborn 'at risk' baby remains a major problem in obstetric practice. This is almost always done indirectly – e.g. from an abnormal heart rate or absence of fetal movements – and it is never known with confidence if a fetus is already hypoxic, is in danger of becoming hypoxic, or perhaps is chronically hypoxic but satisfactorily adapted to this low oxygen condition. Much of the adaptive response to hypoxia (and to infection and inflammation) is cardiovascular in nature (Giussani, 2016), which is critical in ensuring the fetal heart and brain are supplied with increased blood flow despite the subnormal oxygen content of fetal blood. Hence, any proposed treatment given prophylactically to pregnant women should not interfere with these critical physiological defences, and any treatment is likely to be given in the presence of co-morbidities such as chorioamnionitis, poor placental function and FGR. Finally, such treatments should not affect the major transformation of the fetal circulation at birth, when the placental circulation is lost and pulmonary blood flow must increase rapidly.

In addition to magnesium sulphate (discussed further below), a number of antenatally applied treatments that aim to prevent brain damage arising from chronic and/or intrapartum hypoxia have been proposed, and include the use of ascorbic acid, tetrahydrobiopterin, phenobarbital, *N*-acetylcysteine (NAC), xenon and argon (Bel & Groenendaal, 2016). However, these have either provided disappointing results (ascorbic acid, xenon), or require specialized ventilatory equipment (xenon, argon), or revealed unfavourable safety profiles (NAC), or, while showing promise (e.g. allopurinol, melatonin, argon), currently lack full clinical evaluation. NAC administration is known to reduce sequelae such as perinatal brain injury in offspring, but nevertheless it has limitations when given systemically (Buhimschi *et al.* 2003; Lee *et al.* 2005; Beloosesky *et al.* 2012). Nanoparticle-based drug delivery systems, such as dendrimer-based NAC (DNAC) offer many advantages compared to the free drugs, including improved efficacy and reduce side effects such as nausea, vomiting, stomatitis and fever (Kurtoglu *et al.* 2009; Kannan *et al.* 2014; Lei *et al.* 2017). Systemic administration of DNAC prenatally and postnatally in rodents targets activated microglia in the injured pup's brain and prevents perinatal neurobehavioral outcomes (Kannan *et al.* 2012; Burd *et al.* 2014). Targeted drug delivery using nanoparticles thus represents a new way forward, providing that careful pre-clinical experiments have produced evidence for the critical role of the pathway in the genesis of perinatal brain injury.

We will discuss the injurious pathways that contribute to perinatal brain injury with the intention of drawing out the common pathways that are amenable to prophylaxis, but three introductory comments are offered. Firstly, perinatal brain injury is clearly multifactorial, whether the birth is preterm or at term (Volpe, 2009). Secondly, nearly all the medications in use or proposed for protecting the perinatal brain have short half-lives (minutes to a few hours), and when given during labour or immediately after birth may not provide benefits over the critical hours and days of early postnatal life. Finally, the focus on neuroprotection has obscured the importance of other systemic sequelae of hypoxia, metabolic acidosis and/or inflammation on the fetus and neonate. It is in a minority of cases (perhaps <15%) that the brain exhibits dysfunction after hypoxia–ischaemia at birth; injury to the lungs, myocardium, kidney and gut can have devastating effects on neonatal homeostasis (Hankins *et al.* 2002; Antonucci *et al.* 2014; Singh & Sengar, 2016). Low oxygen may not be the direct or only cause of neonatal HIE – it may develop secondary to renal, hepatic and cardiac dysfunction following birth asphyxia – and therefore a therapy that minimizes tissue injury globally may have advantages over one that is specifically targeted to neuronal processes and mechanisms.

Dealing with infection and inflammation

Intrauterine infection. Chorioamnionitis, caused by intrauterine infection and/or inflammation, is an important risk factor for perinatal brain injury and neurodevelopmental disability (Dammann & Leviton, 1997, 2014; Strunk *et al.* 2014). Intrauterine infections often involve the genital *Ureaplasma* species (e.g. *U. urealyticum* and *U. parvum*), which can be isolated from amniotic fluid, cord blood, the respiratory tract and cerebrospinal fluid of infants born prematurely (Goldenberg *et al.* 2008).

Extensive evidence has established a link between infection with *Ureaplasma* spp. and the development of perinatal lung disease (Viscardi & Kallapur, 2015). However, the specific role of these species in perinatal brain injury has been less clear. Importantly, serum *Ureaplasma*-positive infants have a 2.3-fold increased risk of intraventricular haemorrhage (Morency & Bujold, 2007). *Ureaplasma* spp. have also been isolated from cases of neonatal meningitis, particularly in infants born prematurely (reviewed by Glaser & Speer, 2015). Experimental studies with rodents and sheep have provided evidence for the effects of *Ureaplasma* infection on the perinatal brain, including microglial activation (Normann *et al.* 2009; Gussenhoven *et al.* 2017). More recently, non-human primate models that have shown a causal link between intrauterine *Ureaplasma* infection and preterm birth (Novy *et al.* 2009) have been employed to examine effects of infection and prematurity on the developing brain (Kelleher *et al.* 2017).

