Effects of micronutrients on placental function: evidence from clinical studies to animal models

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Abstract

Micronutrient deficiencies are common in pregnant women due to low dietary intake and increased requirements for fetal development. Low maternal micronutrient status is associated with a range of pregnancy pathologies involving placental dysfunction, including fetal growth restriction (FGR), small-for-gestational age (SGA), pre-eclampsia and preterm birth. However, clinical trials commonly fail to convincingly demonstrate beneficial effects of supplementation of individual micronutrients, attributed to heterogeneity and insufficient power, potential interactions and lack of mechanistic knowledge of effects on the placenta. We aimed to provide current evidence of relationships between selected micronutrients (vitamin D, vitamin A, iron, folate, vitamin B12) and adverse pregnancy outcomes, combined with understanding of actions on the placenta. Following a systematic literature search, we reviewed data from clinical, in vitro and in vivo studies of micronutrient deficiency and supplementation. Key findings are potential effects of micronutrient deficiencies on placental development and function, leading to impaired fetal growth. Studies in human trophoblast cells and rodent models provide insights into underpinning mechanisms. Interestingly, there is emerging evidence that deficiencies in all micronutrients examined induce a pro-inflammatory state in the placenta, drawing parallels with the inflammation detected in FGR, pre-eclampsia, stillbirth and preterm birth. Beneficial effects of supplementation are apparent in vitro and in animal models and for combined micronutrients in clinical studies. However, greater understanding of the roles of these micronutrients, and insight into their involvement in placental dysfunction, combined with more robust clinical studies, is needed to fully ascertain the potential benefits of supplementation in pregnancy.


Introduction

Adequate maternal nutritional status in pregnancy is important for fetal growth and development and for long-term health of the offspring. In high-income countries, macronutrient imbalances are relatively infrequent, however, deficiency in micronutrients (vitamins and minerals) are more common. In low- and middle-income countries micronutrient deficiencies are more common; a systematic review of micronutrient intake in women in developing countries found micronutrient intake, most commonly folate, to be below the estimated average requirement in over half of the included studies (Torheim et al. 2010). Micronutrient deficiencies are common in pregnant women due to insufficient dietary intake and increased requirements related to pregnancy (Darnton-Hill & Mkparu 2015) Micronutrients are vital to the body’s proper functioning and have extensive roles in cellular metabolism, proliferation, differentiation and signalling, and thus, inadequate levels can have wide-ranging effects. Normal development and function of the placenta is fundamental to a healthy, normally grown foetus and placental dysfunction is a major contributor to adverse pregnancy outcomes, particularly fetal growth restriction (FGR), small-for-gestational age infants (SGA), low birthweight (LBW), pre-eclampsia (PE) and stillbirth. Abnormalities in placental growth, cell survival, angiogenesis, vascular function and nutrient transport play key roles in the pathogenesis of these conditions (Worton et al. 2014). The placenta also mediates micronutrient partitioning between mother and foetus and thus is an important determinant of fetal micronutrient availability. The current review will focus on key micronutrients (iron, vitamin D, vitamin A, folate), selected based on their association with adverse outcomes related to placental dysfunction. We provide current understanding of their actions in the placenta following a systematic literature search strategy of clinical, in vivo and in vitro studies.

Iron

Iron is essential for the production of haemoglobin, cellular metabolism and immune system function. It is...
obtained in the diet as haem (meat, fish, eggs) or non-haem forms (grains, pulses, nuts, fruit and vegetables), as well as fortified cereal products. Iron homeostasis is tightly regulated, with increased intestinal absorption and cellular uptake mediated by elevated divalent metal transporter 1 (DMT1) and transferrin receptor in deficient states (Fisher & Nemeth 2017). Similar placental adaptive mechanisms exist that partially protect the foetus from maternal deficiency (Gambling et al. 2009, Cetin et al. 2011, Best et al. 2016). DMT1 uptake of transferrin-bound iron is the major route of placental iron uptake (McArdle et al. 2014), although additional mechanisms are implied by the viability of Dmt1−/− mouse foetuses (Gunshin et al. 2005, Cao & O’Brien 2013). Iron depletion leads to iron-deficient anaemia (IDA), estimated to affect 15–20% of pregnant women worldwide (Gernand et al. 2016), with higher rates of up to 55% of pregnant women in low income countries (Casanueva & Viteri 2003). IDA is associated with low birthweight (RR 1.29, 95% CI 1.09–1.53) and preterm birth (RR 1.21, 95% CI 1.13–1.30) (Haider et al. 2013), and cohort studies show an association with PE (Scholl 2005).

**Clinical studies**

A systematic review of randomised controlled trials (RCTs) of iron supplementation in pregnancy reported a positive dose-dependent effect on birth weight (15.1 g for every 10 mg increase in dose/day, \( P = 0.005 \)) and a reduction in incidence of LBW (OR 0.81, 95% CI 0.71–0.93), with no significant influence on length of gestation (Haider et al. 2013). In this review, most trials were performed in iron-replete populations; subgroup analysis to examine neonatal outcomes according to anaemia status was not performed. Iron supplementation is recommended to pregnant women at risk of anaemia, generally from the second trimester to birth, continuing postpartum in populations where IDA is highly prevalent (Casanueva & Viteri 2003). However, controversy exists regarding prophylactic iron supplementation in low-risk populations due to potential adverse effects of iron overload, associated with increased oxidative stress and cellular toxicity (Friedrich & Friedrich 2017) (see later section).

