



Antenatal melatonin as an antioxidant in human pregnancies complicated by fetal growth restriction – a phase I pilot clinical trial: study protocol

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5 by fetal growth restriction – a phase I pilot clinical trial: study protocol
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41 antenatal neuroprotection.
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ABSTRACT

Background: Fetal growth restriction complicates about 5% of pregnancies and is commonly caused by placental dysfunction. It is associated with increased risks of perinatal mortality and short- and long-term morbidity, such as cerebral palsy. Chronic *in utero* hypoxiaemia, inflammation and oxidative stress are likely culprits contributing to the long-term neurological sequelae of fetal growth restriction. In this regard we propose that melatonin, a powerful antioxidant, might mitigate morbidity and/or mortality associated with fetal growth restriction. Melatonin has an excellent biosafety profile and crosses the placenta and blood brain barrier. We present the protocol for a phase I clinical trial to investigate the efficacy of maternal oral melatonin administration in women with a pregnancy complicated by fetal growth restriction.

Methods and analysis: The proposed trial is a single-arm open-label clinical trial involving 12 women. Severe, early onset fetal growth restriction will be diagnosed by estimated fetal weight $\leq 10^{\text{th}}$ centile in combination with abnormal fetoplacental Doppler studies, occurring before 34 weeks of pregnancy. Baseline measurements of maternal and fetal wellbeing, levels of oxidative stress and ultrasound and Doppler measurements will be obtained at the time of diagnosis of fetal growth restriction. Women will then commence melatonin treatment (4mg) twice daily until birth. The primary outcomes are the levels of oxidative stress in the maternal and fetal circulation and placenta. Secondary outcomes are fetoplacental Doppler studies (uterine artery, middle cerebral artery, and ductus

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3 venous), fetal biometry, fetal biophysical profile and a composite determination
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5 of neonatal outcome. A historical cohort of gestational matched fetal growth
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7 restriction and a healthy pregnancy cohort will be used as comparators.
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12 **Ethics and dissemination:** Ethical approval has been obtained from Monash
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14 Health Human Research Ethics Committee B (HREC12133B). Data will be
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16 presented at international conferences and published in peer-reviewed journals.
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21 **Trial registration number:** Clinical Trials, protocol registration system:
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23 NCT01695070.
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ARTICLE SUMMARY

Article focus:

- This trial aims to establish the clinical and/or biochemical benefit of maternally administered melatonin as an antioxidant treatment in pregnancies affected by fetal growth restriction.
- Specific aims are:
 - To determine the effect of daily maternal oral melatonin on levels of oxidative stress in the placenta and in the maternal and fetal circulation in pregnancies affected by early onset fetal growth restriction.
 - To determine the effect of daily maternal oral melatonin on the clinical outcomes of pregnancies affected by early onset fetal growth restriction.
 - To determine the effect of daily maternal oral melatonin on brain injury and neurologic development in growth restricted newborn babies.

Key messages:

- Fetal growth restriction is a serious pregnancy complication and is associated with significant fetal and neonatal morbidity and mortality. Fetal brain injury is common in fetal growth restriction.

- Brain injury in survivors of fetal growth restriction is likely to originate antenatally as a consequence of oxidative stress.
- There are currently no established treatments for fetal growth restriction or for neuroprotection in fetal growth restriction.
- Numerous animal studies have established the use of melatonin as an antenatal antioxidant and neuroprotectant in acute and chronic hypoxia. In this study we aim to explore whether melatonin is an effective antioxidant in the treatment of human fetal growth restriction.

Strengths and limitations of this study:

- This pilot study has an appropriate design, and includes sufficient participants, to achieve measureable outcomes. Extensive previous human and animal studies form the basis for the design of this trial.
- This pilot study is the first of its kind in the world. Being a pilot study, this trial lacks randomisation and a placebo group, although comparator groups are included. If this trial is successful, results will be used to inform future randomized controlled trials.

INTRODUCTION

Fetal growth restriction (FGR) defines the fetus that does not realise its normal growth potential. This significant pregnancy complication accounts for about 5 percent of all pregnancies(1). FGR is most commonly caused by placental dysfunction(2). FGR is strongly associated with increased risks of preterm birth, perinatal death, and, among survivors, significant cardiovascular and neurological morbidities, including cerebral palsy(3-7).

State-of-the-art fetal surveillance techniques are important in the diagnosis and surveillance of the growth restricted fetus, and afford timely delivery. However, with the exception of antenatal glucocorticoids there are currently no treatments to reduce the short- and long-term morbidities associated with FGR(8). In FGR, injury to the developing fetal brain is likely to principally occur *in utero*, emphasizing that, if the burden of neurological morbidity is to be reduced, then antenatal neuroprotective treatment, as opposed to postnatal therapy, will be necessary. Thus, future treatment should be directed at protecting fetal brain development and reducing the incidence of brain injury before birth rather than solely focusing on care in the nursery.

Although many *in utero* events can perturb normal fetal brain development, it is thought that chronic fetoplacental hypoxaemia and oxidative stress are the likely key mechanisms initiating pathways leading to brain injury(9). In placental dysfunction, the placenta is exposed to recurrent ischaemia-reperfusion injury, ultimately leading to decreased placental oxygen transfer capacity.

Consequently, the placenta cannot adequately meet the growing metabolic needs

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3 of the fetus, which in turn becomes progressively hypoxaemic. Compensatory
4 fetal mechanisms attempt to maintain adequate oxygenation, particularly of the
5 heart and brain. Nevertheless, these eventually fail and a complex cascade of
6 metabolic events is initiated that involves the generation of nitrogen and oxygen
7 free radicals(10).
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12 The central nervous system, and in particular the fetal brain, is particularly
13 susceptible to free radical damage(11). Low antioxidant defenses and abundant
14 iron within the fetal brain render it vulnerable to oxidative stress. Further, the
15 fetal brain is rich in membrane lipids that are sensitive to free radical attack.
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17 Indeed, lipid peroxidation plays an important role in neuronal and white matter
18 damage directly and indirectly. Byproducts of lipid peroxidation can trigger
19 vasoconstriction(12), and are cytotoxic, mutagenic and teratogenic(13–15).
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21 Magnetic resonance imaging of preterm infants with white matter injury show
22 significantly higher levels of protein and lipid peroxidation(16). Furthermore, in
23 an autopsy study on human brain tissue, activated microglia, significant protein
24 nitration, lipid peroxidation and cell injury were all detected in premyelinating
25 oligodendrocytes in babies diagnosed with periventricular leukomalacia(17).
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29 Melatonin (5-methoxy-N-acetyltryptamine) is an endogenous lipid-soluble
30 hormone, predominantly produced in the pineal gland that is best recognized for
31 its role in providing circadian and seasonal timing cues. It is also a strong
32 antioxidant. It effectively scavenges free radicals, induces endogenous
33 antioxidant enzymes, such as glutathione peroxidase and glutathione
34 reductase(18), and inhibits the pro-oxidative enzyme nitric oxide synthase(19).
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38 Melatonin also stabilizes cell membranes, enhancing their resistance to oxidative
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3 damage(20). Due to its strong antioxidant capacity and its ability to cross the
4
5 human placenta(21) and blood-brain barrier(22), melatonin is of particular
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7 interest as a candidate antenatal neuroprotectant. The neuroprotective potential
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9 of melatonin is augmented by the fact that melatonin inhibits norepinephrine-
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11 induced middle cerebral artery constriction(23) and induces umbilical
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13 vasodilation(24) in ovine models of fetal growth restriction. We have shown that
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15 melatonin treatment before and during transient severe fetal asphyxia
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17 prevented the formation of hydroxyl radicals within the fetal brain(25), reduced
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19 lipid peroxidation and cell death and stabilized the blood-brain-barrier(26).
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21 Administration of melatonin at the time of induced hypoxia in the fetal sheep
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23 brain also reduced inflammation and cell death(27). Using an established ovine
24
25 model of fetal growth restriction(28), we have now shown that maternal
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27 administration of melatonin reduced fetal hypoxia, improved
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29 neurodevelopment and decreased brain injury and oxidative stress in newborn
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31 lambs(*SL Miller, unpublished data 2013*).