As to treatment of infection, the antenatal use of antibiotics to prevent early labour, prolong gestation and improve immediate neonatal outcomes remains controversial and the subject of vigorous debate (McDonald *et al.* 2007; Morency & Bujold, 2007; Swadpanich *et al.* 2008). Conflicting outcomes of published studies may be attributed to potential confounders and variations embedded in the study designs, which differ substantially (Stetzer & Mercer, 2000; King & Flenady, 2002; Waites *et al.* 2009). For example, a study that has gained a great deal of attention is the 7-year follow-up of the ORACLE II trial, which suggests an increased (*albeit small*) risk of adverse neurological impairment following prescription of erythromycin during pregnancy (Kenyon *et al.* 2008). This trial highlights the importance of ensuring that antenatal treatments do not cause long-term postnatal harm. However, evidence from ovine and primate studies (Grigsby *et al.* 2012) also suggest that with the appropriate safeguards – such as correct patient selection, early diagnosis of infection and appropriate choice, route and timing of antimicrobial treatment – treatments for infection that delay or prevent preterm labour may also provide immediate benefit by reducing preterm neonatal morbidity with the potential for improved long-term outcomes as well.

In addition, the use of the newer macrolide azithromycin and other related agents has proven more effective in animal studies at treating *Ureaplasma* infections than erythromycin, potentially due to intrinsic anti-inflammatory actions and pharmacokinetics that allow it to accumulate in target tissues and amniotic fluid (Ramsey *et al.* 2003; Dando *et al.* 2010; Acosta *et al.* 2014; Keelan *et al.* 2014; Kemp *et al.* 2014). Azithromycin eradicates *U. parvum* from the amniotic cavity and fetal tissues in the rhesus monkey (Grigsby *et al.* 2012) and fetal sheep (Miura *et al.* 2014). This maternal treatment targets inflammatory signalling, as shown by decreased levels of pro-inflammatory cytokines in amniotic fluid, prolongs gestation and reduces fetal lung injury (Grigsby *et al.* 2012). The effect of these treatments on the fetal brain is an important and ongoing research question. Employing relevant pre-clinical models that allow functional outcomes to be studied (Kelleher *et al.* 2017) will be essential to establishing the long-term safety and efficacy of antimicrobial treatment of prematurity of infectious aetiology. As we consider the need for personalized medicine so must we consider unique approaches for the different micro-organisms and viruses that have been implicated in the pathophysiology of premature birth, requiring precise selection of antimicrobial agents, potential adjuvant therapies with anti-inflammatories (Keelan, 2011) and therefore the need for development of rapid diagnostic tests and identification of biomarkers for early detection.

Is it plausible to administer a macrolide antibiotic during pregnancy not only to delay preterm birth, but also reduce the severity of fetal sequelae to intra-amniotic infection? Recent data on functional neurodevelopmental indices suggests that it is, with preliminary observations suggesting no long-term neurobehavioral and cognitive deficits following *in utero* treatment of intra-amniotic *U. parvum* infection with azithromycin (Grigsby *et al.* 2012). This therapeutic approach may reduce CNS compromise and injury that sometimes follows prolonged intra-amniotic exposure to *U. parvum*, which has been shown to include cognitive, sensorimotor and attention deficits in preterm rhesus infants (Kelleher *et al.* 2017).

While clinical resistance to the use of antenatal antibiotics remains, the direct treatment of fetal inflammation and infection is an approach that needs to be considered. *In utero* infection necessarily involves the placenta, and both placental and fetal inflammation involves dysregulation of key pro-inflammatory cytokines such as IL-1 and IL-6, inappropriate anti-inflammatory and regulatory responses (e.g. IL-4, IL-10) in the fetal brain (Lei *et al.* 2015) and up-regulation of the tryptophan–kynurenine pathway with production of cytotoxic quinolate metabolites that potentially damage the fetal brain (Manuelpillai *et al.* 2005). While the use of single cytokine antagonists such as the interleukin-1 (IL-1) receptor antagonist kineret has shown some promise as an immunomodulatory therapy in animal models of maternal inflammation (Rosenzweig *et al.* 2014), it may not easily translate to an *in utero* therapy.

Immunomodulation in utero using mesenchymal stem cells. Mesenchymal stem cells (MSCs) are an attractive therapeutic option because of their inherent low immunogenicity, potential to treat graft-*versus*-host disease (Newman *et al.* 2009; Herrero & Perez-Simon, 2010;

Han *et al.* 2012) and their suitability for cross-species experiments (Karussis *et al.* 2008; Hoogduijn *et al.* 2010; Soleymaninejadian *et al.* 2012). MSCs have been shown to promote postnatal neurological recovery from perinatal stroke and hypoxia–ischaemia (Phillips *et al.* 2013; Verina *et al.* 2013), and their promise as a *maternal cell therapy* to ameliorate perinatal morbidity follows from their inherent immunomodulatory property; e.g. they provoke increased production of the anti-inflammatory cytokine IL-10 (Nemeth *et al.* 2009). MSCs have been used in US Food and Drug Administration (FDA)-approved clinical trials for myocardial infarction, stroke, limb ischaemia, graft-*versus*-host disease, autoimmune disorders and, by virtue of their capacity to regulate inflammation, MSCs decrease apoptosis, promote endogenous neuronal growth and encourage the formation of synaptic connections in the brain damaged by ischaemia and other interventions (Ohtaki *et al.* 2008; Titomanlio *et al.* 2011; Dalous *et al.* 2012).

