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Reproductive Sciences

Intrauterine *Candida albicans* infection causes systemic fetal candidiasis with progressive cardiac dysfunction in a sheep model of early pregnancy

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Manuscripts

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3 1 Intrauterine *Candida albicans* infection causes systemic fetal candidiasis with progressive
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7 3 SHORT TITLE: Fetal candidiasis in a sheep model of early pregnancy
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5 2 candidiasis.
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3 1 ABSTRACT

4 2 INTRODUCTION: Several recent studies have identified a potential role for intrauterine
5 3 *Candida albicans* in adverse pregnancy outcomes, including preterm birth. There is, however,
6 4 a limited understanding of the impact of intrauterine candida infection on fetal well-being in
7 5 early pregnancy. Using a sheep model of early pregnancy, the aims of this study were to
8 6 determine: **i)** the ability of experimentally-induced intrauterine *C. albicans* to infect the fetus;
9 7 **ii)** if *C. albicans* exposure in early pregnancy is associated with alterations in fetal cardiac
10 8 function, as measured by spectral Tissue Doppler Imaging analysis of fetal cardiac function.
11 9

12 10 METHODS: Merino ewes carrying singleton pregnancies at 89 days gestation (term is ~150
13 11 days) received *C. albicans* (n=8) via ultrasound-guided intraamniotic injection. Saline-
14 12 exposed fetuses served as controls (n=6). Spectral Tissue Doppler Imaging echocardiography
15 13 and amniotic fluid collection was performed at baseline, 24 and 72 hours intrauterine *C.*
16 14 *albicans* injection. Fetal tissues were collected at post-mortem for analysis of infection and
17 15 inflammation.
18 16

19 17 RESULTS: Relative to saline control, intrauterine *C. albicans* infection resulted in
20 18 pronounced increases in amniotic fluid TNF- α ; (p<0.05) and cytokine / chemokine mRNA
21 19 (IL-1 β , IL-6, TNF- α and MCP-1; p<0.05) in the fetal myocardium, lung, skin and liver at 72
22 20 and 96 h post-infection. Spectral tissue Doppler imaging showed diastolic dysfunction at 24 h
23 21 and severe biventricular diastolic dysfunction 72 h post-infection.
24 22

25 23 CONCLUSIONS: Intrauterine *C. albicans* infection in a sheep model of early pregnancy
26 24 causes systemic fetal candidiasis, which is associated with a robust systemic inflammatory
27 25 response and progressive cardiac dysfunction detectable by spectral Tissue Doppler Imaging.
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1 INTRODUCTION

2 With advances in perinatal care, infants born early preterm (<32 weeks' gestation) frequently
3 survive to reach adulthood, and up to 1 in 10 young adults will now have been born preterm
4 (<37 weeks' gestation). Preterm birth has a number of different aetiologies, but intrauterine
5 inflammation is a common clinical finding. Infection is frequent, occurring in up to 60% of
6 early preterm births (<32 weeks gestation)¹. Other causes of preterm birth (e.g. intrauterine
7 distension and hemorrhage) are also mediated, at least in part, through an inflammatory
8 response. A significant body of literature serves to demonstrate a strong association between
9 intrauterine infection caused by a number of microorganisms including *Ureaplasma*,
10 *Fusobacterium*, *Prevotella*, *Bacteroides*, and *Streptococcus* spp. and preterm birth.^{2,3} There is
11 also an emerging body of evidence to suggest that intrauterine infection caused by *Candida*
12 *albicans*, is associated with preterm birth and adverse perinatal outcomes. *C. albicans* has
13 been detected in amniotic fluid samples from cases of spontaneous preterm birth, and
14 intrauterine *C. albicans* is associated with fetal death and sub-optimal neuro-development.^{4,5}
15 Interestingly, the fetal responses to *C.albicans* in fetal sheep in mid-late pregnancy⁶ (minimal
16 chorioamnionitis in association with profound fetal inflammatory responses and candidiasis)
17 are distinct from those driven by *Escherichia coli* lipopolysaccharides (LPS)^{7,8} or *Ureaplasma*
18 *parvum* serovar 3.⁹ These data suggest that the intrauterine response to infection, and thus the
19 potential short- and long-term effects on infant well-being may have some degree of organism
20 specificity.