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38 Melatonin treatment during human pregnancy has been studied for a large range
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40 of conditions and at different times during gestation. Predominantly, melatonin
41
42 has been studied in assisted reproductive technology, aiming to improve oocyte
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44 quality and pregnancy rates following *in vitro* fertilization (IVF). Melatonin
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46 administration, commenced prior to IVF-cycles and continued during pregnancy,
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48 was associated with modestly improved pregnancy outcomes(29–31).
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50 Importantly, all babies that were born from melatonin-treated pregnancies were
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52 born healthy and with no congenital abnormalities. (*V. Unfer, personal*

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3 *communication, 2012*). Furthermore, maternal treatment with melatonin
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5 significantly boosts placental antioxidant enzyme gene expression(21).
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9 Except for its known, benign effects on sleep dynamics, melatonin does not exert
10 acute pharmacological effects on the nervous or vascular system. A median lethal
11 dose in mice could not be calculated because an increased mortality rate was not
12 observed, even following extremely high doses of up to 800mg/kg
13 melatonin(32). Long term nightly oral treatment with 75mg melatonin in 1400
14 non-pregnant women did not cause any serious adverse effects(33). To our
15 knowledge, no maternal and/or developmental toxicity effects, due to melatonin
16 treatment, have been reported. The maternal no adverse effect level (NOAEL)
17 and lowest observed adverse effect level were established at 100 and
18 200mg/kg/day, respectively, for maternally administered melatonin in pregnant
19 rats. The developmental toxicity NOAEL was ≥ 200 mg/kg/day(34).
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34 **Rationale:** Fetal growth restriction is a serious pregnancy complication,
35 affecting up to 5% of pregnancies. It is associated with significant fetal and
36 neonatal mortality and morbidity, including neurological impairment. Even
37 though modern obstetrics allows for careful fetal monitoring, there are neither
38 effective therapies for fetal growth restriction nor established options for
39 antenatal neuroprotection in fetal growth restriction. Numerous animal studies
40 have established the use of melatonin as an effective antenatal antioxidant and
41 neuroprotectant in acute and chronic hypoxia. In this trial we aim to explore, for
42 the first time, whether melatonin is an effective antioxidant in human fetal
43 growth restriction.
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3 **Aims:** We will establish the clinical and/or biochemical benefit of maternally
4 administered melatonin as an antioxidant treatment in pregnancies affected by
5 fetal growth restriction.
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10 Specific aims are:

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14 1. To determine the effect of daily maternal oral melatonin on markers of
15 oxidative stress in the placenta, and maternal and fetal circulations in
16 pregnancies affected by early onset fetal growth restriction.
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19 2. To determine the effect of daily maternal oral melatonin on the clinical
20 outcomes of pregnancies affected by early onset fetal growth restriction.
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23 3. To determine the effect of daily maternal oral melatonin on brain injury and
24 neurodevelopment in growth restricted newborn babies.
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34 **METHODS AND ANALYSIS**

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37 **Study design:** Phase I single-arm open label clinical trial, with historic cohorts of
38 FGR and healthy pregnancies as comparators.
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44 **Subjects:** Twelve women (pregnancies) with fetal growth restriction will be
45 recruited and studied to completion of the trial. A historical cohort of gestational
46 matched fetal growth restriction and a healthy pregnancy cohort will be used as
47 comparators.
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3 **Patient eligibility:** Pregnant women with a singleton growth restricted fetus at
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5 less than 34 weeks. Fetal growth restriction will be defined as a sonographic
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7 estimated fetal weight at or below the 10th centile for gestational age in
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9 combination with at least one abnormal fetoplacental Doppler study of the
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11 uterine artery, umbilical artery, middle cerebral artery or ductus venosus.
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13 Women with a multiple pregnancy, or a pregnancy affected by maternal or fetal
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15 infection, or with a fetus with a chromosomal or major structural anomaly or
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17 non-placental fetal growth restriction will be excluded from this trial.
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23 **Recruitment, interventions and procedures:** Patients will be recruited from
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25 the maternity services of Monash Health and Jessie McPherson Private Hospital,
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27 Melbourne, Australia. The study will be staffed with a clinical research team, who
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29 will inform potential participants about the aims, methods and potential risks
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31 and benefits of this study. Subjects will be informed that their participation is
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33 voluntary and that they may withdraw consent to participate at any time during
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35 the study, and for any reason, and that choosing not to participate or withdraw
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37 will not affect their care. An information form about the trial and opportunities
38
39 to ask questions will be offered to the woman and any other person(s) she
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41 chooses. Women will be recruited to the study following written informed
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43 consent. Clinical details of pregnancy, delivery and neonatal outcomes will be
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45 recorded in pre-specified case record forms.
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52 Following recruitment, and until delivery, women will receive oral melatonin
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54 (Circadin®, Neurim Pharmaceuticals), 4mg twice daily (a total daily dose of 8mg)
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56 approximately 12 hours apart. Prior to starting melatonin, a maternal blood
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3 sample will be taken and ultrasound and Doppler measurements will be
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5 obtained. This will be repeated once per week until birth. Immediately prior to
6
7 birth, a maternal blood sample will be obtained. At the time of birth, samples of
8
9 the amniotic fluid, umbilical cord blood and placenta will be collected.
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11 Clinical data regarding maternal wellbeing, pregnancy and fetal and neonatal
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13 outcomes will be recorded as the trial proceeds.
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19 **Outcome measures:** The primary outcome will be the level of oxidative stress in
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21 the placenta and in the maternal (before and during treatment and at delivery)
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23 and fetal (umbilical artery and vein at delivery) circulations, as reflected by the
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25 measurement of malondialdehyde, 8-isoprostane, total antioxidant capacity and
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27 superoxide dismutase. Serum levels of melatonin will be measured in maternal
28
29 and fetal blood.
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35 The secondary outcome measures will include clinical parameters about the
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37 pregnancy and its duration, fetal and neonatal wellbeing, mortality and
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39 morbidity (Tables 1-4).
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Table 1: Ultrasound and Doppler measurements.

Doppler and ultrasound measurements: at recruitment and then weekly until delivery.

Doppler velocimetry: umbilical artery, middle cerebral artery, ductus venosus, and maternal uterine arteries

Biometry (fortnightly)

Amniotic fluid index

Fetal behaviour: heart rate, tone, breathing, gross body movements

Table 2: Maternal morbidity.

Markers of maternal morbidity: at recruitment and at least weekly until delivery.

Blood pressure

Complete blood count

Liver function tests (AST, ALT, GTT, bilirubin)

Renal function tests (urea, creatinine)

Table 3: Pregnancy end-points**Pregnancy outcomes:**

Gestational age at birth

Diagnosis to delivery interval

Abnormal cardiotocogram

Indication for delivery (if not spontaneous)

Mode of birth

Labour analgesia/anaesthesia

Duration of labour stages

Duration of membrane rupture to birth

Use of antihypertensives

Use of magnesium sulphate

Use of corticosteroids

Table 4: Neonatal outcomes.**Neonatal outcomes:**

Sex

Apgar scores (1, 5, 10 minutes)

Birth weight

Cord artery S100B

Head circumference at birth

Composite neonatal outcome: admission to neonatal intensive care unit,
duration of admission, need for and duration of respiratory support,
intraventricular haemorrhage, necrotizing enterocolitis, abnormal neurology,

mortality prior to discharge

Follow up of women and infants: Participants will be followed weekly for the duration of the trial. The routine clinical care of participants, including ultrasound scans, will not be affected by participation in the study. During the weekly follow-up, fetal and maternal wellbeing will be assessed and women will discuss any issues that they have experienced. Medication will be supplied weekly to meet the requirements for the following week. Women will be asked to keep a medication and symptom diary (drowsiness, sleepiness, general physical well-being, time of melatonin intake), which will be reviewed weekly by the principal investigator. Melatonin treatment will cease as soon as birth has occurred. Postnatal clinical care of the women will be the responsibility of the attending obstetric team. The principal investigator will assess the clinical condition of participants daily during the first three days post-partum, to ensure that no problems become apparent in response to withdrawal from treatment. Clinical data will be obtained from neonates who require admission to the neonatal intensive care unit. Long-term follow-up using neurodevelopmental examination and questionnaires and MRI scans, are desirable but will depend on funding.

Sample size and statistical analysis of data: This study is a proof-of-principle phase I trial. As such, there are no current data available to undertake a formal power calculation to determine the sample size. If successful, this exploratory study will provide the measures needed to perform the power calculations

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3 required for the planned phase II randomized-controlled trial. This trial will
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5 provide data for the establishment of the use of maternal melatonin treatment in
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7 fetal growth restriction.
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10 The results obtained in this trial will be compared to historic cohorts of
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12 untreated fetal growth restriction and healthy pregnancies, which we have been
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14 collecting since 2011, using the same protocol.
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17 Biomarkers of oxidative stress in the maternal blood samples will be analysed
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19 using a repeated measures analysis of variance, including the baseline value as a
20
21 covariate. It is assumed that the effect of treatment with melatonin on levels of
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23 oxidative stress will not depend on duration of treatment. However, secondary
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25 analysis will be performed to determine the effect of treatment duration on
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27 biomarkers of oxidative stress. If the treatment duration significantly impacts on
28
29 the effect of melatonin on the oxidative stress status, this will be further
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31 investigated using the least-square measure. Data will be plotted as mean scores
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33 over time.
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40 Biomarkers of oxidative stress in the maternal blood samples will be analysed
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42 using a repeated measures analysis of variance, including the baseline value as a
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44 covariate. Levels of oxidative stress in the maternal blood sample obtained
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46 immediately prior to birth will be compared to samples derived from the historic
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48 cohorts using a one-way ANOVA. Levels of oxidative stress and brain injury will
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50 be compared to data obtained from the previously described historic cohorts.
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52 Clinical data regarding maternal wellbeing, pregnancy outcome and neonatal
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54 outcome will be compared to the same cohorts. These data will be analysed using
55
56 a one-way ANOVA.
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5 A p-value ≤ 0.05 is considered to be statistically significant.
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10 **Adverse events and safety monitoring:** During the treatment period, clinical
11 care will remain unchanged and will be the responsibility of uninvolved medical
12 staff. The principal investigator will monitor all participants weekly, take a brief
13 medical history, measure blood pressure and interpret ultrasound scan results
14 (obtained by uninvolved staff) to ensure melatonin treatment can be safely
15 continued. A weekly blood sample will be obtained to perform a full blood
16 examination, assess liver and renal function and assess C-reactive protein. For
17 the duration of the trial, the principal investigator will be contactable by
18 telephone at all times.
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30 Any adverse medical events reported by the patient will be recorded in the
31 patients' medical records at each visit. Serious adverse events occurring after
32 initiation of the trial will be reported to the Monash Health Human Research
33 Ethics committee, Therapeutics Committee and Therapeutic Goods
34 Administration of Australia's Office of Scientific Evaluation within 72 hours.
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44 **Trial discontinuation or modification**