MSCs probably act, in part, via Toll-like receptor 4 (TLR4) pathways (Lombardo *et al.* 2009; Wang *et al.* 2012; Guijarro-Munoz *et al.* 2014). In pregnant mice and rabbits, exposure to the TLR4 ligand lipopolysaccharide (LPS) induces intrauterine inflammation (Burd *et al.* 2012), and well-defined phenotypes of preterm birth including fetal neuroinflammation, fetal neuronal injury and death as well as short- and long-term grey and white matter damage, and neurological sequelae in the offspring (Burd *et al.* 2010; Dada *et al.* 2014). In a study of LPS-induced intrauterine inflammation in mice, maternal MSC administration increased levels of the anti-inflammatory cytokine IL-10 (maternal serum) and the Th2 anti-inflammatory cytokine IL-4 (placenta) but decreased the pro-inflammatory cytokine IL-6 in the fetal brain in response to intrauterine inflammation (Lei *et al.* 2015). This study provides the proof-of-principle evidence that maternal MSC administration can alleviate fetal brain injury, specifically in the cortex via decreased microglial activation, with the translational effect that the maternal MSC treatment improved the neurobehavioral performance of the pups after birth (Lei *et al.* 2015).

Dealing with hypoxia and oxidative stress

Tissue hypoxia. A primary manifestation of hypoxia–ischaemia in all organs is derangement of the mitochondrial electron transport chain (ETC), disruption to oxidative phosphorylation, and the generation of reactive oxygen (ROS) and reactive nitrogen (RNS) species. This results in rapid depletion of intracellular energy (in particular, ATP), damage to nuclear and mitochondrial DNA, and acute induction of inflammatory and apoptotic cascades, followed for many days by waves of cell death, ultimately resulting in permanent injury in multiple organs (Saikumar & Venkatachalam, 2003; Sun *et al.* 2008). The hydroxyl radical (•OH), generated largely in mitochondria, is one of the most toxic of all the ROS and should be considered a key target for therapeutic intervention (Murphy, 2009; Miller *et al.* 2012).

When cells become hypoxic, mitochondria eventually are unable to maintain adequate levels of oxidative phosphorylation, and along with functional demise they become susceptible to structural damage (Murphy, 2009). As a result of the failure of ATP-dependent processes and the loss of function of plasma membrane ionic pumps, the intracellular accumulation of calcium ions causes osmotic oedema, cell swelling, opening of the mitochondrial membrane transition pore (mMTP), and release of mitochondrial proteins (including cytochrome *c*) into the cytosol, which then initiates nuclear processes that start the apoptotic cascade. Partial loss of mitochondria places increased demands on surviving mitochondria, and many studies have shown that the increased metabolic activity of mitochondria results in further production of reactive oxygen species (ROS), including •OH. Mitochondria are also highly susceptible to ROS attack due, in part, to alterations of intra-mitochondrial proteins, lipids and nucleic acids that then further compromise mitochondrial function. Thus, a vicious cycle of ROS formation and mitochondrial dysfunction is initiated (Murphy, 2009).

Oxidative stress. Normal human pregnancy is a state prone to oxidative stress compared to non-pregnancy, predominantly due to a high metabolic rate of the placenta (Miller *et al.* 2012), placing demand on anti-oxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase to prevent the accumulation of ROS. The ability of maternal and placental anti-oxidant defences to mitigate ROS is obviously important for normal placental function, and therefore for normal fetal growth, but pregnancies complicated by placental insufficiency and FGR are associated with decreased activity of placental SOD and glutathione peroxidase (Wiktor & Kankofer, 1998; Wiktor *et al.* 2000). Furthermore, there is clear evidence of increased oxidative stress in maternal, placental and fetal tissues (Myatt & Cui, 2004). For example, 8-hydroxy-2′-deoxyguanosine, generated by oxidative breakdown of DNA, is significantly elevated in maternal urine (Scholl & Stein, 2001) and the placenta (Takagi *et al.* 2004; Wiktor *et al.* 2004) in FGR pregnancies. In pregnant sheep oxidative stress arising from placental insufficiency is a significant contributor to abnormal fetal brain development and neurobehavioural deficits in the lambs (Miller *et al.* 2007, 2014). The asymmetric FGR induced in fetal sheep by the surgical technique of single umbilical artery ligation at ∼0.7 gestation causes not only chronic fetal hypoxia and

hypoglycaemia (Supramaniam *et al.* 2006; Miller *et al.* 2009), but also significant up-regulation of the lipid peroxidation product 4-hydroxynonenal in the brain (Miller *et al.* 2014). This is an important observation because the fetal/neonatal brain has low endogenous antioxidant defences (Mishra & Delivoria-Papadopoulos, 1999; Back, 2006). Furthermore, this lipid peroxidation causes breakdown of myelin proteins and leads to axonal damage (Miller *et al.* 2014).

Thus it is pertinent to ask, can this oxidative damage be prevented or ameliorated antenatally? Therapies that support mitochondrial respiration under conditions of hypoxia and high levels of oxidative stress could potentially be important, but what are they?