**Animal studies**

The effect of iron deficiency on pregnancy outcome has been tested in rat models, induced by dietary depletion. Whilst a consistent negative effect on fetal weight is reported, differences in outcome have been attributed to length of deficiency, with a reduction of litter size only apparent with extended deficiency (8 vs 0.5 weeks preconceptionally) (Tojyo 1983, Sherman & Moran 1984, Gambling et al. 2002, Toblli et al. 2012). Reduced placental weight occurs in severe models, whereas a compensatory increase in placental weight and labyrinth volume was observed with milder deficiency, leading to a distorted fetal/placental weight ratio (Lewis et al. 2001, Gambling et al. 2009). Impaired placental vascularisation has also been reported, which may impair maternal–fetal nutrient transfer (Lewis et al. 2001). In particular, these placental defects coupled with changes in placental lipid handling (including Fabp1) and maternal lipid metabolism, may contribute to the reduced fetal plasma amino acid and fatty acid concentrations apparent with maternal iron deficiency (Hay et al. 2016). Disrupted placental vascular development may also impact on fetoplacental blood flow, as observed by increased umbilical artery resistance and pulsatility in a rat model of severe iron deficiency (Woodman et al. 2017). A systems biology approach involving whole embryo microarray and proteomic analysis identified key pathways affected by maternal iron deficiency. These included pathways regulating developmental processes, e.g. cytoskeletal remodelling, cell adhesion and regulation of cell cycle. Validation studies focussed on potential gatekeepers of developmental programming; many of these are transcription factors (e.g. Sp1, C-myc, Tp53, Hnf4) and regulators of cell cycle checkpoints (Ccnb1 and proteasome complex components). Transcriptomic changes in regulatory genes were linked with increased expression of pro-apoptotic genes (Casp3 and Ppmd1), which may contribute to abnormal development of multiple organs and reduced expression of cytoskeletal proteins actin and tubulin. Whether similar pathways and regulatory genes are altered in placentas remains unexplored (Swali et al. 2011).

As in other organs, anaemia induces oxidative stress and inflammation in rat placentas, with elevated pro-inflammatory Tnfa, Tnf, Il6 and Lep, and oxidative damage (lipid peroxidation) and reduced antioxidant status (Gambling et al. 2009, Toblli et al. 2012). Fetal hypoxia is also apparent, but variable evidence for placental hypoxia exists (Lewis et al. 2001, Toblli et al. 2012, Woodman et al. 2017). Placental oxidative stress and inflammation have detrimental effects on placental function in humans and rodents (Girard et al. 2014, Burton & Jauniaux 2018), providing potential mechanistic insights into the association between IDA and reduced fetal growth and PE.

The effects of anaemia on adverse fetal outcomes and placenta can be recovered by iron supplementation, but only with IPC forms (iron(III)-hydroxide polymaltose complex with folic acid). IPC supplementation normalises anaemia-induced oxidative stress in the foetus and placenta and reduces IL6 and TNFA protein overexpression in the labyrinth region of the placenta (Toblli et al. 2012). In contrast, oral ferrous preparations that can by-pass physiological iron uptake mechanisms leading to elevated non-transferrin-bound iron, exacerbate inflammation and oxidative stress (Toblli et al. 2012).
et al. 2013). Free iron is highly reactive and can lead to free radical damage; this is seen in the intestinal mucosa, liver and placenta with excess iron supplementation, with corresponding increases in lipid peroxidation and mitochondrial dysfunction (Srigiridhar et al. 2001, Walter et al. 2002). There is some evidence that co-treatment of non-pregnant rats with antioxidants, e.g. vitamin E and C, is protective against oxidative damage, suggesting more research is needed into multi-therapeutic approaches (Srigiridhar & Nair 2000). An inflammatory environment can further affect iron homeostasis, providing evidence for a feedback loop to worsen cellular iron status (Ross 2017). This may be relevant to PE, where there is evidence for iron dysregulation and an established inflammatory pathogenic component (Kell & Kenny 2016).

**In vitro studies**

There have been very few/no analyses of placentalas from human pregnancies with maternal IDA. A study of ex vivo placental perfusion indicated potential beneficial effects of ferric carboxymaltose on placental capillary integrity and a reduction in apoptosis (Malek 2010). However, in vitro treatment of placental explants with iron (FeCl$_3$) augmented elevated trophoblast apoptosis in PE pregnancies (Shaker & Sadik 2013). Further studies are needed to elucidate the relevance of animal studies on the human placenta, particularly the potential risk/benefits of different iron formulations.