21
22 Being born early conveys a potential for detrimental effects on a number of organ systems,
23 including the central nervous system, lungs and cardiovascular system.¹⁰ Children and adults
24 born preterm have an increased risk of death compared with those who were born at full term,
25 with a 2-fold risk of mortality in young adults born extremely preterm (<28 weeks
26 gestation).¹¹ Deaths from cardiovascular disease contribute to this mortality, with the risk
27 inversely proportional to gestational age at birth. Furthermore, prematurity has been linked to
28 cardiovascular risk factors such as hypertension, abnormal glucose regulation and

1
2
3 1 cerebrovascular disease.^{12,13,14} The mechanisms underlying this increased cardiovascular risk
4
5 2 are not clear, but recently published studies using cardiac magnetic resonance imaging have
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7 3 revealed profound differences in cardiac function associated with preterm birth.^{15,16} These
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9 4 findings in adults are consistent with observations that left ventricular mass increases more in
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11 5 the first month of life in preterm infants than those born at term.¹⁷ In sheep, abnormal
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13 6 maturation and accelerated postnatal cardiomyocyte hypertrophy has been related to preterm
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15 7 birth.¹⁸

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19 9 A recent study found that cardiomyocyte growth in the fetal heart was markedly affected by
20
21 10 *in utero* inflammation in a sheep model of pregnancy.¹⁹ This study also showed that
22
23 11 intrauterine inflammation resulted in compromised cardiac performance, vulnerability to
24
25 12 damage and arrhythmias in *ex vivo* experiments. Other studies have shown cardiac
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27 13 dysfunction in association with chorioamnionitis in human pregnancy,²⁰ and in response to
28
29 14 intra-amniotic LPS in mice.²¹ Together, inflammation and preterm birth can result in cardiac
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31 15 dysfunction and vulnerability to ischemic injury that may persist through adult life.

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35 17 With continued high preterm birth rates in developed countries (7-13%), long-term
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37 18 cardiovascular health effects will have an increasing clinical and public health impact.
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39 19 Understanding the effects of intrauterine infection on the fetus is crucial to developing
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41 20 strategies to mitigate the impact on fetal well-being, as well as to allow for the development
42
43 21 of novel diagnostic and preventative approaches. We have previously demonstrated that
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45 22 intrauterine exposure to *E.coli* LPS, *U. parvum* and *C. albicans*, generates distinct
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47 23 inflammatory responses in fetal sheep at early and late gestations.^{6,17} To date, however, our
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49 24 ability to perform direct evaluation of the effects of intrauterine infection and inflammation
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51 25 on fetal cardiac function, and thus correlate real-time functional changes in fetal physiology
52
53 26 with changes in infection status and intrauterine inflammation, have been restricted by
54
55 27 limitations in the ability of imaging technologies to resolve functional changes in the small,
56
57 28 rapidly pulsing fetal heart. Furthermore, although the fetal immunological capacity changes

1 markedly across gestation²², there have been very few studies in clinically relevant, early
2 gestation models of intrauterine infection and inflammation.²³ With recent methodological
3 advances spectral Tissue Doppler Imaging (TDI) of the fetal heart is now both feasible and
4 reliable in human pregnancy.²⁴ In the present report, we used a well-validated sheep-model
5 of early pregnancy and aimed to determine: **i)** the ability of experimentally-induced
6 intrauterine *C. albicans* to infect the fetus; **ii)** the fetal capacity to mount an inflammatory
7 response to *C. albicans* exposure; and **iii)** if *C. albicans* exposure in early pregnancy is
8 associated with alterations in fetal cardiac function, as measured by spectral tissue Doppler
9 imaging of fetal cardiac function.

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2
3 1 METHODS AND MATERIALS

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5 2 *Animal model*

6
7 3 Animal studies were approved by The University of Western Australia's Animal Ethics
8
9 4 Committee (RA/3/100/1289). All animal work was performed in Perth, Western Australia and
10
11 5 complied with the National Health and Medical Research Council Australian Code for the
12
13 6 care and use of animals for scientific purposes.²⁵ Sheep were housed in a custom-built sheep
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15 7 research facility that allows sheep to remain in established flock structures, in well-contained
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17 8 paddocks with access to shelter.
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21 10 All intrauterine interventions were performed under ultrasound guidance as previously
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23 11 published⁸. Briefly, the wool covering the abdomen was clipped close to the skin and then
24
25 12 thoroughly cleaned with an alcohol / chlorhexidine solution (non-irritant) and then coated in
26
27 13 iodine. The fetus and amniotic cavity were visualised by ultrasound and amniocentesis
28
29 14 performed under direct ultrasound guidance. Procedures took less than two minutes on
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31 15 average.
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35 17 For the *C. albicans* group, date-mated merino ewes each carrying a single fetus were sampled
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37 18 for amniotic fluid and received a single intra-amniotic injection of 10^7 colony-forming units
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39 19 (CFU) of *C. albicans* in 2 mL sterile saline under ultrasound guidance (n=8). For the saline
40
41 20 control group, date-mated merino ewes each carrying a single fetus were sampled for
42
43 21 amniotic fluid and received a single intra-amniotic injection of 2 mL sterile saline under
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45 22 ultrasound guidance (n=6).
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47 23