Creatine and tissue hypoxia. Creatine is an amino acid derivative obtained from an omnivorous diet that includes meat, fish and dairy, and it is also synthesized endogenously from the amino acids arginine, glycine and methionine (Wallimann *et al.* 1992; Wyss & Kaddurah-Daouk, 2000). Creatine drives the creatine kinase circuit, a phosphogen pathway responsible for the regeneration of ATP from ADP in an oxygen-*independent* manner. Once in the cell, a proportion of creatine is rapidly phosphorylated, and phosphocreatine acts as a phosphate donor to produce ATP from ADP. The rephosphorylation of ADP by PCr via the reversible chemical reaction driven by creatine kinase (CK) utilizes a proton [H^+], giving the creatine/phosphocreatine reaction the ability to prevent the cytosol of the cell from becoming acidic, particularly under hypoxic conditions (Wallimann *et al.* 1992). Oral creatine supplementation in rats attenuated ROS in neural tissue, including accumulation of superoxide anion radical O_2^-, hydrogen peroxide (H_2O_2) and •OH, and by amounts similar to those affected by conventional antioxidants (Matthews *et al.* 1998). In skeletal muscle, creatine had no effect on the redox-driven expression of antioxidant enzymes, supporting the hypothesis that creatine acts as a ROS scavenger in its own right (Guimaraes-Ferreira *et al.* 2012). Creatine has been shown to protect mitochondrial DNA (mtDNA) from damage associated with hypoxia and oxidative stress by stabilization of cellular pH and mitochondrial membrane potential (Guidi *et al.* 2008), which appears to be dose-dependent, with intracellular concentrations of creatine at 3 mM being ineffective, but 10 mM protecting mtDNA for up to 24 h after a hypoxic insult (Sestili *et al.* 2006).

The ability of creatine to maintain ATP turnover, acid–base balance and mitochondrial function, together with its antioxidant, vasodilator and anti-excitotoxic properties (Gualano *et al.* 2012), make it a candidate for the treatment of the ischaemic–reperfusion injury that occurs in neonates who develop HIE. Indeed, in brain slices prepared from immature mouse and fetal guinea pigs, increased creatine in the external medium sustained ATP turnover and reduced neuronal cell injury when the slices were exposed to hypoxia (Wilken *et al.* 1998; Berger *et al.* 2004). These results support the concept that antenatally administered creatine may act to protect the neonatal brain from HIE induced by intrapartum asphyxia (Ireland *et al.* 2011). However, the animal experiments showing increased survival of offspring of creatine-fed dams after birth asphyxia also suggest that prenatal creatine loading is protective for other major fetal organs (Ireland *et al.* 2008), as shown by reduction of damage to the diaphragm muscle, axial skeletal muscles and kidneys following birth asphyxia (Cannata *et al.* 2010; Ellery *et al.* 2013, 2017; LaRosa *et al.* 2016*b*). A particular benefit in relation to CP is not only the decrease in severity of brain damage, but also the protection of skeletal muscle fibres and improved resistance to fatigue in the diaphragm (LaRosa *et al.* 2016*a*). Neonatal acute kidney injury, which has a high prevalence in neonates that have experienced intrapartum hypoxia and is a co-morbidity associated with poor neurological outcomes due to the acid–base and electrolyte imbalance (Perlman *et al.* 1989), is also prevented by antenatal creatine treatment (Ellery *et al.* 2013). This body of work has led to a Cochrane Review on creatine supplementation for women in pregnancy for neuroprotection of the fetus (Dickinson *et al.* 2014), and is the basis of a proposed randomized control trial of maternal creatine supplementation for neuroprotection of the fetus, but it remains that we still know little about creatine biosynthesis and homeostasis in human pregnancy. A pre-clinical study in the non-human primate (macaque) has demonstrated some benefits to the fetus of antenatal maternal creatine loading (M. Kelleher, P. Grigsby, S. Ellery, D. Walker and L. Sherman, unpublished observations), and this study has not identified any unwanted side effects of creatine treatment in the mother.

Allopurinol. Allopurinol has clear neuro- and cardiovascular protective effects for the fetus and neonate, and the ease of it use, and its rapid onset of action and clearance are reasons to persist with evaluation of it as a treatment to be used at the delivery of high-risk obstetric patients. Allopurinol is an inhibitor of xanthine oxidase, and its administration reduces ROS production by preventing the conversion of hypoxanthine to uric acid. It also has the effect of scavenging •OH by chelating non-protein-bound iron, although this effect is seen only at the higher doses. The experimental data supporting the neuroprotective benefit of allopurinol is compelling. For example, allopurinol given to pregnant sheep prevented neuronal loss in the fetal brain in the event of hypoxia–ischaemia (Saugstad, 1996; Kaandorp *et al.* 2014), and maternal allopurinol administration also improved fetal cardiac function and allowed these

fetuses to maintain umbilical blood flow during the period of ischaemia–reperfusion (Derks *et al.* 2010). Improved cardiac function due to allopurinol is also known from paediatric cardiac surgery (Clancy *et al.* 2001), and its intrapartum use is therefore likely to be beneficial for the fetus undergoing hypoxic stress at birth.