**Vitamin D**

Vitamin D is a fat-soluble hormone with widespread actions, including calcium homeostasis and bone metabolism, cellular proliferation, differentiation and immunomodulatory effects (Anderson et al. 2003). Vitamin D3 is synthesised in skin following exposure to sunlight and obtained through diet from oily fish, liver, egg yolks and some plants (in the form of vitamin D2). Infant formula milk, margarine and some breakfast cereals are fortified with vitamin D. Vitamin D is metabolised in the liver by cytochrome P450 27A1 (also known as CYP27A1) to 25(OH)D, which is the major stable circulating form (bound to vitamin D-binding protein, DBP). Its actions are tightly regulated at the paracrine level, with local metabolism to the biologically active form (1,25(OH)$_2$D) by CYP27B1 and inactivation by CYP24A1. 1,25(OH)$_2$D acts via the vitamin D receptor, VDR, a member of the steroid hormone receptor family, which acts as a transcription factor in a heterodimer with retinoid X receptor (RXR) (Ryan et al. 2015).

There remain inconsistencies in the definition of vitamin D deficiency, ranging from serum concentrations of 25(OH)D of 25–75 nmol/L, based solely on calcium homeostatic/skeletal actions. However, low vitamin D status is prevalent in pregnant women (reports vary between 20 and 60%), with geographical, seasonal and ethnic influences (Looker et al. 2008, Bodnar & Simhan 2010). A systematic review found significantly increased risk of PE (two-fold), gestational diabetes mellitus (GDM;1.4-fold), preterm birth (PTB;1.6-fold) and SGA (1.5-fold) with suboptimal vitamin D status (defined as <50 nmol/L) (Wei et al. 2013).

Fetal tissues highly express VDR, and there is evidence that fetal calcium levels and bone mineralisation are protected with maternal vitamin D deficiency (Kovacs et al. 2005). The placenta is also a target for vitamin D, with strong expression of VDR in syncytiotrophoblast and extravillous trophoblast (EVT). CYP27B1 is abundantly expressed by trophoblast and decidua, whilst CYP24A1 is silenced, indicating high local bioactive vitamin D at the maternal–fetal interface (Evans et al. 2008). Reduced placental VDR expression and disrupted metabolism have been reported in pathological pregnancies, e.g. PE (Tamblyn et al. 2017), FGR (Nguyen et al. 2015) and recurrent miscarriage (Wang et al. 2016). Stimuli associated with placental dysfunction, such as oxidative stress and inflammation, can alter expression of VDR and CYP27B1, resulting in lower bioactive placental vitamin D (Shin et al. 2010, Barrera et al. 2015, Tamblyn et al. 2015).

**Clinical studies**

A 2017 systematic review and meta-analysis of vitamin D supplementation in pregnancy identified a modest beneficial effect on birthweight (mean increase of 58.33 g, 95% CI 18.88–97.78 g) and a reduced risk of SGA (rRR 0.60, 95% CI 0.40–0.90) (Roth et al. 2017); in ten included trials, the mean baseline vitamin D status of participants was low (defined as 25(OH)D <30 nmol/L), but these studies were not analysed separately. No protective effect against PE was detected, except in an analysis of three trials in which women were co-supplemented with vitamin D and calcium; these women had a reduced risk of PE (RR 0.51, 95% CI 0.32–0.80) (De-Regil et al. 2016). The review also found an increased risk of preterm birth (RR 1.57, 95% CI 1.02–2.43) in a subgroup analysis.

**Animal studies**

Female Vdr-knockout mice have reduced fertility and litter sizes (Kovacs et al. 2005). Conflicting effects on fetal weights have been reported, with weights either lower (Kovacs et al. 2005) or unchanged from WT mice (Wilson et al. 2015). Calcium supplementation improved fertility, but had no impact on fetal outcomes. Furthermore, fetal mineral levels and skeletal mineralisation were normal in unsupplemented knockouts, consistent with evidence for adaptive increased placental calcium transport (Kovacs et al. 2005).

Dietary restriction in mice preconceptionally and during pregnancy altered the maternal renal...
renin-angiotensin system and elevated blood pressure (Liu et al. 2013). Placental morphological changes were also apparent, most notably a reduction in labyrinth vascularisation. Fetal weights were higher from vitamin D-deficient dams in late pregnancy, but this effect was reversed by 14 days postpartum, suggesting fetal and neonatal growth trajectories are altered. Vitamin D supplementation partially rescued both maternal and fetal phenotypes. A similar model identified a reduction in placental weight and vascularisation, accompanied by reduced expression of angiogenic Vegfa (Tesic et al. 2015). Placental expression of 11β-hydroxysteroid dehydrogenase-2 (11bhsd2) was also reduced, suggesting wider deleterious effects of deficiency through compromised protection from glucocorticoids.

Vitamin D has known anti-inflammatory and immunomodulatory actions in other organs, and this has been reinforced by placental studies. Treatment of mice with the inflammatory stimulus LPS induces placental Vdr and Cyp27b1 mRNA, indicating an endogenous anti-inflammatory response (Liu et al. 2011). Knockout of Vdr or Cyp27b1 resulted in a pro-inflammatory bias in placentas and heightened responses to LPS challenge ex vivo, with elevated expression of Tlr2 and pro-inflammatory cytokines (including Il1b, Tnfa and Il6) and chemokines (including Ccl2, Cxcl10, Cxcl6) (Liu et al. 2011, 2017). Interestingly, a more severe phenotype was observed in placentas from female pups, indicating a degree of sexual dimorphism. Ex vivo treatment of WT placentas with vitamin D had anti-inflammatory effects, reducing cytokine and chemokine expression.