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46 The participants can end participation at their own request at any time.
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48 Participants will be withdrawn from the trial if they or their fetus suffer from an
49 unexpected serious adverse event. Worsening of fetoplacental Doppler
50 velocimetry or fetal growth are within the natural history of fetal growth
51 restriction and are not part of the discontinuation criteria.
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3 **Ethics and dissemination:** This study has been approved by the Monash Health
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5 Human Research Ethics Committee B (HREC 12133B). Data will be presented at
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7 relevant conferences and published in peer-reviewed journals.
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10 11 12 **DISCUSSION**

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17 The use of melatonin as an antenatal antioxidant therapy in fetal growth
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19 restriction has been proposed as a promising and safe option to protect the fetal
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21 brain from injury. This trial is essential to confirm the antioxidant capacity and
22
23 safety of melatonin treatment. If this study confirms that maternal melatonin can
24
25 decrease maternal, fetal and/or placental levels of oxidative stress, we will
26
27 proceed to a phase II randomized-controlled trial to assess the effect of maternal
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29 melatonin treatment on brain injury and neurodevelopment. In addition,
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31 melatonin treatment could also potentially be used as an antenatal
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33 neuroprotectant in other compromised pregnancies or in threatened preterm
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35 birth to protect the mother and/or fetus. Finally, if melatonin treatment indeed
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37 strengthens antioxidant defenses during pregnancy, it might be a suitable new
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39 strategy in the management of pre-eclampsia. A phase I study to study the
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41 clinical and biochemical benefit of melatonin treatment in preeclampsia has been
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43 initiated(35).
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51 **AUTHORS' CONTRIBUTIONS**

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55 NOA: research, contribution of original material, editing and approval of final
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57 manuscript. SLM, GJ, EMW: editing and approval of final manuscript.
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COMPETING INTERESTS

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Antenatal melatonin as an antioxidant in human pregnancies complicated by fetal growth restriction – a phase I pilot clinical trial: study protocol

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5 by fetal growth restriction – a phase I pilot clinical trial: study protocol
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39 **Keywords:** Fetal growth restriction, melatonin, clinical trial, oxidative stress,
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41 antenatal neuroprotection.
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ABSTRACT

Background: Fetal growth restriction complicates about 5% of pregnancies and is commonly caused by placental dysfunction. It is associated with increased risks of perinatal mortality and short- and long-term morbidity, such as cerebral palsy. Chronic *in utero* hypoxaemia, inflammation and oxidative stress are likely culprits contributing to the long-term neurological sequelae of fetal growth restriction. In this regard we propose that melatonin, a powerful antioxidant, might mitigate morbidity and/or mortality associated with fetal growth restriction. Melatonin has an excellent biosafety profile and crosses the placenta and blood brain barrier. We present the protocol for a phase I clinical trial to investigate the efficacy of maternal oral melatonin administration in women with a pregnancy complicated by fetal growth restriction.

Methods and analysis: The proposed trial is a single-arm open-label clinical trial involving 12 women. Severe, early onset fetal growth restriction will be diagnosed by estimated fetal weight $\leq 10^{\text{th}}$ centile in combination with abnormal fetoplacental Doppler studies, occurring before 34 weeks of pregnancy. Baseline measurements of maternal and fetal wellbeing, levels of oxidative stress and ultrasound and Doppler measurements will be obtained at the time of diagnosis of fetal growth restriction. Women will then commence melatonin treatment (4mg) twice daily until birth. The primary outcomes are the levels of oxidative stress in the maternal and fetal circulation and placenta. Secondary outcomes are fetoplacental Doppler studies (uterine artery, middle cerebral artery, and ductus venosus), fetal biometry, fetal biophysical profile and a composite determination

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3 of neonatal outcome. A historical cohort of gestational matched fetal growth
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5 restriction and a healthy pregnancy cohort will be used as comparators.
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10 **Ethics and dissemination:** Ethical approval has been obtained from Monash
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12 Health Human Research Ethics Committee B (HREC12133B). Data will be
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14 presented at international conferences and published in peer-reviewed journals.
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19 **Trial registration number:** Clinical Trials, protocol registration system:
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21 NCT01695070.
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ARTICLE SUMMARY

Article focus:

- This trial aims to establish the clinical and/or biochemical benefit of maternally administered melatonin as an antioxidant treatment in pregnancies affected by fetal growth restriction.
- Specific aims are:
 - To determine the effect of daily maternal oral melatonin on levels of oxidative stress in the placenta and in the maternal and fetal circulation in pregnancies affected by early onset fetal growth restriction.
 - To determine the effect of daily maternal oral melatonin on the clinical outcomes of pregnancies affected by early onset fetal growth restriction.
 - To determine the effect of daily maternal oral melatonin on brain injury and neurologic development in growth restricted newborn babies.

Key messages:

- Fetal growth restriction is a serious pregnancy complication and is associated with significant fetal and neonatal morbidity and mortality. Fetal brain injury is common in fetal growth restriction.

- Brain injury in survivors of fetal growth restriction is likely to originate antenatally as a consequence of oxidative stress.
- There are currently no established treatments for fetal growth restriction or for neuroprotection in fetal growth restriction.
- Numerous animal studies have established the use of melatonin as an antenatal antioxidant and neuroprotectant in acute and chronic hypoxia. In this study we aim to explore whether melatonin is an effective antioxidant in the treatment of human fetal growth restriction.

Strengths and limitations of this study:

- This pilot study has an appropriate design, and includes sufficient participants, to achieve measureable outcomes. Extensive previous human and animal studies form the basis for the design of this trial.
- This pilot study is the first of its kind in the world. Being a pilot study, this trial lacks randomisation and a placebo group, although comparator groups are included. If this trial is successful, results will be used to inform future randomized controlled trials.

INTRODUCTION

Fetal growth restriction (FGR) defines the fetus that does not realise its normal growth potential. This significant pregnancy complication accounts for about 5 percent of all pregnancies(1). FGR is most commonly caused by placental dysfunction(2). FGR is strongly associated with increased risks of preterm birth, perinatal death, and, among survivors, significant cardiovascular and neurological morbidities, including cerebral palsy(3-7).

State-of-the-art fetal surveillance techniques are important in the diagnosis and surveillance of the growth restricted fetus, and afford timely delivery. However, with the exception of antenatal glucocorticoids there are currently no treatments to reduce the short- and long-term morbidities associated with FGR(8). In FGR, injury to the developing fetal brain is likely to principally occur *in utero*, emphasizing that, if the burden of neurological morbidity is to be reduced, then antenatal neuroprotective treatment, as opposed to postnatal therapy, will be necessary. Thus, future treatment should be directed at protecting fetal brain development and reducing the incidence of brain injury before birth rather than solely focusing on care in the nursery.

Although many *in utero* events can perturb normal fetal brain development, it is thought that chronic fetoplacental hypoxaemia and oxidative stress are the likely key mechanisms initiating pathways leading to brain injury(9). In placental dysfunction, the placenta is exposed to recurrent ischaemia-reperfusion injury, ultimately leading to decreased placental oxygen transfer capacity.