Over 25 years ago, it was shown that allopurinol treatment of newborn preterm babies reduced mortality associated with respiratory distress syndrome, an effect possibly mediated by a decrease in the production of superoxide radicals. The recently published ALLO-Trial in humans examined the administration of allopurinol to pregnant women at term when acute severe intrauterine fetal hypoxia during labour was suspected (Kaandorp *et al.* 2015). The ALLO-Trial aimed to reduce HIE after birth by muting the burden of early oxidative stress in the fetal brain during or after hypoxia. Although the ALLO trial showed there was a slight reduction in the serum values of S-100B and neuroketal (markers of neuronal damage), this was only significant for girls and not for boys, thereby suggesting that pathways of apoptosis and neuroprotection are more sensitive to treatment in females. In addition, the fetuses included in the ALLO trial only had a relatively mild hypoxia, consistent with the fact that current fetal monitoring has a very low sensitivity for identifying the really hypoxic fetus. Although the results from the ALLO trial were inconclusive, a major grant was awarded by the European Union for the ALBINO trial, in which allopurinol is to be administered to asphyxiated newborns in combination with hypothermia, with the aim of providing new data on neonatal outcomes, and possibly, on longer-term cardiovascular and neurological outcomes in childhood. The outcome of this trial will hopefully give more evidence about the efficacy of allopurinol as a protective agent for perinatal HIE.

Melatonin. Melatonin is known for regulating circadian rhythms, but it is also a very efficient antioxidant, acting both as a scavenger of oxygen free radicals (including the highly destructive hydroxyl radical) and by up-regulation of endogenous antioxidant processes. Melatonin readily crosses the placenta and fetal blood–brain barrier (Miller *et al.* 2005), and is safe for human pregnancy (Tamura *et al.* 2008), making it very suitable as an *in utero* antioxidant therapy. The neuroprotective potential of antenatal melatonin for FGR has been shown by treating pregnant sheep carrying a growth restricted fetus, with continuous I.V. infusion of melatonin commencing soon after the onset of placental insufficiency and for the remainder of the pregnancy, a duration of up to 30 days. Melatonin was very effective in reducing the hypomyelination and axonal damage in FGR sheep, and it also improved motor and cognitive function of growth-restricted lambs after birth (Miller *et al.* 2014). These neuroprotective actions of melatonin have been attributed to its antioxidant ability, but antenatal melatonin also improved fetal oxygen saturation and fetal cardiac function (Miller *et al.* 2014; Tare *et al.* 2014), which may be the result of increased uteroplacental blood flow (Thakor *et al.* 2010). These pre-clinical results suggest that melatonin is an effective antenatal treatment for reducing brain injury associated with FGR. In a small clinical trial of women with moderate to severe FGR, antenatal melatonin reduced the serum markers of placental oxidative stress (Miller *et al.* 2014). Thus, there is justification for considering melatonin as a neuroprotective therapy whenever moderate to severe human FGR is detected antenatally.

Magnesium sulphate. Considerable examination of multiple trials of $MgSO_4$ leads to the conclusion that it is highly effective in preventing the brain damage that may arise with preterm birth (Shepherd *et al.* 2016; Crowther *et al.* 2017), and it is now standard practice in many Western countries. The overall effect is modest, with the 'need to treat' of about 1 in 46, but this is nevertheless a valuable outcome in terms of health economics. Careful consideration about dose and dosing regime has led to avoidance of the risk of magnesium toxicity in the mother, and the worries about neonatal respiratory depression not being confirmed (Johnson *et al.* 2012). Provided that neonatal serum magnesium concentrations are <4.5 mEq dl^{-1}, $MgSO_4$ has powerful neuroprotective effects (Narasimhulu *et al.* 2017). Neonatal (and presumably, fetal) serum concentrations correlate closely with the total maternal dose and duration of therapy, and serum concentrations between 2.5 and 4.5 mEq dl^{-1} are optimal for neuroprotection, whereas concentrations >4.5 mEq dl^{-1} may be associated with periventricular leukomalacia (Narasimhulu *et al.* 2017). Of note, this study also identified increased risk of grade 3–4 intraventricular haemorrhage in neonates with serum concentrations <2.5 mEq dl^{-1}, suggesting there are some pregnancies that would benefit from maternal magnesium supplementation. However, measurement of serum magnesium in the mother is not always practical when preterm birth is imminent, nor is the obtaining of an early sample from the neonate.

While it is likely that the use of $MgSO_4$ will be restricted to preterm birth because the imminence of delivery means that the duration of exposure to this cation is limited, the clinical success of $MgSO_4$ has obscured other issues about its mechanisms of action, and concerns about its use at later stages of pregnancy, particularly at term. For example, it is unlikely that magnesium concentrations in the CNS increase to the levels that fully block the Ca^{2+} channel on the NMDA receptor as shown to be necessary *in vitro* (Galinsky *et al.* 2014), and therefore other mechanisms of action that lead to the apparent 'neuroprotection'

need to be considered, e.g. vasodilatation (Altura *et al.* 1987), anti-inflammation (Sugimoto *et al.* 2012) and/or the confounding effect of spontaneous hypothermia arising from the use of $MgSO_4$ (Zhu *et al.* 2004). These considerations have importance beyond the argument of whether $MgSO_4$ should be restricted to impending preterm birth, because it may be that increased Mg^{2+} ion in the fetal and neonatal circulation in increasing peripheral vascular conductance has protective effects for the renal and mesenteric circulations, thereby decreasing the impact of peripheral inflammation that arises from post-asphyxial ischaemia on cerebral tissue (Galinsky *et al.* 2016).

Dealing with preterm birth and fetal growth restriction

As mentioned above, FGR is a major risk factor for poor neurodevelopmental outcomes into childhood, including cerebral palsy (Miller *et al.* 2016), and this is even greater if they have experienced 'oxygen starvation' at birth, have been exposed to intrauterine infection or inflammation, or are born preterm (McIntyre *et al.* 2013). FGR is primarily caused by chronic placental insufficiency, which exposes the fetus to long-term and progressive hypoxia and hypoglycaemia, particularly in the second and third trimesters as fetal demand for nutrients outstrips placental capacity. At the same time (24–32 weeks' human gestation), the brain is beginning the critical process of white matter development (Volpe, 2009). Accordingly, it is not surprising that white matter brain injury is a common neuropathological finding in FGR and preterm infants, and is the principal aetiology associated with CP.