A central role for vitamin D in regulating placental inflammatory pathways was reinforced in a mouse model of LPS-induced FGR and fetal death (Chen et al. 2015a). In this model, LPS treatment did not affect VDR expression, but prevented its activation and target gene expression. Pre-treatment of mice with vitamin D effectively rescued fetal and placental growth and increased fetal viability. This was associated with attenuated LPS-induced pro-inflammatory cytokine (Tnfa, Il1b and Il6) and chemokine (Ccl2, Cxcl1, Cxcl2) levels in both maternal and placental compartments. These anti-inflammatory effects were attributed to a blockade of NFkB activation by vitamin D. This represents a distinct mechanistic pathway to that in immune cells, whereby vitamin D exerts anti-inflammatory effects via PI3K/Akt signalling pathways. LPS treatment also reduced placental folate transporters in mice, with a concordant rise in incidence of neural tube defects in offspring (Chen et al. 2015b). Supplementation with vitamin D rescued folate transport and prevented neural tube defects, signifying a link between the anti-inflammatory effects of vitamin D and folate biology (see later section). Additional mechanistic pathways altered by vitamin D in mouse placenta have been identified by gene array studies, including those regulating autophagy, cell signalling and the mTOR pathway (Wilson et al. 2015).

The therapeutic potential of vitamin D was also been explored in a rat model of placental ischaemia, induced by ligation of the uterine artery (Tian et al. 2016). Vitamin D prevented preeclamptic-type symptoms in pregnant dams, reduced fetal mortality and prevented effects of ischaemia on placental morphology and cell survival, associated with a decrease in placental oxidative and endoplasmic reticulum stress. In vitro analyses indicated normalisation of placental sFLT1 release as a primary mechanism underpinning the prevention of maternal endothelial activation (Ma et al. 2017).

Studies in the 1960s and 70s raised concerns about excessive antenatal supplementation with vitamin D2. High-dose supplementation (up to 40,000IU/day) in pregnant rats resulted in reduced placental size, coupled with histological features of abnormal placental development (including delayed trophoblast differentiation and vascularisation), placental calcification and inflammation (Potvliege 1962). Extended supplementation in late pregnancy had additional adverse fetal effects, with reduced fetal weights and high rates of fetal loss at term (Ornoy et al. 1968, Nebel & Ornoy 1971, 1972). There is no current evidence for similar effects in humans; however, vitamin D3 is more effective at raising serum vitamin D concentrations and thus is now the favoured form (Houghton & Vieth 2006).

In vitro studies

In vitro studies of human placental cell lines and primary trophoblasts have confirmed and extended our understanding of potential roles for vitamin D in the placenta. As in the mouse, vitamin D exerts anti-inflammatory effects via VDR in JEG3 cells, attenuating LPS-induced nuclear translocation of NFkB p65 subunit and pro-inflammatory cytokine expression (Il6 and TNFA) (Chen et al. 2015a). Similarly, vitamin D acting through VDR dose dependently abolishes pro-inflammatory effects of TNFA in trophoblast cells, preventing the expression of Il6, IFNG and TNFA itself (Noyola-Martinez et al. 2013). These effects may be relevant to PE; all three cytokines are expressed at higher levels by placental cells from preeclamptic women and are decreased by vitamin D treatment in vitro. Anti-inflammatory effects of vitamin D were also observed in an in vitro model of anti-phospholipid syndrome (APS) – another immunological pregnancy disorder – including suppression of IL1B and IL8, together with reduced microparticle release (Barrera et al. 2015, Gysler et al. 2015). Vitamin D has also been reported to have antimicrobial effects by stimulating the expression of β-defensins and cathelicidin in trophoblast cells (Olmos-Ortiz et al. 2015).

Vitamin D may be beneficial during the establishment of pregnancy, by promoting an immunologically tolerant environment in the decidua and regulating a
number of genes critical for implantation, e.g. *CABP9K* and *HOXA10* (reviewed elsewhere Evans et al. 2004, Ganguly et al. 2018). Vitamin D also promotes EVT invasion in vitro (Chan et al. 2015) and can modulate the inhibitory effects of locally active lipid metabolite sphingosine-1-phosphate (SIP) on EVT migration (Hay et al. 2016). These findings provide insight into the biological significance of high local vitamin D levels in the decidua and the increased susceptibility of vitamin D-deficient women to PE, where early placentation is defective. Further potential actions in PE include effects on maternal endothelial cells, as noted in rodent studies, including negative regulation of anti-angiogenic sFLT-1, stimulation of VEGF and e-NOS, and enhanced antioxidant defences (reviewed in detail in Barrera et al. 2015).

Vitamin D can influence cell proliferation and survival and in trophoblasts regulate cell cycle regulatory genes (*TP53*, cyclin-dependent kinase inhibitors), inhibits pro-apoptotic caspase 3 and alters expression of growth factors, including *TGFB*, important for trophoblast proliferation and differentiation (Nguyen et al. 2015, Xu et al. 2017). In addition to regulating placental calcium transport (Tuan 1991), vitamin D may enhance general fetal nutrient availability, e.g. a positive association has been reported between maternal vitamin D status and placental amino acid transport via system A (Cleal et al. 2015). Vitamin D can also stimulate placental hCG, hPL and steroidogenesis and thus could have profound effects on maternal metabolism and pregnancy maintenance (Stephanou et al. 1994, Barrera et al. 2007, 2008). Low maternal vitamin D status may therefore predispose to FGR and PE via multiple actions on maternal and placental physiology.