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49 24 Amniotic fluid placement was confirmed by the presence of chloride in samples prior to
50
51 25 injection of either *C. albicans* or sterile saline, using a Rapidlab 1265 blood gas analyser
52
53 26 (Siemens, Australia). For *C. albicans*-exposed animals, ultrasound assessment of fetal cardiac
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55 27 function (see below) and amniotic fluid collection was performed prior to, 24 and 72 h after
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57 28 intraamniotic injection of 10^7 CFU *C. albicans*. Animals in the *C. albicans* group were
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1 euthanized by intravenous injection of pentobarbitone (160 mg/kg) and the fetus surgically
2 delivered either 72 (n=4) or 96 (n=4) h after intraamniotic infection was established. Animals
3 in the saline control group were euthanized by intravenous injection of pentobarbitone (160
4 mg/kg) and the fetus surgically delivered. Gestational age at delivery was 94-95 d for both *C.*
5 *albicans* and saline control groups.

6
7 Amniotic fluid, cerebrospinal fluid and tissues for mRNA expression analyses were collected
8 at necropsy and snap frozen in liquid nitrogen. Tissue (fetal myocardium, skin,
9 chorioamnion, liver and lung) from 72 and 96 h *C. albicans* animals were pooled for cytokine
10 mRNA and histological analyses. Previous experiments in mid-late pregnancy demonstrated
11 *C. albicans* infection caused significant fetal compromise; accordingly, the length of infection
12 was staggered as the investigators were uncertain *a priori* as to the length of time a
13 comparatively immature fetus might survive exposure to intrauterine *C. albicans*.

14
15 Fetal blood (2 mL) was collected from the umbilical cord and inoculated into a BACTEC™
16 Peds Plus culture vial (Becton Dickinson, Franklin Lakes, NJ) for microbiological culture.
17 Fetal lung (right upper lobe) for histological analysis was perfusion fixed (30 cmH₂O) in 10%
18 neutral buffered formalin for 24 h before paraffin embedding. Fetal skin tissues for
19 histological analysis were cryo-preserved in optimum cutting temperature (OCT) compound
20 (Sakura Finetek, the Netherlands). 5-6 animals were analysed from each group.

21 *Candida albicans* culture for intra-amniotic injection

22
23 A single clinical isolate of *C. albicans* was cultured on Difco™ Sabouraud Dextrose Agar
24 (Becton Dickinson) at 37°C for 48 h. Single colonies were inoculated into sterile 1 x
25 phosphate-buffered saline (PBS) (Sigma-Aldrich, St Louis, MO). *C. albicans* colony
26 morphology was confirmed by growth on Brilliance Candida Agar (Oxoid, Adelaide,
27 Australia). Inoculums were quantified using a plate dilution series as per standard
28 microbiological methods and recorded as CFU/mL. Quantified inoculums (10⁷ CFU in 2 mL

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3 1 1 x PBS) were stored at -80°C prior to use.
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7 3 *Nucleic Acid Extraction*
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9 4 Total RNA was extracted from liquid nitrogen-homogenized fetal tissues using TRIzol as
10 previously reported.²⁶ Extracted RNA was treated with Turbo-DNase (Life Technologies,
11 Carlsbad, CA) to remove any residual DNA and subsequently quantified on a Qubit 2.0
12 fluorometer (Life Technologies) using a broad-range RNA quantitation kit (Life
13 Technologies). RNA yields from fetal tissues were normalized to 100 ng/ μL using nuclease-
14 free water (Life Technologies).
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25 12 *Candida albicans* detection
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27 13 The presence of viable *C. albicans* in amniotic fluid samples was determined using a
28 Sabaraud-Dextrose agar plate dilution series as described above. Three single colonies from
29 positive plates were subsequently inoculated onto Candida Brilliance agar (Oxoid) for
30 confirmation of isolate identification. The presence of viable *C. albicans* in fetal blood
31 samples collected at post-mortem was determined by incubation of 2 mL of fetal blood in
32 BACTECTM Peds Plus culture vials at 37°C for up to 96 h. At 24 h intervals, a 100 μL sample
33 of blood culture was aseptically drawn from the culture vials and inoculated onto 5% sheep
34 blood agar and Sabaraud-Dextrose agar. Positive cultures were confirmed as *C. albicans* by
35 inoculation of three single colonies onto Candida Brilliance agar (Oxoid).
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48 23 *Histology*
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50 24 Fetal skin was dissected, embedded in OCT and immediately frozen on dry ice before thin (9
51 μm) cryo-sectioning. Immunofluorescence staining for the identification of hyphal structures
52 in the epidermis was performed with rabbit anti-*C. albicans* (Meridian Life Sciences,
53 Memphis, TN) and Alexa Fluor 488 anti-rabbit IgG antibodies (Life Technologies) diluted in
54 1x PBS containing 0.1% Tween 20 (Life Technologies) and 5% fetal calf serum to 1:600 and
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1 1:10,000, respectively. Sections were blocked for 2 h in 1 x PBS containing 5% fetal calf
2 serum at 4°C in a humidified chamber. Sections were incubated with primary antibody
3 overnight at 4°C in a humidified chamber and secondary antibody for 2 h at room temperature
4 in a darkened humidified chamber with washing between steps as appropriate. Washed
5 sections were coated in Vectashield with DAPI (Vector Laboratories, Burlingame, CA),
6 cover-slipped and sealed. Imaging was performed on a Zeiss LSM510 microscope (Carl Zeiss
7 Microscopy, Oberkochen, Germany) using an EC Plan-Neofluor 20 x objective (Carl Zeiss
8 Microscopy). Skin and chorioamnion sections were stained with haematoxylin and eosin as
9 previously described.²⁷