Animal studies show that perturbation of white matter development in the FGR brain is often the result of impaired maturation of oligodendrocytes, in addition to astrogliosis and microgliosis (Segovia *et al.* 2008; Tolcos *et al.* 2011; Reid *et al.* 2012; Miller *et al.* 2014; Castillo-Melendez *et al.* 2015). Oligodendrocytes mature according to a well-defined sequence in which four stages of oligodendrocytes development have been defined, as shown in Fig. 1. Oligodendrocyte precursor cell (OPCs) are highly proliferative, and mature oligodendrocytes develop in sequential waves from multi-potent progenitor cells found in restricted proliferative zones during fetal and early postnatal life. However, critically ill preterm neonates are highly susceptible to white matter injury due, in part, to poor and incomplete myelination, and at least one mechanism behind this is a failure of OPCs to mature into myelin-forming oligodendrocytes (Segovia *et al.* 2008; Buser *et al.* 2012). In number, the pre-oligodendrocytes and OPCs predominate in the human brain at 23–32 weeks of gestation prior to the onset of myelination proper, and it is the vulnerability of the immature oligodendrocytes to hypoxia and inflammation in particular that causes the delay, or even *blockage*, of the myelination process in the preterm and FGR brain. Preventing the accumulation of ROS provides significant benefits in terms of limiting the advance of brain injury and, as discussed above in relation to melatonin, this not only decreases the markers of oxidative stress such as lipid peroxidation and DNA/RNA fragmentation, but also promotes myelination when given to the FGR fetus.

In specifically addressing the impact of FGR on white matter, we discuss three further approaches based on

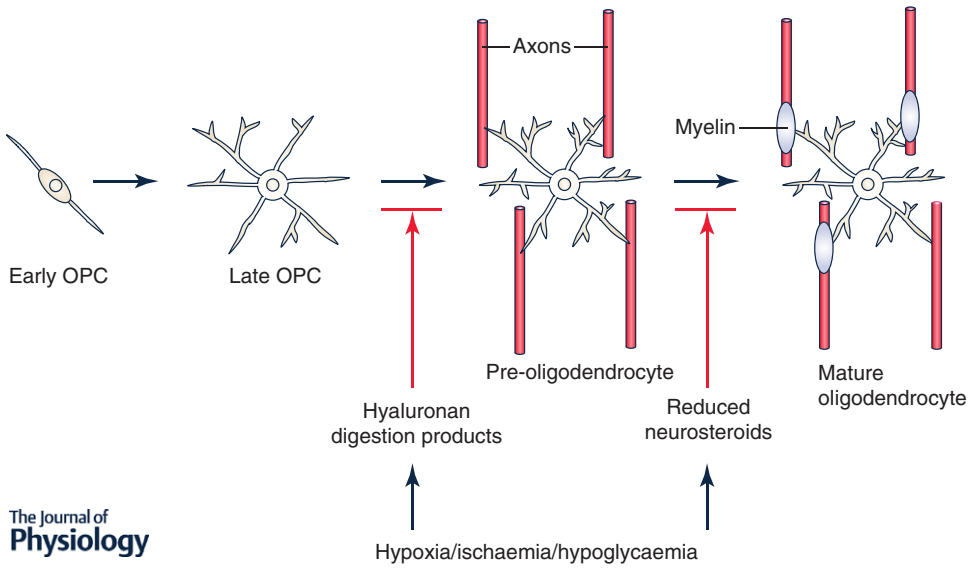

Figure 1. Oligodendrocyte progenitor cells (OPCs) undergo distinct changes as they mature into mature, myelinating oligodendrocytes
Different signals in the injured perinatal nervous system (e.g. hyaluronan, changes in neurosteroids) influence maturation at different stages.

interactions with pathways known to be critical to the maturation of oligodendrocytes.

Hyaluronic acid and myelination. Myelination failure is consistently associated with astrogliosis, accumulation of the glycosaminoglycan hyaluronan (HA) and elevated expression of the transmembrane HA receptor, CD44 (Buser *et al.* 2012). HA is synthesized predominantly by reactive astrocytes as a high molecular mass (>1 MDa) molecule. It accumulates in demyelinating lesions from patients with multiple sclerosis, in mice with the inflammatory demyelinating disease experimental autoimmune encephalomyelitis (EAE) and in traumatic spinal cord injuries (Back *et al.* 2005; Struve *et al.* 2005; Sherman *et al.* 2015), coincident with elevated expression of CD44. Overexpression of CD44 by OPCs leads to HA accumulation and *dys*myelination (Tuohy *et al.* 2004; Back *et al.* 2005), while the addition of HA to lysolecithin-demyelinated corpus callosum lesions inhibits remyelination by promoting the accumulation of OPCs that fail to become myelin basic protein-positive (MBP^+) oligodendrocytes. The administration of HA to OPC cultures reversibly inhibits their maturation.