**Vitamin A**

Vitamin A consists of a group of naturally occurring fat-soluble compounds obtained from the diet. Retinol and retinyl esters are present in animal sources and betacarotene or other provitamin A carotenoids in plants, with the total vitamin A content of the diet expressed as retinol activity equivalents (RAEs). Biologically active forms of vitamin A (retinol, retinal and retinoic acid) have essential roles in normal vision, immune function, cell differentiation and reproduction, with actions exerted through nuclear receptors (*RAR*α, β and γ) which, like VDR, heterodimerise with RXR and alter gene transcription. Recent reviews describe vitamin A uptake, transport and storage in general (Chelstowska et al. 2016) and by the placenta (Spiegler et al. 2012).

Vitamin A deficiency is assessed by biochemical measurement of serum retinol, clinical indication of eye disease and functional indicators such as night blindness. Adult serum retinol concentrations <0.7 µmol/L define biochemical deficiency and in pregnancy/lactation concentrations <1.05 µmol/L are considered deficient (Wiseman et al. 2017). Vitamin A deficiency is common in low-income countries, with ~15% of pregnant women globally being biochemically deficient (World Health Organization 2009). However, even in the United Kingdom and United States particular subpopulations are at risk of deficiency, including adolescent girls, African-Americans and those in low socioeconomic groups (Hanson et al. 2016). Vitamin A deficiency is associated with increased incidence of preterm birth (OR 1.99, 95% CI 1.12–3.53) (Radhika et al. 2002). However, current guidance is to avoid excessive vitamin A intake in pregnancy from supplement and dietary forms (no more than 800 µg RAE/day) (FAO/WHO 2005) due to potential teratogenic effects. Supplementation is only recommended for populations where vitamin A deficiency is a severe public health problem.

**Clinical studies**

A recent Cochrane review evaluating RCTs of vitamin A supplementation in areas endemic for deficiency (17 of 19 included studies were conducted in populations considered to be deficient) found a decreased risk of maternal night blindness (ocular sign of vitamin A deficiency), anaemia and clinical infection in women, but no beneficial effect on fetal/neonatal outcomes (McCauley et al. 2015). Vitamin A has potent immune regulatory effects and can alter the balance between Th1/Th2 responses, with a tendency to suppress pro-inflammatory responses (Villamor & Fawzi 2005). Pregnancy is associated with altered immune responses; a bias towards Th2 responses by vitamin A would be hypothesised to be favourable to pregnancy outcomes; however, concerns regarding susceptibility to maternal and fetal infection warrant further investigation, particularly given the prevalence of vitamin A deficiency in populations at high risk of malaria and HIV infection (Cox et al. 2006, Cañete et al. 2017).

**Animal studies**

The role of vitamin A on placental and fetal development has been addressed in animal models of deficiency and excess, delineating different roles for retinoic acid and retinol during pregnancy. A total vitamin A-deficient diet results in extensive embryonic resorption by mid-pregnancy in rats (Takahashi et al. 1975); this can be rescued by supplementation with retinol, but not retinoic acid, demonstrating a non-redundant role in embryonic development (Wellik & DeLuca 1995). Supplementation of vitamin A-deficient rats with all-trans-retinoic acid (the highest affinity ligand for RAR) supports survival of offspring to parturition (White et al. 1998).

More moderate models of deficiency have identified effects on the placenta. Restricted dietary intake of retinyl acetate (acetylated form of retinol) led to decreased litter size and pup viability. Fetal growth
rates were impaired and defects in placental growth and histological appearance were detected from mid-pregnancy (Takahashi et al. 1975). A similar phenotype was observed in rats fed a retinol-free diet 8 weeks before and throughout pregnancy, with lower numbers of viable foetuses, and increased placental weight and placental/fetal weight ratio for surviving pups (Antipatis et al. 2002). Placentas had increased neutrophil infiltration and higher LEPTIN, TNFA and TNFR expression, together with increased apoptosis and altered BCL2/BAX ratio in adjacent trophoblast cells.

Mice with mutations in more than one of the Rar or Rxr subtype exhibit many of the same embryonic defects as dams maintained on marginal vitamin A intakes (reviewed in Mark et al. 2009). Of note, embryonic death in Rxa- and Rxb-null mice is attributed to defective development of the placental labyrinthine zone (Wendling et al. 1999).

Hypervitaminosis A results in congenital malformations, due to wide-ranging developmental actions, including axial polarity and cell morphogenesis (Clagett-Dame & Knutson 2011). A single dose of retinoic acid equivalent to 240 mg/kg (800,000 IU/kg) in early rat pregnancy resulted in almost all embryos dying with cardiovascular and nervous system defects and hydramia 24 h after treatment (Love & Vickers 1976). There was suppression of allantotic leading to placental agenesis. A lower single dose of all-trans-retinoic acid (120 mg/kg) on gestational day 10 induced a clubfoot-like deformity and altered placental morphology, with increased apoptosis and altered BCL2:BAX ratio (Jiang et al. 2014). No effects on placental or fetal weight were observed.