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3 Consequently, the placenta cannot adequately meet the growing metabolic needs
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5 of the fetus, which in turn becomes progressively hypoxaemic. Compensatory
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7 fetal mechanisms attempt to maintain adequate oxygenation, particularly of the
8
9 heart and brain. Nevertheless, these eventually fail and a complex cascade of
10
11 metabolic events is initiated that involves the generation of nitrogen and oxygen
12
13 free radicals(10).
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17 The central nervous system, and in particular the fetal brain, is particularly
18
19 susceptible to free radical damage(11). Low antioxidant defenses and abundant
20
21 iron within the fetal brain render it vulnerable to oxidative stress. Further, the
22
23 fetal brain is rich in membrane lipids that are sensitive to free radical attack.
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27 Indeed, lipid peroxidation plays an important role in neuronal and white matter
28
29 damage directly and indirectly. Byproducts of lipid peroxidation can trigger
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31 vasoconstriction(12), and are cytotoxic, mutagenic and teratogenic(13–15).
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35 Magnetic resonance imaging of preterm infants with white matter injury show
36
37 significantly higher levels of protein and lipid peroxidation(16). Furthermore, in
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39 an autopsy study on human brain tissue, activated microglia, significant protein
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41 nitration, lipid peroxidation and cell injury were all detected in premyelinating
42
43 oligodendrocytes in babies diagnosed with periventricular leukomalacia(17).
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47 Melatonin (5-methoxy-N-acetyltryptamine) is an endogenous lipid-soluble
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49 hormone, predominantly produced in the pineal gland that is best recognized for
50
51 its role in providing circadian and seasonal timing cues. It is also a strong
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53 antioxidant. It effectively scavenges free radicals, induces endogenous
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55 antioxidant enzymes, such as glutathione peroxidase and glutathione
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57 reductase(18), and inhibits the pro-oxidative enzyme nitric oxide synthase(19).
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3 Melatonin also stabilizes cell membranes, enhancing their resistance to oxidative
4 damage(20). *In vitro*, melatonin treatment protects the villous trophoblast
5
6 against hypoxia-reperfusion induced oxidative stress and apoptosis(21). Due to
7
8 its strong antioxidant capacity and its ability to cross the human placenta(22)
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10 and blood-brain barrier(23), melatonin is of particular interest as a candidate
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12 antenatal neuroprotectant. The neuroprotective potential of melatonin is
13
14 augmented by the fact that melatonin inhibits norepinephrine-induced middle
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16 cerebral artery constriction(24) and induces umbilical vasodilation(25) in ovine
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18 models of fetal growth restriction. In animal models, we have shown that
19
20 melatonin treatment before and during transient severe fetal asphyxia
21
22 prevented the formation of hydroxyl radicals within the fetal brain(26), reduced
23
24 lipid peroxidation and cell death and stabilized the blood-brain-barrier(27).
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26 Administration of melatonin at the time of induced hypoxia in the fetal sheep
27
28 brain also reduced inflammation and cell death(28). Using an established ovine
29
30 model of fetal growth restriction(29), we have now shown that maternal
31
32 administration of melatonin reduced fetal hypoxia, improved
33
34 neurodevelopment and decreased brain injury and oxidative stress in newborn
35
36 lambs(*SL Miller, unpublished data 2013*).

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39 Melatonin treatment during human pregnancy has been studied for a large range
40
41 of conditions and at different times during gestation. Predominantly, melatonin
42
43 has been studied in assisted reproductive technology, aiming to improve oocyte
44
45 quality and pregnancy rates following *in vitro* fertilization (IVF). Melatonin
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47 administration, commenced prior to IVF-cycles and continued during pregnancy,
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49 was associated with modestly improved pregnancy outcomes(30–32).
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3 Importantly, all babies that were born from melatonin-treated pregnancies were
4
5 born healthy and with no congenital abnormalities (*V. Unfer. personal*
6
7 *communication, 2012*). Furthermore, maternal treatment with melatonin
8
9 significantly boosts placental antioxidant enzyme gene expression(22).
10

11
12 Except for its known, benign effects on sleep dynamics, melatonin does not exert
13
14 acute pharmacological effects on the nervous or vascular system. A median lethal
15
16 dose in mice could not be calculated because an increased mortality rate was not
17
18 observed, even following extremely high doses of up to 800mg/kg
19
20 melatonin(33). Long term nightly oral treatment with 75mg melatonin in 1400
21
22 non-pregnant women did not cause any serious adverse effects(34). To our
23
24 knowledge, no maternal and/or developmental toxicity effects, due to melatonin
25
26 treatment, have been reported. The maternal no adverse effect level (NOAEL)
27
28 and lowest observed adverse effect level (LOAEL) were established at 100 and
29
30 200mg/kg/day, respectively, for maternally administered melatonin in pregnant
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32 rats. The developmental toxicity NOAEL was $\geq 200\text{mg/kg/day}$ (35).
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39 **Rationale:** Fetal growth restriction is a serious pregnancy complication,
40
41 affecting up to 5% of pregnancies. It is associated with significant fetal and
42
43 neonatal mortality and morbidity, including neurological impairment. Even
44
45 though modern obstetrics allows for careful fetal monitoring, there are neither
46
47 effective therapies for fetal growth restriction nor established options for
48
49 antenatal neuroprotection in fetal growth restriction. Numerous animal studies
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51 have established the use of melatonin as an effective antenatal antioxidant and
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53 neuroprotectant in acute and chronic hypoxia. In this trial we aim to explore, for
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3 the first time, whether melatonin is an effective antioxidant in human fetal
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5 growth restriction.
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8 **Aims:** We will establish the clinical and/or biochemical benefit of maternally
9 administered melatonin as an antioxidant treatment in pregnancies affected by
10 fetal growth restriction.
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16 Specific aims are:
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20 1. To determine the effect of daily maternal oral melatonin on markers of
21 oxidative stress in the placenta, and maternal and fetal circulations in
22 pregnancies affected by early onset fetal growth restriction.
23
24 2. To determine the effect of daily maternal oral melatonin on the clinical
25 outcomes of pregnancies affected by early onset fetal growth restriction.
26
27 3. To determine the effect of daily maternal oral melatonin on brain injury and
28 neurodevelopment in growth restricted newborn babies.
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40 **METHODS AND ANALYSIS**

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43 **Study design:** Phase I single-arm open label clinical trial, with historic cohorts of
44 FGR and healthy pregnancies as comparators.
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50 **Subjects:** Twelve women (pregnancies) with fetal growth restriction will be
51 recruited and studied to completion of the trial. A historical cohort of gestational
52 matched fetal growth restriction and a healthy pregnancy cohort will be used as
53 comparators.
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8 **Patient eligibility:** Pregnant women with a singleton growth restricted fetus
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10 between 23⁺⁰ and 34⁺⁰ weeks (confirmed gestational age). Fetal growth
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12 restriction will be defined as a sonographic estimated fetal weight at or below
13
14 the 10th centile for gestational age in combination with at least one abnormal
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16 fetoplacental Doppler study of the uterine artery (bilateral uterine artery
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18 notching or unilateral uterine artery notching on the ipsilateral side of the
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20 placenta), umbilical artery (systolic:diastolic ratio $\geq 95^{\text{th}}$ centile or absent, or,
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22 reversed end-diastolic flow), middle cerebral artery (pulsatility index $\leq 5^{\text{th}}$
23
24 centile) or ductus venosus (abnormal A wave and/or pulsatility index $\geq 95^{\text{th}}$
25
26 centile). For consenting purposes, mothers must be 18 years or older and have a
27
28 basic understanding of English. Women with an unconfirmed pregnancy
29
30 duration, a multiple pregnancy, or a pregnancy affected by maternal or fetal
31
32 infection, or with a fetus with a chromosomal or major structural anomaly or
33
34 non-placental fetal growth restriction will be excluded from this trial.
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45 **Recruitment:** Patients will be recruited from the maternity services of Monash
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47 Health and Jessie McPherson Private Hospital, Melbourne, Australia. The study
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49 will be staffed with a clinical research team, who will inform potential
50
51 participants about the aims, methods and potential risks and benefits of this
52
53 study. Subjects will be informed that their participation is voluntary and that
54
55 they may withdraw consent to participate at any time during the study, and for
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3 any reason, and that choosing not to participate or withdraw will not affect their
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5 care. An information form about the trial and opportunities to ask questions will
6
7 be offered to the woman and any other person(s) she chooses. Women will be
8
9 recruited to the study following written informed consent.
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14 **Trial treatment:** Following recruitment, and until delivery, women will receive
15
16 oral melatonin prolonged release (Circadin®, Neurim Pharmaceuticals), 4mg
17
18 twice daily (a total daily dose of 8mg) approximately 12 hours apart. This dose
19
20 and regimen are based on results from our own experimental animal research,
21
22 previously published animal research, and clinical trials.
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28 In our experimental FGR-study a maternal, cumulative, intravenous dose of
29
30 6.0mg/day was administered. The human equivalent dose (HED) was calculated
31
32 using the FDA-dose-conversion algorithm(36) and the average Australian height
33
34 and weight. This resulted in a dose between 7.3mg/day (based on pre-pregnancy
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36 weight) and 8.1mg/day (based on end-pregnancy weight). As Circadin® is only
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38 available in 2mg tablets, the chosen dose for this study is 8mg per day.
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44 This dose of melatonin is lower than the used doses in several clinical studies in
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46 neonates. These trials evaluated melatonin as an antioxidant treatment in
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48 newborns with birth asphyxia and other conditions associated with increased
49
50 oxidative stress. Melatonin was administered orally in doses up to 80mg/day in
51
52 these trials without any toxicity(37–41). In clinical trials using melatonin to
53
54 improve assisted reproductive technology, melatonin was administered orally in
55
56 doses of 3mg/day before and during pregnancy(30,32,42). Melatonin treatment,
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3 started prior to conception and continued until birth, did not adversely affect
4 pregnancy outcome and fetal or neonatal health (*V. Unfer. personal*
5 *communication, 2012*). The dose in this trial is higher than previously used doses
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7 in human pregnancy but still falls well below the maternal and fetal NOAEL (HED
8 16 and 32mg/kg respectively) and maternal LOAEL (HED 32mg/kg).
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16 Melatonin will be administered orally as 4mg given twice per day. This dosage
17 regimen is chosen because the terminal half-life of Circadin® is 3.5 to 4 hours. In
18 fetal growth restriction, increased levels of oxidative stress likely occur due to
19 continuous placental generation of reactive oxygen species. By administering
20 melatonin twice daily, we aim to provide a sustained increase in maternal and
21 fetal serum. To minimize daytime sleepiness and severe disturbances of
22 circadian rhythms, melatonin will be given early in the morning and at night.
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35 **Sample collection:** Prior to starting melatonin, a maternal blood sample will be
36 taken and ultrasound and Doppler measurements will be obtained. This will be
37 repeated once per week until birth. Immediately prior to birth, a maternal blood
38 sample will be obtained. At the time of birth, samples of the amniotic fluid,
39 umbilical cord blood and placenta will be collected. Clinical details of pregnancy,
40 including medication and (multi) vitamin use, delivery and neonatal outcomes
41 will be recorded in pre-specified case record forms.
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53 All blood samples will be collected in serum tubes and blood RNA tubes, allowing
54 for measurement of markers of oxidative stress and gene expression studies
55 respectively. Serum tubes will be centrifuged (4400g, 15 minutes). The serum
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3 will be collected and transferred into collection tubes. The collection tubes will
4
5 be stored in a -80°C until further processing.
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9
10 Amniotic fluid will be collected during caesarean section deliveries. The
11
12 myometrium will be bisected sharply and distinctly from the amnion and
13
14 chorion. The amniotic fluid will be aspirated using a sterile syringe and 18 gauge
15
16 drawing-up needle. Samples visibly contaminated with blood will be discarded
17
18 off. Within 30 minutes of the sample collection, specimens will be transferred to
19
20 a laboratory. The samples will be centrifuged (1000g, 15 minutes) and the
21
22 supernatant will be collected, transferred into sterile collection tubes and snap
23
24 frozen. Specimens will be stored at -80°C until further analysis.
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30 Placentas will be obtained at time of delivery and placed in a sterile tray. The
31
32 area at the midpoint of the longest distance between the cord insertion and the
33
34 edge of the placental disc will be sampled. Six full thickness placental samples
35
36 will be cut out using sterile scissors. The placental tissue samples will be
37
38 removed from the membranes using sterile forceps and scissors. The placental
39
40 tissue samples will then be washed in a sterile container using ice cold, sterile
41
42 phosphate-buffered saline. Two tissue samples will be fixed in 4%
43
44 paraformaldehyde and embedded in paraffin for histological analysis. Two tissue
45
46 samples will be placed in sterile tubes containing RNA-later for 24 hours at 4°C.
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48 After immersion in RNA-later, these two samples will be transferred into two
49
50 sterile cryovials, snap frozen in liquid nitrogen and stored at -80°C until further
51
52 processing for gene expression studies. The remaining tissue samples will be
53
54 placed into sterile cryovials and snap frozen in liquid nitrogen. Samples will be
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1
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3 stored in a -80°C freezer until further processing to measure levels of oxidative
4 stress and melatonin.
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12 **Outcome measures:** The primary outcome will be the level of oxidative stress
13 in the placenta and in the maternal (before and during treatment and at
14 delivery) and fetal (umbilical artery and vein at delivery) circulations, as
15 reflected by the measurement of malondialdehyde, 8-isoprostane, total
16 antioxidant capacity and superoxide dismutase. Levels of melatonin will be
17 measured in maternal and fetal blood and in the placenta. Gene expression of
18 pro- and antioxidant enzymes, melatonin synthesizing enzymes, melatonin
19 receptors and clock genes will be measured in the placenta and in the maternal
20 and fetal blood.
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35 The secondary outcome measures will include clinical parameters about the
36 pregnancy and its duration, fetal and neonatal wellbeing, mortality and
37 morbidity (Tables 1-4).
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Table 1: Ultrasound and Doppler measurements.