These findings supported the hypothesis that high molecular mass HA itself prevents remyelination by inhibiting OPC maturation, and that the interaction of HA with oligodendrocytes is a potential therapeutic approach for myelination failure (Back *et al.* 2005). However, OPCs express multiple hyaluronidases (Sloane *et al.* 2010; Preston *et al.* 2013), some of which are transiently induced in white matter lesions following preterm ischaemic white matter injury (Hagen *et al.* 2014), and in reactive astrocytes and OPCs in demyelinating EAE and MS patient lesions (Preston *et al.* 2013). Elevated expression of hyaluronidases by OPCs blocks OPC maturation. Furthermore, digestion products of HA are sufficient to inhibit OPC maturation and remyelination (Preston *et al.* 2013). Pharmacological inhibition of hyaluronidase activity is sufficient to promote OPC maturation and remyelination (Sloane *et al.* 2010; Preston *et al.* 2013) and increased conduction velocities through demyelinating lesions (Preston *et al.* 2013).

Together, these findings support a model in which preterm white matter injury results in increased astrogliosis, accumulation of high molecular mass HA and the concomitant induction of hyaluronidase activity. The resulting HA digestion products then act to inhibit OPC maturation and prevent or delay myelination. This effect is mediated by TLR4 expressed by OPCs (Srivastava *et al.* 2018). Nonetheless, the finding that a hyaluronidase inhibitor promotes OPC maturation and functional remyelination in these clinically relevant models of white matter injury (Sloane *et al.* 2010; Preston *et al.* 2013) suggests that blocking the activities of specific hyaluronidases could be an effective means of promoting myelination in neonates that experience preterm or perinatal white matter injury.

Thyroid hormone signalling as a postnatal therapy for myelination deficits in FGR. Thyroid hormones (T_3, triiodothyronine; T_4, thyroxine) are integral to brain development and, in particular, for oligodendrocyte differentiation, maturation (Barres *et al.* 1994), expression of myelin-specific genes (Younes-Rapozo *et al.* 2006) and eventually myelin production by mature oligodendrocytes (Barres *et al.* 1994; Younes-Rapozo *et al.* 2006). In the brain, the pro-hormone T_4 is de-iodinated to T_3, which then binds to nuclear thyroid hormone receptors TRα and TRβ to regulate the expression of key myelin protein genes such as *MBP*. However, for thyroid hormone to reach the nuclear TRα/β receptors, T_3 and T_4 must first be transported across the cell membrane by a thyroid hormone (monocarboxylate) transporter such as MCT8 or MCT10. Children with a congenital MCT8 mutation show severe and persistent myelination delay (Gika *et al.* 2010; Lopez-Espindola *et al.* 2014), despite the presence of high serum T_3 (Gika *et al.* 2010).

Quantification of MCT8 (Chan *et al.* 2014) and all isoforms of the thyroid hormone receptor (Kilby *et al.* 2000) by immunostaining shows that both are significantly reduced in post-mortem brain tissue from FGR babies compared to babies appropriately grown for gestational age. Given that thyroid hormone can only be effective if functional and sufficient MCT8 is present, T_3 or T_4 therapy is a relatively ineffective treatment for the FGR brain. For example, thyroid hormone supplementation has limited success in improving white matter injury in animal models of perinatal hypoxia–ischaemia (Hung *et al.* 2013), or in preterm infants with intraventricular haemorrhage (Vose *et al.* 2013). Nor does thyroid hormone treatment improve growth or neurological outcomes in children born preterm (Chowdhry *et al.* 1984), nor does it always promote myelin recovery in rodent models of inflammation-induced white matter injury (Schang *et al.* 2014). The ineffectiveness of thyroid hormone treatment in these cases is understandable if the cellular uptake of thyroid hormone is impaired. The MCT8 deficit found in the brains of FGR babies at post-mortem is also replicated in at least one small animal model of induced FGR (rat), giving sufficient evidence to warrant further investigation of the limited ability of endogenous thyroid hormone to initiate the transcription of myelin genes to produce myelin proteins in oligodendrocytes, accounting for the impaired maturation and delay in myelination reported in FGR (Olivier *et al.* 2005; Tolcos *et al.* 2011; Reid *et al.* 2012). Thus, as discussed in detail elsewhere (Tolcos *et al.* 2017), there is a need to identify candidate compounds that mimic the ability of T_3 to promote oligodendrocyte

maturation and myelination, but which do not require MCT8 for cellular uptake, and such compounds could have a major therapeutic impact in the treatment of white matter injury in general, but in the neonate in particular.

Neurosteroids. The placenta produces large amounts of progesterone during pregnancy, much of which is metabolized to precursors for the production of neurosteroids in the fetal brain. The 5α-reduced metabolites of progesterone, including allopregnanolone, have neuroprotective effects because of agonist actions at the $GABA_A$ receptor (Nguyen *et al.* 2003; Hirst *et al.* 2008, 2014). Allopregnanolone levels are higher in the fetal brain than at any other time of life, and decline markedly at birth whether this occurs at term or preterm (Kelleher *et al.* 2013). Because these neurosteroids also have growth stimulating, 'trophic' effects on GABA networks (Hirst *et al.* 2014), preterm birth carries with it not only the loss of a steroid-mediated neuroprotection, but also the loss of an essential neuro-hormone that promotes brain development *in utero*. It has been suggested that the reduced development of GABAergic pathways associated with fetal hypoxia, FGR, infection and even maternal psychosocial stress (Mitchell *et al.* 2008; Elgen *et al.* 2015) arises not only from direct effects on GABAergic networks but also from disruption of the supply of essential neurosteroid precursors from the placenta. In the case of preterm birth, the early loss of these precursors of placental origin compounds all of these effects.