12 *Enzyme-Linked Immunosorbent Assays (ELISA)*

13 Quantification of TNF- α protein concentrations in amniotic fluid and arterial cord blood
14 plasma was performed using Vetset ELISA kits (VS0285B-00. Kingfisher Biotech Inc., St.
15 Paul, MN). Standards (run in triplicate) and samples (run in duplicate) were incubated for 16
16 h at 4°C before the assays were performed in accordance with manufacturer's instructions.
17 100 μ L TMB substrate (T8865; Sigma Aldrich, St. Louis, MO) was added to each well and
18 the plate incubated in the dark at room temperature for 15 min. The chromogenic reaction was
19 halted by the addition of 100 μ L 2% sulfuric acid solution in 1 x PBS (Sigma) to each well.
20 The plate was then immediately read at $\lambda = 450$ nm.

22 *Relative quantification of mRNA expression*

23 Ovine-specific PCR primers and hydrolysis probes for interleukin (IL)-1 β , IL-6, IL-8, IL-17,
24 TNF- α , MCP-1, SMAP-29, SBD-1, and SBD-2 (Life Technologies) were used to perform
25 quantitative PCR reactions on RNA from fetal myocardium, skin, chorioamnion, liver and
26 lung tissue. Reactions were performed using an EXPRESS One-Step SuperScript qRT-PCR
27 kit (Life Technologies) with 25 ng template RNA in a total volume of 20 μ L as follows: 1 x
28 15 min reverse transcription at 50 °C and an initial denaturation at 95 °C for 20 s, followed by

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3 1 40 cycles of 95 °C for 3 s and 60 °C for 30 s. All reactions were performed in 96-well plates
4
5 2 on a ViiA7 real-time PCR thermocycler (Life Technologies). Cq values were normalized to
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7 3 18S rRNA and expressed as fold changes relative to pooled control values. Statistical
8
9 4 analyses were performed on dCq values.
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11 5 12 13 6 *Ultrasound imaging and analysis*

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15 7 Ultrasound assessment was performed by one operator with ewes held by an experienced
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17 8 handler in a dorsal recumbent position similar to that used during shearing. Measurements
18
19 9 were taken using a Philips CX50 system with an S5-1 phased array probe (both Philips
20
21 10 Healthcare, the Netherlands) with TDI program set to pulsed-wave Doppler mode. The
22
23 11 ultrasound beam was focused parallel to the longitudinal myocardial wall and ventricular
24
25 12 myocardial velocities were measured from apical or basal four-chamber views. Doppler
26
27 13 sample volume was placed at the level of the lateral mitral annulus, at the basal level of the
28
29 14 inter-ventricular septum, and the level of the lateral tricuspid annulus. Data were stored in
30
31 15 cineloop format, and analysed at St George's University, London, by an investigator blinded
32
33 16 to treatment.
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36 17
37 18 The TDI data were used to derive cardiac indices of myocardial motion in systole and diastole
38
39 19 using offline analyses (QLAB 10; Philips Healthcare). Annular peak velocity profiles and
40
41 20 their peak values were obtained in systole (S'), early diastole (E'), and atrial contraction (A').
42
43 21 E'/A' indices were calculated. Criteria for image acquisition were applied as follows:
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45 22 maximum sweep speed (15 cm/s); high-pass wall motion filter (WMF) setting; reduced gain
46
47 23 in order to observe echogenic valve clicks and hypoechogenic LV inflow and outflow
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49 24 waveforms. E/A spectral waveforms were visualized as positive in the apical chamber view,
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51 25 or as negative in the basal chamber view.
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53 26 54 55 27 *Statistics*