In vitro studies

In both human and rat trophoblast cells, vitamin A regulates secretion of hormones important for trophoblast differentiation, fetal nutrient availability and maternal energy expenditure. Retinoic acid increases secretion of hCG by human choriocarcinoma cells in a dose-dependent manner, with similar effects on progesterone although this varied between cell lines (Chou 1982, Kato & Braunstein 1991). Similar experiments with rat primary differentiated spongiotrophoblast cells demonstrated effects on morphology with more well-defined nuclei and thinner and fewer cytoplasmic projections in retinoic acid treated cells and reduced placental prolactin levels (Lu et al. 1994). The production of hormone leptin is also stimulated by retinoic acid in primary human trophoblasts mediated by the RXXα receptor (Guibourdenche et al. 2000). This receptor also mediates transcription of pregnancy-specific glycoprotein 5 (PSG5) in JEG-3 cells in response to retinoic acid (Lopez-Diaz et al. 2007). PSGs are thought to be important in mediating fetal protection from the maternal immune system and reduced PSG secretion in vitamin A-deficient animal models may partly explain the observed placental inflammatory responses.

Further effects on placental development and function have been suggested from in vitro analyses of human placentas. Antagonism of RXR stimulated invasion of primary human EVTs through ECM-coated transwells (Tarrade et al. 2001). Ex vivo human placental perfusion studies identified beneficial effects of beta-carotene on placental vascular tone, with an attenuation of peroxide-induced vasoconstriction and reduced lipid peroxide and TXB2 production (Cueto et al. 1997).

Folate and vitamin B12

Folate and vitamin B12 (cobalamin) are naturally occurring, water-soluble vitamins present in biological tissues and foods. Both play critical roles in one carbon metabolism, which generate purines and thymidylate for DNA synthesis/repair and S-adenosylmethionine (SAM), the donor for cellular methylation reactions and remethylates cytotoxic homocysteine to methionine (Molloy 2012). Folate deficiency is characterised by elevated homocysteine and clinically manifests as megaloblastic anaemia. Recently, evidence for a critical role of folate in protection against oxidative stress, via generation of NADPH has emerged (Fan et al. 2014). Vitamin B12 is also essential for myelinogenesis and red blood cell production, thus deficiency leads to neurological damage and anaemia.

Absorption, transport and intracellular storage of folate in non-pregnant adults is tightly controlled and both folate and vitamin B12 are present at higher concentrations in fetal than maternal circulation (Suh et al. 2001). Vitamin B12 is predominantly transported across the placenta by transcobalamin (Fisher & Nemeth 2017), and recent studies propose transplacental folate transport via folate receptor α (FRα), proton-coupled folate transporter (PCFT) and reduced folate carrier (RFC) (Solanky et al. 2010, Rosario et al. 2017a). Folate requirements are increased in pregnancy to meet the needs of the developing foetus and thus pregnant women are at risk of folate deficiency. Low maternal folate status is associated with neural tube defects and other congenital abnormalities (de Benoist 2008), with evidence from observational studies for associations with PE, FGR/SGA and preterm birth (Ek 1982, Scholl & Johnson 2000, Rao et al. 2001, Baker et al. 2009, Bukowski et al. 2009). Current guidance for folic acid supplementation by healthy women is 400–800 µg/day preconceptually and throughout the first trimester. As many pregnancies are unplanned, many countries recommend daily supplementation by all women of reproductive age. However, supplementation rates are low in many groups of women in the United Kingdom and the United States (e.g. adolescents), and higher rates of deficiency (up to 26% of pregnant women) are reported in low-income countries (Gernand et al. 2016). Folate deficiency is
currently defined as serum folate <10nmol/L and red blood cell folate <340nmol/L, based on the increased plasma homocysteine; however, recent guidance suggests RBC folate concentrations >906nmol/L are required to achieve the greatest reduction in neural tube defects occurrence (Cordero et al. 2015). A growing proportion of pregnant women at higher risk of neural tube defects are recommended to supplement with high-dose folic acid (4–5mg/day), far exceeding the tolerable upper limit of 1mg/day for a non-pregnant adult.

Vitamin B12 deficiency (serum concentrations <150pmol/L) is common in countries where a vegan diet is consumed. A recent systematic review estimated a worldwide prevalence of 19–29% (Sukumar et al. 2016); however, deficiency rates in UK and USA are low. In the UK, recommended intake of vitamin B12 in pregnancy is 2.6µg/day. In addition to increased risk of neural tube defects, maternal B12 deficiency is associated with both increased risk of LBW (RR 1.15, 95% CI 1.01–1.31) and preterm birth (RR 1.21, 95% CI 0.99–1.49) (Rogne et al. 2017).