Doppler and ultrasound measurements: at recruitment and then weekly until delivery.

Doppler velocimetry: umbilical artery, middle cerebral artery, ductus venosus, and maternal uterine arteries

Biometry (fortnightly)

Amniotic fluid index

Fetal behaviour: heart rate, tone, breathing, gross body movements

Table 2: Maternal morbidity.

Markers of maternal morbidity: at recruitment and at least weekly until delivery.

Blood pressure

Complete blood count

Liver function tests (AST, ALT, GTT, bilirubin)

Renal function tests (urea, creatinine, uric acid)

Table 3: Pregnancy end-points**Pregnancy outcomes:**

Gestational age at birth

Diagnosis to delivery interval

Abnormal cardiotocogram

Indication for delivery (if not spontaneous)

Mode of birth

Labour analgesia/anaesthesia

Duration of labour stages

Duration of membrane rupture to birth

Used medication such as antihypertensives, magnesium sulphate and corticosteroids.

Use of (multi) vitamins and dietary supplements

Table 4: Neonatal outcomes.**Neonatal outcomes:**

Sex

Apgar scores (1, 5, 10 minutes)

Birth weight

Cord artery S100B

Head circumference at birth

Composite neonatal outcome: admission to neonatal intensive care unit, duration of admission, need for and duration of respiratory support,

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3 intraventricular haemorrhage, necrotizing enterocolitis, abnormal neurology,
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5 mortality prior to discharge
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11 **Follow up of women and infants:** Participants will be followed weekly for the
12 duration of the trial. The routine clinical care of participants, including
13 ultrasound scans, will not be affected by participation in the study. During the
14 weekly follow-up, fetal and maternal wellbeing will be assessed and women will
15 discuss any issues that they have experienced. Medication will be supplied
16 weekly to meet the requirements for the following week. Women will be asked to
17 keep a medication and symptom diary (drowsiness, sleepiness, general physical
18 well-being, time of melatonin intake), which will be reviewed weekly by the
19 principal investigator. Melatonin treatment will cease as soon as birth has
20 occurred. Postnatal clinical care of the women will be the responsibility of the
21 attending obstetric team. The principal investigator will assess the clinical
22 condition of participants daily during the first three days post-partum, to ensure
23 that no problems become apparent in response to withdrawal from treatment.
24 Clinical data will be obtained from neonates who require admission to the
25 neonatal intensive care unit. Long-term follow-up using neurodevelopmental
26 examination and questionnaires and MRI scans, are desirable but will depend on
27 funding.
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53 **Sample size and statistical analysis of data:** This study is a proof-of-principle
54 phase I trial. As such, there are no current data available to undertake a formal
55 power calculation to determine the sample size. If successful, this exploratory
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3 study will provide the measures needed to perform the power calculations
4
5 required for the planned phase II randomized-controlled trial. This trial will
6
7 provide data for the establishment of the use of maternal melatonin treatment in
8
9 fetal growth restriction.

10
11 The results obtained in this trial will be compared to historic cohorts of
12
13 untreated fetal growth restriction and healthy pregnancies, which we have been
14
15 collecting since 2011, using the same protocol.

16
17 Biomarkers of oxidative stress in the maternal blood samples will be analysed
18
19 using a repeated measures analysis of variance, including the baseline value as a
20
21 covariate. It is assumed that the effect of treatment with melatonin on levels of
22
23 oxidative stress will not depend on duration of treatment. However, secondary
24
25 analysis will be performed to determine the effect of treatment duration on
26
27 biomarkers of oxidative stress. If the treatment duration significantly impacts on
28
29 the effect of melatonin on the oxidative stress status, this will be further
30
31 investigated using the least-square measure. Data will be plotted as mean scores
32
33 over time.
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41 Biomarkers of oxidative stress in the maternal blood samples will be analysed
42
43 using a repeated measures analysis of variance, including the baseline value as a
44
45 covariate. Levels of oxidative stress in the maternal blood sample obtained
46
47 immediately prior to birth will be compared to samples derived from the historic
48
49 cohorts using a one-way ANOVA. Levels of oxidative stress and brain injury will
50
51 be compared to data obtained from the previously described historic cohorts.
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53 Clinical data regarding maternal wellbeing, pregnancy outcome and neonatal
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3 outcome will be compared to the same cohorts. These data will be analysed using
4
5 a one-way ANOVA.
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9 A p-value ≤ 0.05 is considered to be statistically significant.
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14 **Adverse events and safety monitoring:** During the treatment period, clinical
15 care will remain unchanged and will be the responsibility of uninvolved medical
16 staff. The principal investigator will monitor all participants weekly, take a brief
17 medical history, measure blood pressure and interpret ultrasound scan results
18 (obtained by uninvolved staff) to ensure melatonin treatment can be safely
19 continued. A weekly blood sample will be obtained to perform a full blood
20 examination, assess liver and renal function and assess C-reactive protein. For
21 the duration of the trial, the principal investigator will be contactable by
22 telephone at all times.
23
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25 Any adverse medical events reported by the patient will be recorded in the
26 patients' medical records at each visit. Serious adverse events occurring after
27 initiation of the trial will be reported to the Monash Health Human Research
28 Ethics committee, Therapeutics Committee and Therapeutic Goods
29 Administration of Australia's Office of Scientific Evaluation within 72 hours.
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35 36 37 38 39 40 41 42 43 44 45 46 47 48 **Trial discontinuation or modification**