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58 28 Spectral TDI data descriptive statistics are presented as mean and standard deviation. The
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3 1 Wilcoxon test was performed to test normality. Normally distributed data were analysed with
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5 2 repeated measure ANOVA to test the null hypothesis that there was no difference in the
6
7 3 means between 0, 24 and 72 h values. Cytokine mRNA and protein concentration data were
8
9 4 assessed for normality with Shapiro-Wilk tests. Parametric data were tested for group mean
10
11 5 differences using one-way ANOVA. Data were analysed using IBM SPSS for Windows,
12
13 6 Version 20.0 (IBM Corporation, Armonk, NY).
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15 7

17 8 RESULTS

19 9 *Necroscopy data*

10 One animal from the *C. albicans* group (96 h infection) was found dead at delivery and
11 excluded from analyses. All other animals were delivered alive. There was no significant
12 difference in delivery weight, arterial cord blood pH, pO₂, or pCO₂ (data not shown).
13 Intrauterine *C. albicans* infection was confirmed by culture, and hyphae were directly
14 visualized in fetal skin in haematoxylin and eosin stained sections (Figure 1, panel ii, B). No
15 hyphal structures were identified in skin from control animals (Figure 1, panel ii, A).
16 Identification of hyphae as *C. albicans* was by immunohistochemistry in treated animals
17 (Figure 1, panel i, A-C). At least 6/7 (one sample was not collected) animals from the *C.*
18 *albicans* group had positive blood cultures for *C. albicans*. 6/7 animals from the *C. albicans*
19 group had positive cerebrospinal fluid *C. albicans* cultures at delivery. The average *C.*
20 *albicans* amniotic fluid culture count in animals delivered at 96 h was 2.2×10^6 CFU/mL. No
21 saline (n=4; two samples were not collected) or *C. albicans* (n=8) animals had positive
22 baseline amniotic fluid *C. albicans* cultures.
23

24 24 *Cytokine expression*

25 Concentrations of TNF- α (Figure 2) were increased in the amniotic fluid in *C. albicans*-
26 exposed animals at 72 h compared to control samples collected at delivery. Relative to saline
27 control, increased TNF- α protein concentration was also identified in cord blood plasma
28 samples from *C. albicans*-infected animals collected at delivery (0.1 ± 0.09 vs. 0.02 ± 0.03)
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3 1 ng/mL, $p < 0.05$). Fetal lung (right upper lobe) from *C. albicans*-infected animals showed
4
5 2 profound airspace consolidation (Figure 1, panel iii, A), whereas saline treated controls had
6
7 3 no evidence of consolidation (Figure 1, panel iii, B).

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9 4
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11 5 Large relative increases in cytokine / chemokine transcript expression were detected in the
12
13 6 fetal lung, and liver (Figure 3 A and B, respectively; $p < 0.05$). mRNA levels for inflammatory
14
15 7 genes *IL-1 β* , *IL-6*, *TNF- α* and *MCP-1* were increased in the myocardium of *C. albicans*
16
17 8 treated animals when compared controls receiving normal saline (Figure 3, C). Limited
18
19 9 changes in cytokine mRNA expression were detected in the fetal skin, with only transcripts
20
21 10 for *IL-1 β* (mean 2.8-fold increase) and *MCP-1* (mean 4.3-fold increase) significantly ($p < 0.05$)
22
23 11 elevated relative to saline control. There was no statistically significant change in the mRNA
24
25 12 transcripts for *IL-1 β* , *IL-6*, *IL-8*, *TNF- α* or *MCP-1* in chorioamnion tissues (data not shown).

26 27 13 28 29 14 *Tissue Doppler Imaging*

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31 15 Results of cardiac TDI examination of the inter-ventricular annulus at 0 (pre-infection), 24
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33 16 and 72 h following inoculation of *C. albicans* are summarized in Table 1 and Figure 4. After
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35 17 24 h, there was evidence of diastolic dysfunction with a significant decrease in
36
37 18 interventricular septum peak annular early diastolic velocity (IV E'; 26% decrease; $p = 0.018$)
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39 19 and early diastolic/late diastolic ratio (IV E'/A'; 31% decrease; $p = 0.016$). At 72 h there was
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41 20 evidence of increased biventricular systolic function, with a significant increase in mitral
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43 21 valve peak systolic velocity (MV S'; 32% increase; $p = 0.022$) and interventricular septum
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45 22 peak systolic velocity (IV S'; 25% increase; $p = 0.050$). These changes were associated with
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47 23 the finding of diastolic dysfunction in both MV (abnormal relaxation) and TV (pseudo-
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49 24 normal) indices (IV A'; 34% increase; $p = 0.023$ and TV E' 21% increase; $p = 0.019$).