**Clinical trials**

Folic acid supplementation is effective in reducing neural tube defects (De-Regil et al. 2015). A recent meta-analysis detected a positive influence of maternal RBC folate concentrations on birthweight and risk of SGA (van Uitert & Steegers-Theunissen 2012). This was reinforced by a systematic review of folic acid supplementation RCTs finding an increase in birthweight (mean 135.75 g, 95% CI 47.85–223.68) with folic acid supplementation, but no effects on other pregnancy outcomes. It is noteworthy that many of these trials tested folic acid in combination with iron supplementation, with a range of comparator control groups and found no overall beneficial effect of supplementation on maternal folate status (Lassi et al. 2013). Both these reviews included studies of mostly replete populations, and in some cases, these studies excluded women with low folate and/or iron levels. A more focussed analysis of folic acid vs placebo also found a small but significant increase in birthweight (2% increase, P<0.0001) with a two-fold increase in folate intake (Fekete et al. 2012). The timing of supplementation may have an impact with two recent analyses suggesting the protection against SGA is dependent on pre-conceptual supplementation (Hodgetts et al. 2014). Supplementing with folic acid may have adverse effects in individuals with low vitamin B12 status and has been associated with increased incidence of SGA birth (Dwarkanath et al. 2013). Vitamin B12 supplementation during pregnancy has been evaluated in a single RCT to date (Duggan et al. 2014), reporting a beneficial effect on maternal and infant plasma B12 concentrations, but with no difference in incidence of SGA.

**Animal studies**

The importance of folate in embryonic development was first realised in animal studies in the 1950s in which rats fed a folate-deficient diet during pregnancy produced offspring with multiple congenital anomalies (Nelson et al. 1952). More recent rodent studies have identified effects of folate deficiency on one carbon metabolism in the foetus, placenta and mother. These include a reduced S-adenosylmethionine:S-adenosylhomocysteine (SAM:SAH) ratio indicative of lower production of methyl donors, accompanied by a global decrease in placental DNA methylation (Kim et al. 2009) and altered expression of specific genes predicted to disrupt numerous metabolic pathways (McKay & Mathers 2016). A similar reduction in SAM:SAH ratio was measured in maternal plasma and liver of folate-deficient mice. This was associated with hyper-homocystenaemia and inflammation, with elevated expression of placental ling (Mikael et al. 2013). Mice fed a folate-deficient diet had decreased fetal weights, with unaltered placental weight, litter size and crown rump length (Rosario et al. 2017b). Folate deficiency led to inhibition of placental mTORC signalling and decreased amino acid transporter expression and activity.

The effects of a number of polymorphisms in one carbon cycle enzymes (MTHFR and MTHFD) that occur in the human population have been investigated in mice, as they may modulate the outcomes of excess or deficient folate supply in pregnancy. Folate-deficient mice or Mthfr heterozygotes (a model for the prevalent 677TT polymorphism) suffered higher rates of embryonic loss, congenital abnormalities, developmental delay and FGR (Pickell et al. 2009). Decreased placental weight and area was also observed in folate-deficient mice with equal decreases in both the junctional and labyrinth zones. A mouse model for the human R653Q variant (Mthfd1s+/−) fed a five-fold higher folic acid diet before and during pregnancy also showed increased embryonic and placental defects at E10.5, particularly in the labyrinth zone (Christensen et al. 2016). In both studies, the combination of dietary deficiency and genotype exacerbated the defects.

Other studies in animal models indicate adverse effect of high levels of folic acid supplementation on fetal growth. Rats supplemented with 40mg/kg vs 2mg/kg throughout pregnancy had offspring with significantly reduced birthweight and crown rump length (Achon et al. 2000). Similarly, embryos from mice fed a high folic acid diet showed embryonic delay and growth retardation and heart defects in early-to-mid pregnancy (Pickell et al. 2011). Excess folic acid administered to pregnant rats (8mg/kg vs 1mg/kg) resulted in sexually dimorphic responses, with reduced 1β-HSD2 expression (related to promoter hypermethylation) in male placentas from male infants only (Penailillo et al. 2015). No alteration was detected...
in female pups, who instead had increased birthweights. This suggests over supply, as well as deficiency of folic acid, can affect offspring responses and programme for future health. Finding equivalent evidence in humans is difficult, but two studies found a strong association with higher folic acid supplementation in pregnancy and impaired respiratory health in children (Haberg et al. 2009, Whitrow et al. 2009). This effect of high methyl donor supplementation during pregnancy on allergic airway disease in offspring has also been observed in mice (Hollingsworth et al. 2008).

The negative effects of vitamin B12 deficiency alone, and in the presence of excess folic acid supplementation, on placental and fetal development has been examined in several animal studies. Maternal vitamin B12 deficiency in rats altered placental phospholipid ratios, particularly phosphatidylcholine and phosphoethanolamine (Khot et al. 2014) and decreased placental expression of Δ5 desaturase and fatty acid transporters Fatp1 and 4 (Wadhwani et al. 2013). Excess FA (8 mg/kg vs 2 mg/kg) combined with B12 deficiency decreased global placental DNA methylation in rats (Kulkarni et al. 2011) and altered expression of one carbon metabolism enzymes including Mthfr and methionine synthase (Khot et al. 2014). In contrast, vitamin B12 attenuated the negative effects of high folic acid (5 mg/day vs 400 µg) on birth and placental weight in rats (Shah et al. 2017).