49 The participants can end participation at their own request at any time.
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51 Participants will be withdrawn from the trial if they or their fetus suffer from an
52 unexpected serious adverse event. Worsening of fetoplacental Doppler
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3 velocimetry or fetal growth are within the natural history of fetal growth
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5 restriction and are not part of the discontinuation criteria.
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10 **Ethics and dissemination:** This study has been approved by the Monash Health
11
12 Human Research Ethics Committee B (HREC 12133B). Data will be presented at
13
14 relevant conferences and published in peer-reviewed journals.
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16

17 18 19 **DISCUSSION**

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24 The use of melatonin as an antenatal antioxidant therapy in fetal growth
25
26 restriction has been proposed as a promising and safe option to protect the fetal
27
28 brain from injury. This trial is essential to confirm the antioxidant capacity and
29
30 safety of melatonin treatment. If this study confirms that maternal melatonin can
31
32 decrease maternal, fetal and/or placental levels of oxidative stress, we will
33
34 proceed to a phase II randomized-controlled trial to assess the effect of maternal
35
36 melatonin treatment on brain injury and neurodevelopment. In addition,
37
38 melatonin treatment could also potentially be used as an antenatal
39
40 neuroprotectant in other compromised pregnancies or in threatened preterm
41
42 birth to protect the mother and/or fetus. Finally, if melatonin treatment indeed
43
44 strengthens antioxidant defenses during pregnancy, it might be a suitable new
45
46 strategy in the management of pre-eclampsia. A phase I study to study the
47
48 clinical and biochemical benefit of melatonin treatment in preeclampsia has been
49
50 initiated.
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AUTHORS' CONTRIBUTIONS

NOA: research, contribution of original material, editing and approval of final manuscript. SLM, GJ, EMW: editing and approval of final manuscript.

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3 **Title:** Antenatal melatonin as an antioxidant in human pregnancies complicated
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5 by fetal growth restriction – a phase I pilot clinical trial: study protocol
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39 **Keywords:** Fetal growth restriction, melatonin, clinical trial, oxidative stress,
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41 antenatal neuroprotection.
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46 **Word count:** 3143.
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ABSTRACT

Background: Fetal growth restriction complicates about 5% of pregnancies and is commonly caused by placental dysfunction. It is associated with increased risks of perinatal mortality and short- and long-term morbidity, such as cerebral palsy. Chronic *in utero* hypoxaemia, inflammation and oxidative stress are likely culprits contributing to the long-term neurological sequelae of fetal growth restriction. In this regard we propose that melatonin, a powerful antioxidant, might mitigate morbidity and/or mortality associated with fetal growth restriction. Melatonin has an excellent biosafety profile and crosses the placenta and blood brain barrier. We present the protocol for a phase I clinical trial to investigate the efficacy of maternal oral melatonin administration in women with a pregnancy complicated by fetal growth restriction.

Methods and analysis: The proposed trial is a single-arm open-label clinical trial involving 12 women. Severe, early onset fetal growth restriction will be diagnosed by estimated fetal weight $\leq 10^{\text{th}}$ centile in combination with abnormal fetoplacental Doppler studies, occurring before 34 weeks of pregnancy. Baseline measurements of maternal and fetal wellbeing, levels of oxidative stress and ultrasound and Doppler measurements will be obtained at the time of diagnosis of fetal growth restriction. Women will then commence melatonin treatment (4mg) twice daily until birth. The primary outcomes are the levels of oxidative stress in the maternal and fetal circulation and placenta. Secondary outcomes are fetoplacental Doppler studies (uterine artery, middle cerebral artery, and ductus venosus), fetal biometry, fetal biophysical profile and a composite determination

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3 of neonatal outcome. A historical cohort of gestational matched fetal growth
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5 restriction and a healthy pregnancy cohort will be used as comparators.
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10 **Ethics and dissemination:** Ethical approval has been obtained from Monash
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12 Health Human Research Ethics Committee B (HREC12133B). Data will be
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14 presented at international conferences and published in peer-reviewed journals.
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19 **Trial registration number:** Clinical Trials, protocol registration system:
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21 NCT01695070.
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ARTICLE SUMMARY

Article focus:

- This trial aims to establish the clinical and/or biochemical benefit of maternally administered melatonin as an antioxidant treatment in pregnancies affected by fetal growth restriction.
- Specific aims are:
 - To determine the effect of daily maternal oral melatonin on levels of oxidative stress in the placenta and in the maternal and fetal circulation in pregnancies affected by early onset fetal growth restriction.
 - To determine the effect of daily maternal oral melatonin on the clinical outcomes of pregnancies affected by early onset fetal growth restriction.
 - To determine the effect of daily maternal oral melatonin on brain injury and neurologic development in growth restricted newborn babies.

Key messages:

- Fetal growth restriction is a serious pregnancy complication and is associated with significant fetal and neonatal morbidity and mortality. Fetal brain injury is common in fetal growth restriction.

- Brain injury in survivors of fetal growth restriction is likely to originate antenatally as a consequence of oxidative stress.
- There are currently no established treatments for fetal growth restriction or for neuroprotection in fetal growth restriction.
- Numerous animal studies have established the use of melatonin as an antenatal antioxidant and neuroprotectant in acute and chronic hypoxia. In this study we aim to explore whether melatonin is an effective antioxidant in the treatment of human fetal growth restriction.

Strengths and limitations of this study:

- This pilot study has an appropriate design, and includes sufficient participants, to achieve measureable outcomes. Extensive previous human and animal studies form the basis for the design of this trial.
- This pilot study is the first of its kind in the world. Being a pilot study, this trial lacks randomisation and a placebo group, although comparator groups are included. If this trial is successful, results will be used to inform future randomized controlled trials.

INTRODUCTION

Fetal growth restriction (FGR) defines the fetus that does not realise its normal growth potential. This significant pregnancy complication accounts for about 5 percent of all pregnancies(1). FGR is most commonly caused by placental dysfunction(2). FGR is strongly associated with increased risks of preterm birth, perinatal death, and, among survivors, significant cardiovascular and neurological morbidities, including cerebral palsy(3-7).

State-of-the-art fetal surveillance techniques are important in the diagnosis and surveillance of the growth restricted fetus, and afford timely delivery. However, with the exception of antenatal glucocorticoids there are currently no treatments to reduce the short- and long-term morbidities associated with FGR(8). In FGR, injury to the developing fetal brain is likely to principally occur *in utero*, emphasizing that, if the burden of neurological morbidity is to be reduced, then antenatal neuroprotective treatment, as opposed to postnatal therapy, will be necessary. Thus, future treatment should be directed at protecting fetal brain development and reducing the incidence of brain injury before birth rather than solely focusing on care in the nursery.

Although many *in utero* events can perturb normal fetal brain development, it is thought that chronic fetoplacental hypoxaemia and oxidative stress are the likely key mechanisms initiating pathways leading to brain injury(9). In placental dysfunction, the placenta is exposed to recurrent ischaemia-reperfusion injury, ultimately leading to decreased placental oxygen transfer capacity.

Consequently, the placenta cannot adequately meet the growing metabolic needs

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2
3 of the fetus, which in turn becomes progressively hypoxaemic. Compensatory
4 fetal mechanisms attempt to maintain adequate oxygenation, particularly of the
5 heart and brain. Nevertheless, these eventually fail and a complex cascade of
6 metabolic events is initiated that involves the generation of nitrogen and oxygen
7 free radicals(10).
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15 The central nervous system, and in particular the fetal brain, is particularly
16 susceptible to free radical damage(11). Low antioxidant defenses and abundant
17 iron within the fetal brain render it vulnerable to oxidative stress. Further, the
18 fetal brain is rich in membrane lipids that are sensitive to free radical attack.
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24 Indeed, lipid peroxidation plays an important role in neuronal and white matter
25 damage directly and indirectly. Byproducts of lipid peroxidation can trigger
26 vasoconstriction(12), and are cytotoxic, mutagenic and teratogenic(13–15).
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Magnetic resonance imaging of preterm infants with white matter injury show
significantly higher levels of protein and lipid peroxidation(16). Furthermore, in
an autopsy study on human brain tissue, activated microglia, significant protein
nitration, lipid peroxidation and cell injury were all detected in premyelinating
oligodendrocytes in babies diagnosed with periventricular leukomalacia(17).

Melatonin (5-methoxy-N-acetyltryptamine) is an endogenous lipid-soluble
hormone, predominantly produced in the pineal gland that is best recognized for
its role in providing circadian and seasonal timing cues. It is also a strong
antioxidant. It effectively scavenges free radicals, induces endogenous
antioxidant enzymes, such as glutathione peroxidase and glutathione
reductase(18), and inhibits the pro-oxidative enzyme nitric oxide synthase(19).