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3 1 COMMENT
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5 2 The primary findings of this study are: **i)** that intrauterine *C. albicans* infection in early
6 pregnancy rapidly involves the fetus, causing systemic infection (blood, tissues and
7 cerebrospinal fluid) characterized by hyphal invasion of the epidermis and dermis, marked
8 alveolar consolidation and profound inflammatory changes in fetal, but not chorioamnion
9 tissues; **ii)** that the magnitude of changes in fetal inflammatory mediator expression was
10 greatest in the lung and liver, but comparatively mild in the heart and **iii)** that intrauterine *C.*
11 *albicans* infection is associated with fetal cardiac dysfunction that is detectable by spectral
12 TDI.
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23 11 These results should be considered with reference to the experimental design. A strength of
24 our study is the use of a large animal model of early gestation pregnancy, which is relevant to
25 clinical practice as infection is the most common cause of early spontaneous preterm labour.
26 Limitations of this study relate to the two time points (72 h and 96 h) selected for fetal
27 delivery post-infection. This experimental design did not impact our ability to perform serial
28 TDI assessment of fetal cardiac function; however it did limit our ability to determine time-
29 dependent (i.e. 72 h vs. 96 h) changes in fetal infection and inflammation.
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39 19 These novel findings are of interest as they provide evidence from a large animal model of
40 pregnancy to demonstrate the ability of *C. albicans* to rapidly and comprehensively infect the
41 fetus, causing significant pathological changes in the fetal lung. No significant changes in
42 cytokine or chemokine transcript expression were detected in the chorioamnion, which is in
43 keeping with our earlier work with *C. albicans* in mid-gestation sheep pregnancy²⁸.
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51 25 Histological analysis of fetal skin revealed invasion of hyphal structures deep into the dermis.
52 Surprisingly, the *C. albicans*-infected fetal skin exhibited comparatively little obvious
53 changes in terms of immunocyte infiltration in H&E sections and only minor changes in
54 cytokine and chemokine expression. These observations are in contrast to fetal ovine skin
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3 1 responses to *C. albicans* in mid-gestation pregnancy⁶, and also to stimulation with *E.coli* LPS
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5 2 in instrumented fetal sheep at a similar gestational age.²³ Although this difference may be due
6
7 3 to differences in the length of agonist exposure (at least in regards to LPS-stimulation), it is
8
9 4 also possible that the fetal skin is partly deficient in its ability to mount a pro-inflammatory
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11 5 response to some microorganisms, such as *C. albicans*, in early pregnancy. Such a conclusion
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13 6 is in keeping with the observation that fetal immunological capacity develops with increasing
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15 7 gestational age.²²
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19 9 Of particular interest, is the finding that it is feasible to perform real-time visualization of the
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21 10 effects of systemic infection and inflammation on the developing heart with non-invasive TDI
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23 11 ultrasound analysis. Intrauterine *C. albicans* infection in the fetus is rare in clinical practice,
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25 12 although there are data to suggest that its impact may be underreported.⁶ Nevertheless, these
26
27 13 experiments show that intrauterine *C. albicans* causes rapid and systemic fetal infection in
28
29 14 association with fetal cardiac dysfunction that is detectable by TDI. It is possible that the
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31 15 cardiac findings were due to overwhelming fetal sepsis. With diastolic dysfunction identified
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33 16 at 24 h, future studies would need to include groups of fetuses delivered for necroscopy and
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35 17 infection analysis at a selection of earlier time-points in order to determine whether the
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37 18 pathological changes in cardiac function were in response to systemic fetal inflammation or
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39 19 sepsis.
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43 21 Our results demonstrate that the study of the mechanisms leading to the persistent increased
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45 22 cardiovascular risk seen in children and adults born preterm is feasible. More importantly,
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47 23 development of non-invasive diagnostic tests for fetal infection and studies of therapies to
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49 24 improve cardiac function may be possible. Translating this work into clinical practice is a
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51 25 goal of future studies.
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6
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8
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10
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For Peer Review