Some of the placental effects may relate to modulation of placental microRNAs, with high folate decreasing, and vitamin B12 increasing, miR-16 and miR-21 expression.

**In vitro studies**

*In vitro* studies provide evidence of adverse effects of folic acid supplementation on placental function. Culture in low folic acid conditions increased apoptosis in primary trophoblasts from human term placentas (Steegers-Theunissen et al. 2000), and reduced viability, proliferation and invasive capacity in JEG-3 (Moussa et al. 2015) and HTR-8/SVneo (Ahmed et al. 2016) cell lines. Homocysteine treatment increased trophoblast apoptosis and reduced hCG secretion (Di Simone et al. 2004). These negative effects on trophoblast survival and function were reversed by addition of folic acid in a dose-dependent manner, and folic acid treatment alone increased hCG production. Metabolic effects have also been reported with inhibition of mTOR signalling and reduced amino acid transport via system A and system L in primary human cytotrophoblasts cultured in folate-deficient media (Rosario et al. 2017a) concordant with evidence in mice (Rosario et al. 2017b). *In vitro* studies also provide supportive evidence for detrimental effects of excess folic acid on the placenta, with a reduction in BeWo and JEG3 cell viability.
Micronutrient deficiencies are prevalent in pregnant women and have been linked to a range of adverse pregnancy outcomes. Despite this, RCTs of supplementation studies of individual micronutrients have frequently failed to show consistent improvements in most fetal outcomes, with common issues of insufficient statistical power, poor quality and substantial heterogeneity identified by systematic reviews. A major source of heterogeneity is the baseline characteristics of the study populations; all the systematic reviews include studies of pregnant women who are deficient and replete in the micronutrient of interest, with very few performing sub-analyses of deficient participants alone. In addition, many of the studies include participants with co-morbidities, both obstetric or pre-existing, introducing further confounding effects that complicate the analysis of individual micronutrient effects on pregnancy outcome.

However, strong evidence for the beneficial effects of micronutrients on fetal/neonatal outcomes comes from a systematic review of multi-micronutrient (MMN) supplementation, showing a reduction in the incidence of LBW (RR 0.88 (0.85–0.91)) and SGA (RR 0.92 (0.86–0.98)) (Haider et al. 2013), leading to a recommendation for MMN to replace supplementation with individual micronutrients (most commonly iron and folate) in pregnant women at high risk of deficiencies. The beneficial effects of MMN are probably due to interactions between micronutrients and the fact that single micronutrient deficiencies are rarely seen in isolation (Haider et al. 2013). Several interactions have been described in this review, including between folate and other micronutrients, but also there are other established interactions between vitamin A, iron and zinc that impact on bioavailability of these nutrients.

Notwithstanding the issues with clinical studies, mechanistic studies have identified significant effects of micronutrient deficiency on the placenta and have begun to decipher mechanistic pathways affected by micronutrients. In vivo animal models provide the opportunity to dissect the effects of stark maternal deficiency on pregnancy outcome in a whole body system, enabling analysis of effects on fetal growth and survival. These models are more extreme than the clinical scenario and enable manipulation of single micronutrients through dietary depletion or gene knockout of key signalling components, whilst controlling for confounding factors. These are useful to identifying mechanistic pathways of action, but need careful interpretation given the previously mentioned co-existence of micronutrient deficiency, common signalling pathways (e.g. vitamin A and D, B vitamins) and the inherent limitations in comparisons of rodent and human pregnancies (including differences in placental structure, fetal number and length of pregnancy) (Bonney 2013). However, coupled with in vitro analyses of human placentas, these studies enable construction of hypotheses to be tested in clinical studies.

The micronutrients discussed have wide-ranging actions, and these are reflected in diverse effects on the placenta. However, an emerging common theme is their effects on placental inflammation (Fig. 1), which is relevant to numerous pregnancy pathologies, including FGR, PE and stillbirth (Nadeau-Vallee et al. 2016). All four micronutrients studied in this review have immunomodulatory effects, and in vitro/in vivo models of deficiency have demonstrated pro-inflammatory effects in the placenta including upregulation of pro-inflammatory cytokines (Antipatis et al. 2002, Gambling et al. 2009, Liu et al. 2011, 2017, Toblli et al. 2012, Mikael et al. 2013). Interestingly, vitamin D supplementation can have anti-inflammatory effects on placental inflammation induced by other stimuli, such as LPS, TNFA or auto-antibodies (Noyola-Martinez et al. 2013, Barrera et al. 2015, Chen et al. 2015b, Gysler et al. 2015), suggesting therapeutic uses in non-deficient women. Whether vitamin A has similar effects on placental inflammation has not been studied, but the commonalities in signalling pathways via RXR warrant further investigation. Iron supplementation can overcome the inflammatory effects of anaemia, but this is dependent on the form used (Toblli et al. 2012, 2013), with ferrous forms exacerbating inflammation and oxidative stress in rat models. Further research is needed to fully elucidate the immunomodulatory and other actions of micronutrients on the placenta, and their contribution to placental dysfunction and pregnancy pathologies. More robust and adequately powered clinical studies will enable translation of knowledge from animal and in vitro experimental studies and better targeting to obstetric populations most likely to benefit from individual micronutrient supplementation.

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