Melatonin also stabilizes cell membranes, enhancing their resistance to oxidative

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3 damage(20). *In vitro*, melatonin treatment protects the villous trophoblast
4 against hypoxia-reperfusion induced oxidative stress and apoptosis(21). Due to
5
6 its strong antioxidant capacity and its ability to cross the human placenta(22)
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8 and blood-brain barrier(23), melatonin is of particular interest as a candidate
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10 antenatal neuroprotectant. The neuroprotective potential of melatonin is
11
12 augmented by the fact that melatonin inhibits norepinephrine-induced middle
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14 cerebral artery constriction(24) and induces umbilical vasodilation(25) in ovine
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16 models of fetal growth restriction. *In animal models, we* have shown that
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18 melatonin treatment before and during transient severe fetal asphyxia
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20 prevented the formation of hydroxyl radicals within the fetal brain(26), reduced
21
22 lipid peroxidation and cell death and stabilized the blood-brain-barrier(27).
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24 Administration of melatonin at the time of induced hypoxia in the fetal sheep
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26 brain also reduced inflammation and cell death(28). Using an established ovine
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28 model of fetal growth restriction(29), we have now shown that maternal
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30 administration of melatonin reduced fetal hypoxia, improved
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32 neurodevelopment and decreased brain injury and oxidative stress in newborn
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34 lambs(*SL Miller, unpublished data 2013*).

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37 Melatonin treatment during human pregnancy has been studied for a large range
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39 of conditions and at different times during gestation. Predominantly, melatonin
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41 has been studied in assisted reproductive technology, aiming to improve oocyte
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43 quality and pregnancy rates following *in vitro* fertilization (IVF). Melatonin
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45 administration, commenced prior to IVF-cycles and continued during pregnancy,
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47 was associated with modestly improved pregnancy outcomes(30-32).
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50 Importantly, all babies that were born from melatonin-treated pregnancies were
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3 born healthy and with no congenital abnormalities (*V. Unfer. personal*
4 *communication, 2012*). Furthermore, maternal treatment with melatonin
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6 significantly boosts placental antioxidant enzyme gene expression(22).
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10 Except for its known, benign effects on sleep dynamics, melatonin does not exert
11 acute pharmacological effects on the nervous or vascular system. A median lethal
12 dose in mice could not be calculated because an increased mortality rate was not
13 observed, even following extremely high doses of up to 800mg/kg
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15 melatonin(33). Long term nightly oral treatment with 75mg melatonin in 1400
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17 non-pregnant women did not cause any serious adverse effects(34). To our
18
19 knowledge, no maternal and/or developmental toxicity effects, due to melatonin
20
21 treatment, have been reported. The maternal no adverse effect level (NOAEL)
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23 and lowest observed adverse effect level (LOAEL) were established at 100 and
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25 200mg/kg/day, respectively, for maternally administered melatonin in pregnant
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27 rats. The developmental toxicity NOAEL was $\geq 200\text{mg/kg/day}$ (35).
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37 **Rationale:** Fetal growth restriction is a serious pregnancy complication,
38 affecting up to 5% of pregnancies. It is associated with significant fetal and
39 neonatal mortality and morbidity, including neurological impairment. Even
40
41 though modern obstetrics allows for careful fetal monitoring, there are neither
42
43 effective therapies for fetal growth restriction nor established options for
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45 antenatal neuroprotection in fetal growth restriction. Numerous animal studies
46
47 have established the use of melatonin as an effective antenatal antioxidant and
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49 neuroprotectant in acute and chronic hypoxia. In this trial we aim to explore, for
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51 the first time, whether melatonin is an effective antioxidant in human fetal
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53 growth restriction. In this trial we aim to explore, for
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55 the first time, whether melatonin is an effective antioxidant in human fetal
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57 growth restriction.
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3 **Aims:** We will establish the clinical and/or biochemical benefit of maternally
4 administered melatonin as an antioxidant treatment in pregnancies affected by
5 fetal growth restriction.
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10 Specific aims are:

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14 1. To determine the effect of daily maternal oral melatonin on markers of
15 oxidative stress in the placenta, and maternal and fetal circulations in
16 pregnancies affected by early onset fetal growth restriction.
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19 2. To determine the effect of daily maternal oral melatonin on the clinical
20 outcomes of pregnancies affected by early onset fetal growth restriction.
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23 3. To determine the effect of daily maternal oral melatonin on brain injury and
24 neurodevelopment in growth restricted newborn babies.
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34 **METHODS AND ANALYSIS**

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37 **Study design:** Phase I single-arm open label clinical trial, with historic cohorts of
38 FGR and healthy pregnancies as comparators.
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44 **Subjects:** Twelve women (pregnancies) with fetal growth restriction will be
45 recruited and studied to completion of the trial. A historical cohort of gestational
46 matched fetal growth restriction and a healthy pregnancy cohort will be used as
47 comparators.
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3 **Patient eligibility:** Pregnant women with a singleton growth restricted fetus
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5 between 23⁺⁰ and 34⁺⁰ weeks (confirmed gestational age). Fetal growth
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7 restriction will be defined as a sonographic estimated fetal weight at or below
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9 the 10th centile for gestational age in combination with at least one abnormal
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11 fetoplacental Doppler study of the uterine artery (bilateral uterine artery
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13 notching or unilateral uterine artery notching on the ipsilateral side of the
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15 placenta), umbilical artery (systolic:diastolic ratio $\geq 95^{\text{th}}$ centile or absent, or,
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17 reversed end-diastolic flow), middle cerebral artery (pulsatility index $\leq 5^{\text{th}}$
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19 centile) or ductus venosus (abnormal A wave and/or pulsatility index $\geq 95^{\text{th}}$
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21 centile). For consenting purposes, mothers must be 18 years or older and have a
22
23 basic understanding of English. Women with an unconfirmed pregnancy
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25 duration, a multiple pregnancy, or a pregnancy affected by maternal or fetal
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27 infection, or with a fetus with a chromosomal or major structural anomaly or
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29 non-placental fetal growth restriction will be excluded from this trial.
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40 **Recruitment:** Patients will be recruited from the maternity services of Monash
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42 Health and Jessie McPherson Private Hospital, Melbourne, Australia. The study
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44 will be staffed with a clinical research team, who will inform potential
45
46 participants about the aims, methods and potential risks and benefits of this
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48 study. Subjects will be informed that their participation is voluntary and that
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50 they may withdraw consent to participate at any time during the study, and for
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52 any reason, and that choosing not to participate or withdraw will not affect their
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54 care. An information form about the trial and opportunities to ask questions will
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3 be offered to the woman and any other person(s) she chooses. Women will be
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5 recruited to the study following written informed consent.
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10 **Trial treatment:** Following recruitment, and until delivery, women will receive
11 oral melatonin prolonged release (Circadin®, Neurim Pharmaceuticals), 4mg
12 twice daily (a total daily dose of 8mg) approximately 12 hours apart. This dose
13 and regimen are based on results from our own experimental animal research,
14 previously published animal research, and clinical trials.
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23 In our experimental FGR-study a maternal, cumulative, intravenous dose of
24 6.0mg/day was administered. The human equivalent dose (HED) was calculated
25 using the FDA-dose-conversion algorithm(36) and the average Australian height
26 and weight. This resulted in a dose between 7.3mg/day (based on pre-pregnancy
27 weight) and 8.1mg/day (based on end-pregnancy weight). As Circadin® is only
28 available in 2mg tablets, the chosen dose for this study is 8mg per day.
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39 This dose of melatonin is lower than the used doses in several clinical studies in
40 neonates. These trials evaluated melatonin as an antioxidant treatment in
41 newborns with birth asphyxia and other conditions associated with increased
42 oxidative stress. Melatonin was administered orally in doses up to 80mg/day in
43 these trials without any toxicity(37–41). In clinical trials using melatonin to
44 improve assisted reproductive technology, melatonin was administered orally in
45 doses of 3mg/day before and during pregnancy(30,32,42). Melatonin treatment,
46 started prior to conception and continued until birth, did not adversely affect
47 pregnancy outcome and fetal or neonatal health (V. Unfer. personal
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3 communication, 2012). The dose in this trial is higher than previously used doses
4 in human pregnancy but still falls well below the maternal and fetal NOAEL (HED
5 16 and 32mg/kg respectively) and maternal LOAEL (HED 32mg/kg).
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11 Melatonin will be administered orally as 4mg given twice per day. This dosage
12 regimen is chosen because the terminal half-life of Circadin® is 3.5 to 4 hours. In
13 fetal growth restriction, increased levels of oxidative stress likely occur due to
14 continuous placental generation of reactive oxygen species. By administering
15 melatonin twice daily, we aim to provide a sustained increase in maternal and
16 fetal serum. To minimize daytime sleepiness and severe disturbances of
17 circadian rhythms, melatonin will be given early in the morning and at night.
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30 **Sample collection:** Prior to starting melatonin, a maternal blood sample will be
31 taken and ultrasound and Doppler measurements will be obtained. This will be
32 repeated once per week until birth. Immediately prior to birth, a maternal blood
33 sample will be obtained. At the time of birth, samples of the amniotic fluid,
34 umbilical cord blood and placenta will be collected. Clinical details of pregnancy,
35 including medication and (multi) vitamin use, delivery and neonatal outcomes
36 will be recorded in pre-specified case record forms.
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48 All blood samples will be collected in serum tubes and blood RNA tubes, allowing
49 for measurement of markers of oxidative stress and gene expression studies
50 respectively. Serum tubes will be centrifuged (4400g, 15 minutes). The serum
51 will be collected and transferred into collection tubes. The collection tubes will
52 be stored in a -80°C until further processing.
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5 Amniotic fluid will be collected during caesarean section deliveries. The
6 myometrium will be bisected sharply and distinctly from the amnion and
7 chorion. The amniotic fluid will be aspirated using a sterile syringe and 18 gauge
8 drawing-up needle. Samples visibly contaminated with blood will be discarded
9 off. Within 30 minutes of the sample collection, specimens will be transferred to
10 a laboratory. The samples will be centrifuged (1000g, 15 minutes) and the
11 supernatant will be collected, transferred into sterile collection tubes and snap
12 frozen. Specimens will be stored at -80°C until further analysis.