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3 1 References
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- 5 2 1. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of
6 3 preterm birth. *Lancet*. 2008;371(9606):75-84.
- 7 4 2. DiGiulio DB. Diversity of microbes in amniotic fluid. *Seminars in fetal & neonatal
8 5 medicine*. 2012;17(1):2-11.
- 9 6 3. Jones HE, Harris KA, Azizia M, et al. Differing prevalence and diversity of bacterial
10 7 species in fetal membranes from very preterm and term labor. *PloS one*.
11 8 2009;4(12):e8205.
- 12 9 4. Benjamin DK, Jr., Stoll BJ, Fanaroff AA, et al. Neonatal candidiasis among
13 10 extremely low birth weight infants: risk factors, mortality rates, and
14 11 neurodevelopmental outcomes at 18 to 22 months. *Pediatrics*. 2006;117(1):84-92.
- 15 12 5. Darmstadt GL, Dinulos JG, Miller Z. Congenital cutaneous candidiasis: clinical
16 13 presentation, pathogenesis, and management guidelines. *Pediatrics*. 2000;105(2):438-
17 14 444.
- 18 15 6. Payne MS, Kemp MW, Kallapur SG, et al. Intrauterine *Candida albicans* infection
19 16 elicits severe inflammation in fetal sheep. *Pediatric Research*. 2014;75(6):716-722.
- 20 17 7. Snyder CC, Wolfe KB, Gisslen T, et al. Modulation of lipopolysaccharide-induced
21 18 chorioamnionitis by *Ureaplasma parvum* in sheep. *American Journal of Obstetrics
22 19 and Gynecology*. 2013;208(5):399.e391-399.e398.
- 23 20 8. Zhang L, Saito M, Jobe A, et al. Intra-amniotic administration of *e coli*
24 21 lipopolysaccharides causes sustained inflammation of the fetal skin in sheep.
25 22 *Reproductive Sciences*. 2012;19(11):1181-1189.
- 26 23 9. Kemp MW, Miura Y, Payne MS, et al. Repeated maternal intramuscular or
27 24 intraamniotic erythromycin incompletely resolves intrauterine *Ureaplasma parvum*
28 25 infection in a sheep model of pregnancy. *American Journal of Obstetrics and
29 26 Gynecology*. 2014;211(2):134.e131-134.e139.
- 30 27 10. Saigal S, Doyle LW. An overview of mortality and sequelae of preterm birth from
31 28 infancy to adulthood. *Lancet*. 2008;371(9608):261-269.
- 32 29 11. Crump C, Sundquist K, Sundquist J, Winkleby MA. Gestational age at birth and
33 30 mortality in young adulthood. *JAMA*. 2011;306(11):1233-1240.
- 34 31 12. de Jong F, Monuteaux MC, van Elburg RM, Gillman MW, Belfort MB. Systematic
35 32 review and meta-analysis of preterm birth and later systolic blood pressure.
36 33 *Hypertension*. 2012;59(2):226-234.
- 37 34 13. Irving RJ, Belton NR, Elton RA, Walker BR. Adult cardiovascular risk factors in
38 35 premature babies. *Lancet*. 2000;355(9221):2135-2136.
- 39 36 14. Ueda P, Cnattingius S, Stephansson O, Ingelsson E, Ludvigsson JF, Bonamy AK.
40 37 Cerebrovascular and ischemic heart disease in young adults born preterm: a
41 38 population-based Swedish cohort study. *European journal of epidemiology*.
42 39 2014;29(4):253-260.
- 43 40 15. Lewandowski AJ, Augustine D, Lamata P, et al. Preterm heart in adult life:
44 41 cardiovascular magnetic resonance reveals distinct differences in left ventricular
45 42 mass, geometry, and function. *Circulation*. 2013;127(2):197-206.
- 46 43 16. Lewandowski AJ, Bradlow WM, Augustine D, et al. Right ventricular systolic
47 44 dysfunction in young adults born preterm. *Circulation*. 2013;128(7):713-720.
- 48 45 17. Kozak-Barany A, Jokinen E, Saraste M, Tuominen J, Valimaki I. Development of left
49 46 ventricular systolic and diastolic function in preterm infants during the first month of
50 47 life: a prospective follow-up study. *The Journal of pediatrics*. 2001;139(4):539-545.
- 51 48 18. Bensley JG, Stacy VK, De Matteo R, Harding R, Black MJ. Cardiac remodelling as a
52 49 result of pre-term birth: implications for future cardiovascular disease. *European
53 50 heart journal*. 2010;31(16):2058-2066.
- 54 51 19. Tare M, Bensley JG, Moss TJ, et al. Exposure to intrauterine inflammation leads to
55 52 impaired function and altered structure in the preterm heart of fetal sheep. *Clinical
56 53 science*. 2014;127(9):559-569.