Neurosteroids promote myelination by stimulating maturation of pre-oligodendrocytes, and upregulating myelin production by mature oligodendrocytes (Gago *et al.* 2001, 2004). For example, in pregnant guinea pigs the 5α-reductase inhibitor finasteride was used to reduce allopregnanolone levels over the last 8 days of gestation. This treatment caused a marked reduction in myelination in the CA1 region of the hippocampus and cerebellum at term (Cumberland *et al.* 2017). Thus, we have proposed that loss of neurosteroids is one of the reasons that preterm birth is so disruptive for brain development, and as a consequence, replacement of neurosteroids after preterm birth should ameliorate the long-term harm caused by neurosteroid deficiency in the period from the inappropriately early birth to term-age equivalence (Hirst *et al.* 2014). Indeed, it can be shown that treating prematurely delivered guinea pig neonates with progesterone from birth until term equivalent age markedly increases allopregnanolone and progesterone concentrations in plasma and the brain at term equivalent age (Palliser *et al.* 2015). However, plasma cortisol levels were also markedly increased by the progesterone treatment (Palliser *et al.* 2015), and as cortisol may have negative effects on development, this finding suggests treatment with neurosteroid analogues less likely to raise cortisol might be a more sensible approach. It was previously shown that the allopregnanolone analogue alfaxalone is neuroprotective in the fetus, but its short half-life renders it unsuitable as a neonatal therapy (Yawno *et al.* 2009). In contrast, ganaxolone is a synthetic, $GABA_A$ receptor agonist neurosteroid that has a longer half-life and is thus likely to be effective in replacing the loss of brain neurosteroids following preterm birth. An alternative approach may be the stimulation of the mitochondrial translocator protein (TSPO), which is located in the inner mitochondrial membrane and mediates the metabolism of cholesterol to pregnenolone. The TSPO ligand emapunil (XBD173) increases pregnenolone synthesis and thus provides increased substrate for the downstream neurosteroids such as allopregnanolone (Rupprecht *et al.* 2010). Treatment with this agent during the period between preterm delivery and normal term may raise steroid synthesis in the neonatal brain and replace neurosteroid-induced stimulation of myelination that is disrupted with the premature loss of placental support for neurosteroid synthesis. In preliminary studies we found have that emapunil treatment of neonates that had been exposed to stress in the perinatal period reversed stress-induces changes in $GABA_A$ receptor pathways (Crombie *et al.* 2017).

Conclusions

We have discussed prospects for antenatal and very early post-birth treatments that could dramatically decrease, and perhaps even eliminate, the brain damage arising from acute fetal hypoxia and infection at term, or from chronic fetal hypoxia due to placental insufficiency and intrauterine infection leading to FGR, or from preterm birth. The manipulation of neurosteroids, thyroid status and hyaluronidases may each provide options for treatments that prevent devastating injury to white matter in the perinatal brain.

Several of the experimental approaches we discuss have been evaluated in non-human primates and shown to be effective (macrolide antibiotics, creatine). And many of the potential therapies have been evaluated with respect to their effectiveness in ameliorating or even preventing the onset of abnormal postnatal behaviours (melatonin, creatine, neurosteroids), showing that early treatment can have enduring and curative effects.

It should also be noted that the treatments we have discussed are either inexpensive dietary interventions (e.g. creatine), or simple treatments that effectively decrease oxidative stress (e.g. allopurinol, melatonin). This illustrates the suitability of these approaches for use in environments other than tertiary level hospital care – for example, underdeveloped and developing countries – where the burden of intrapartum and

postpartum brain damage and cerebral palsy is the greatest. In developed countries CP is a defined set of phenotypes that has provoked substantial investigation into cost–benefit analyses in the health system, and engendered significant investment into the treatment of complications arising from CP (i.e. learning disabilities, vision, hearing and speech impairments). In under-developed countries CP does not attract this support, and the need for the approach we outline here is of even greater importance. In these settings, the priority may be on treatments that deal with damage that has already occurred, such as with neurosteroids, hyaluronidase inhibitors, or analogues of thyroid hormone as discussed above. Thus, while the burden of perinatal brain injury remains high in all medical settings, and the priorities will differ between the 'have' and 'have not' economies, on the basis of new understandings of the mechanistic pathways that lead to perinatal brain damage, there has been excellent progress towards finding targeted therapies that could even prove the impossible to be possible – preventing cerebral palsy.

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Additional information

Competing interests

All authors declare there are no competing interests.

Author contributions

This review and opinion piece is based on presentations given by each of the authors at a symposium at the 5th International Conference of Cerebral Palsy, held in Stockholm, 1–4 June 2016. S.E. and D.W. contributed the section on creatine; M.K., P.G. and I.B. contributed the section Infection and inflammation; J.D. and S.L.M. contributed the section on hypoxia and oxidative stress; J.J.H. and D.W. contributed the section on neurosteroids; M.T. and L.S. contributed the section on oligodendrocytes and thyroid hormone. All authors contributed to the overall structure of the review, and were involved in all revisions of the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Acknowledgements

The authors acknowledge their various funding agencies and institutions for support in the primary studies that provide the material that is drawn from their primary publications that is discussed in this review.