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26 Placentas will be obtained at time of delivery and placed in a sterile tray. The
27 area at the midpoint of the longest distance between the cord insertion and the
28 edge of the placental disc will be sampled. Six full thickness placental samples
29 will be cut out using sterile scissors. The placental tissue samples will be
30 removed from the membranes using sterile forceps and scissors. The placental
31 tissue samples will then be washed in a sterile container using ice cold, sterile
32 phosphate-buffered saline. Two tissue samples will be fixed in 4%
33 paraformaldehyde and embedded in paraffin for histological analysis. Two tissue
34 samples will be placed in sterile tubes containing RNA-later for 24 hours at 4°C.
35 After immersion in RNA-later, these two samples will be transferred into two
36 sterile cryovials, snap frozen in liquid nitrogen and stored at -80°C until further
37 processing for gene expression studies. The remaining tissue samples will be
38 placed into sterile cryovials and snap frozen in liquid nitrogen. Samples will be
39 stored in a -80°C freezer until further processing to measure levels of oxidative
40 stress and melatonin.
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8 **Outcome measures:** The primary outcome will be the level of oxidative stress
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10 in the placenta and in the maternal (before and during treatment and at
11 delivery) and fetal (umbilical artery and vein at delivery) circulations, as
12 reflected by the measurement of malondialdehyde, 8-isoprostane, total
13 antioxidant capacity and superoxide dismutase. Levels of melatonin will be
14 measured in maternal and fetal blood and in the placenta. Gene expression of
15 pro- and antioxidant enzymes, melatonin synthesizing enzymes, melatonin
16 receptors and clock genes will be measured in the placenta and in the maternal
17 and fetal blood.
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30 The secondary outcome measures will include clinical parameters about the
31 pregnancy and its duration, fetal and neonatal wellbeing, mortality and
32 morbidity (Tables 1-4).
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Table 1: Ultrasound and Doppler measurements.

Doppler and ultrasound measurements: at recruitment and then weekly until delivery.

Doppler velocimetry: umbilical artery, middle cerebral artery, ductus venosus, and maternal uterine arteries

Biometry (fortnightly)

Amniotic fluid index

Fetal behaviour: heart rate, tone, breathing, gross body movements

Table 2: Maternal morbidity.

Markers of maternal morbidity: at recruitment and at least weekly until delivery.

Blood pressure

Complete blood count

Liver function tests (AST, ALT, GTT, bilirubin)

Renal function tests (urea, creatinine, uric acid)

Table 3: Pregnancy end-points**Pregnancy outcomes:**

Gestational age at birth

Diagnosis to delivery interval

Abnormal cardiotocogram

Indication for delivery (if not spontaneous)

Mode of birth

Labour analgesia/anaesthesia

Duration of labour stages

Duration of membrane rupture to birth

Used medication such as antihypertensives, magnesium sulphate and corticosteroids.

Use of (multi) vitamins and dietary supplements

Table 4: Neonatal outcomes.**Neonatal outcomes:**

Sex

Apgar scores (1, 5, 10 minutes)

Birth weight

Cord artery S100B

Head circumference at birth

Composite neonatal outcome: admission to neonatal intensive care unit,

duration of admission, need for and duration of respiratory support,

intraventricular haemorrhage, necrotizing enterocolitis, abnormal neurology,

mortality prior to discharge

Follow up of women and infants: Participants will be followed weekly for the duration of the trial. The routine clinical care of participants, including ultrasound scans, will not be affected by participation in the study. During the weekly follow-up, fetal and maternal wellbeing will be assessed and women will discuss any issues that they have experienced. Medication will be supplied weekly to meet the requirements for the following week. Women will be asked to keep a medication and symptom diary (drowsiness, sleepiness, general physical well-being, time of melatonin intake), which will be reviewed weekly by the principal investigator. Melatonin treatment will cease as soon as birth has occurred. Postnatal clinical care of the women will be the responsibility of the attending obstetric team. The principal investigator will assess the clinical condition of participants daily during the first three days post-partum, to ensure that no problems become apparent in response to withdrawal from treatment. Clinical data will be obtained from neonates who require admission to the neonatal intensive care unit. Long-term follow-up using neurodevelopmental examination and questionnaires and MRI scans, are desirable but will depend on funding.

Sample size and statistical analysis of data: This study is a proof-of-principle phase I trial. As such, there are no current data available to undertake a formal power calculation to determine the sample size. If successful, this exploratory study will provide the measures needed to perform the power calculations

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3 required for the planned phase II randomized-controlled trial. This trial will
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5 provide data for the establishment of the use of maternal melatonin treatment in
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7 fetal growth restriction.
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10 The results obtained in this trial will be compared to historic cohorts of
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12 untreated fetal growth restriction and healthy pregnancies, which we have been
13
14 collecting since 2011, using the same protocol.
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16 Biomarkers of oxidative stress in the maternal blood samples will be analysed
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18 using a repeated measures analysis of variance, including the baseline value as a
19
20 covariate. It is assumed that the effect of treatment with melatonin on levels of
21
22 oxidative stress will not depend on duration of treatment. However, secondary
23
24 analysis will be performed to determine the effect of treatment duration on
25
26 biomarkers of oxidative stress. If the treatment duration significantly impacts on
27
28 the effect of melatonin on the oxidative stress status, this will be further
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30 investigated using the least-square measure. Data will be plotted as mean scores
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32 over time.
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39 Biomarkers of oxidative stress in the maternal blood samples will be analysed
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41 using a repeated measures analysis of variance, including the baseline value as a
42
43 covariate. Levels of oxidative stress in the maternal blood sample obtained
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45 immediately prior to birth will be compared to samples derived from the historic
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47 cohorts using a one-way ANOVA. Levels of oxidative stress and brain injury will
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49 be compared to data obtained from the previously described historic cohorts.
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51 Clinical data regarding maternal wellbeing, pregnancy outcome and neonatal
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53 outcome will be compared to the same cohorts. These data will be analysed using
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55 a one-way ANOVA.
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5 A p-value ≤ 0.05 is considered to be statistically significant.
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10 **Adverse events and safety monitoring:** During the treatment period, clinical
11 care will remain unchanged and will be the responsibility of uninvolved medical
12 staff. The principal investigator will monitor all participants weekly, take a brief
13 medical history, measure blood pressure and interpret ultrasound scan results
14 (obtained by uninvolved staff) to ensure melatonin treatment can be safely
15 continued. A weekly blood sample will be obtained to perform a full blood
16 examination, assess liver and renal function and assess C-reactive protein. For
17 the duration of the trial, the principal investigator will be contactable by
18 telephone at all times.
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30 Any adverse medical events reported by the patient will be recorded in the
31 patients' medical records at each visit. Serious adverse events occurring after
32 initiation of the trial will be reported to the Monash Health Human Research
33 Ethics committee, Therapeutics Committee and Therapeutic Goods
34 Administration of Australia's Office of Scientific Evaluation within 72 hours.
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44 **Trial discontinuation or modification**

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46 The participants can end participation at their own request at any time.
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48 Participants will be withdrawn from the trial if they or their fetus suffer from an
49 unexpected serious adverse event. Worsening of fetoplacental Doppler
50 velocimetry or fetal growth are within the natural history of fetal growth
51 restriction and are not part of the discontinuation criteria.
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3 **Ethics and dissemination:** This study has been approved by the Monash Health
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5 Human Research Ethics Committee B (HREC 12133B). Data will be presented at
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7 relevant conferences and published in peer-reviewed journals.
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10 11 12 **DISCUSSION**

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17 The use of melatonin as an antenatal antioxidant therapy in fetal growth
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19 restriction has been proposed as a promising and safe option to protect the fetal
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21 brain from injury. This trial is essential to confirm the antioxidant capacity and
22
23 safety of melatonin treatment. If this study confirms that maternal melatonin can
24
25 decrease maternal, fetal and/or placental levels of oxidative stress, we will
26
27 proceed to a phase II randomized-controlled trial to assess the effect of maternal
28
29 melatonin treatment on brain injury and neurodevelopment. In addition,
30
31 melatonin treatment could also potentially be used as an antenatal
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33 neuroprotectant in other compromised pregnancies or in threatened preterm
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35 birth to protect the mother and/or fetus. Finally, if melatonin treatment indeed
36
37 strengthens antioxidant defenses during pregnancy, it might be a suitable new
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39 strategy in the management of pre-eclampsia. A phase I study to study the
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41 clinical and biochemical benefit of melatonin treatment in preeclampsia has been
42
43 initiated.
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51 52 **AUTHORS' CONTRIBUTIONS**

53
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55 NOA: research, contribution of original material, editing and approval of final
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57 manuscript. SLM, GJ, EMW: editing and approval of final manuscript.
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