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2
3 1 20. Yanowitz TD, Jordan JA, Gilmour CH, et al. Hemodynamic disturbances in
4 2 premature infants born after chorioamnionitis: association with cord blood cytokine
5 3 concentrations. *Pediatric research*. 2002;51(3):310-316.
6 4 21. Rounioja S, Rasanen J, Glumoff V, Ojaniemi M, Makikallio K, Hallman M. Intra-
7 5 amniotic lipopolysaccharide leads to fetal cardiac dysfunction. A mouse model for
8 6 fetal inflammatory response. *Cardiovasc Res*. 2003;60(1):156-164.
9 7 22. Kemp MW. Preterm birth, intrauterine infection, and fetal inflammation. *Frontiers in*
10 8 *Immunology*. 2014;5(DEC):574.
11 9 23. Kemp MW, Molloy TJ, Usuda H, et al. Outside-in? Acute fetal systemic
12 10 inflammation in very preterm chronically catheterized sheep fetuses is not driven by
13 11 cells in the fetal blood. *American Journal of Obstetrics and Gynecology*. 2015.
14 12 24. Cruz-Martinez R, Figueras F, Benavides-Serralde A, Crispi F, Hernandez-Andrade E,
15 13 Gratacos E. Sequence of changes in myocardial performance index in relation to
16 14 aortic isthmus and ductus venosus Doppler in fetuses with early-onset intrauterine
17 15 growth restriction. *Ultrasound in obstetrics & gynecology : the official journal of the*
18 16 *International Society of Ultrasound in Obstetrics and Gynecology*. 2011;38(2):179-
19 17 184.
20 18 25. Australian code for the care and use of animals for scientific purposes. In: Council
21 19 NHaMR, ed. 8th ed. Canberra: National Health and Medical Research Council; 2013.
22 20 26. Snyder CC, Wolfe KB, Gisslen T, et al. Modulation of lipopolysaccharide-induced
23 21 chorioamnionitis by *Ureaplasma parvum* in sheep. *American journal of obstetrics and*
24 22 *gynecology*. 2013;208(5):399 e391-398.
25 23 27. Miura Y, Payne MS, Keelan JA, et al. Maternal intravenous treatment with either
26 24 azithromycin or solithromycin clears *Ureaplasma parvum* from the amniotic fluid in
27 25 an ovine model of intrauterine infection. *Antimicrobial Agents and Chemotherapy*.
28 26 2014;58(9):5413-5420.
29 27 28. Payne MS, Kemp MW, Kallapur SG, et al. Intrauterine *Candida albicans* infection
30 28 elicits severe inflammation in fetal sheep. *Pediatric research*. 2014;75(6):716.
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1 TABLES

2 Table 1

	T0 (mean SD)	T24 (mean SD)	P	T72 (mean SD)	P
MV S'	4.74 (1.06)	4.99 (0.87)	0.112	6.27 (1.85)	0.022 *
MV E'	5.23 (1.42)	5.31 (1.88)	0.099	7.48 (2.45)	0.206
MV A'	7.75 (1.63)	10.6 (2.59)	0.053	10.37 (2.52)	0.093
MV EA'	0.67 (0.09)	0.5 (0.12)	0.084	0.73 (0.17)	0.561
IV S'	4.33 (0.86)	5.15 (0.30)	0.253	5.77 (1.41)	0.050*
IV E'	4.94 (1.41)	3.9 (1.28)	0.018*	6.49 (1.58)	0.236
IV A'	6.63 (1.17)	6.88 (2.17)	0.851	8.91(1.27)	0.023 *
IV EA'	0.75 (0.19)	0.57 (0.05)	0.016*	0.73 (0.15)	0.803
TV S'	6.04 (1.06)	6.71 (2.47)	0.982	6.12 (0.93)	0.888
TV E'	5.79 (0.95)	6.58 (1.33)	0.623	6.95 (1.02)	0.019 *
TV A'	8.87 (1.42)	9.75 (2.05)	0.909	9.36 (2.08)	0.379
TV EA'	0.65 (0.04)	0.68 (0.07)	0.169	0.76 (0.15)	0.166

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4 Table 1: Mean (standard deviation) peak annular Systolic (S'), Early Diastolic (E') and Late
5 Diastolic (A') velocities and E'/A' Ratio at Left annulus (MV), Right annulus (TV) and
6 Interventricular Septum (IV).

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1 FIGURE LEGENDS

2 Figure 1: Representative images of histological analysis. Panel **i**, confocal images fetal skin
3 from *C. albicans* infected fetus. Hyphae probed with *C. albicans*-specific primary antibody
4 (green) with DAPI nuclear staining (blue). Panel **ii**, H&E images of fetal skin from control
5 (A) and *C. albicans*-exposed (B) animals. Panel **iii**, H&E images of perfusion-fixed fetal lung
6 from control (A) and *C. albicans*-exposed (B) animals.

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8 Figure 2: Mean concentration of TNF- α in amniotic fluid (ng/mL). Error bars represent 1 SD.
9 h, hours post-*C. albicans* exposure; Ctrl, control. *, $p < 0.05$, vs. control.

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11 Figure 3: Mean fold change in cytokine / chemokine transcript expression in *C. albicans*-
12 exposed tissues (red bars), relative to control tissues (blue bars). A, fetal lung; B, fetal liver;
13 and C, fetal myocardium. Error bars represent 1 SD. *, $p < 0.05$, vs. control.

14
15 Figure 4: Diagram representing normal PW-TDI wave form representing mechanical events
16 in cardiac cycle and summarizing changes seen in peak annular Systolic (S'), Early Diastolic
17 (E') and Late Diastolic (E'/A' Ratio at Left annulus (MV), Right annulus (TV) and
18 Interventricular Septum (IV) in fetal sheep at 24 hours and 72 hours after intra-amniotic
19 injection of *C. albicans*.

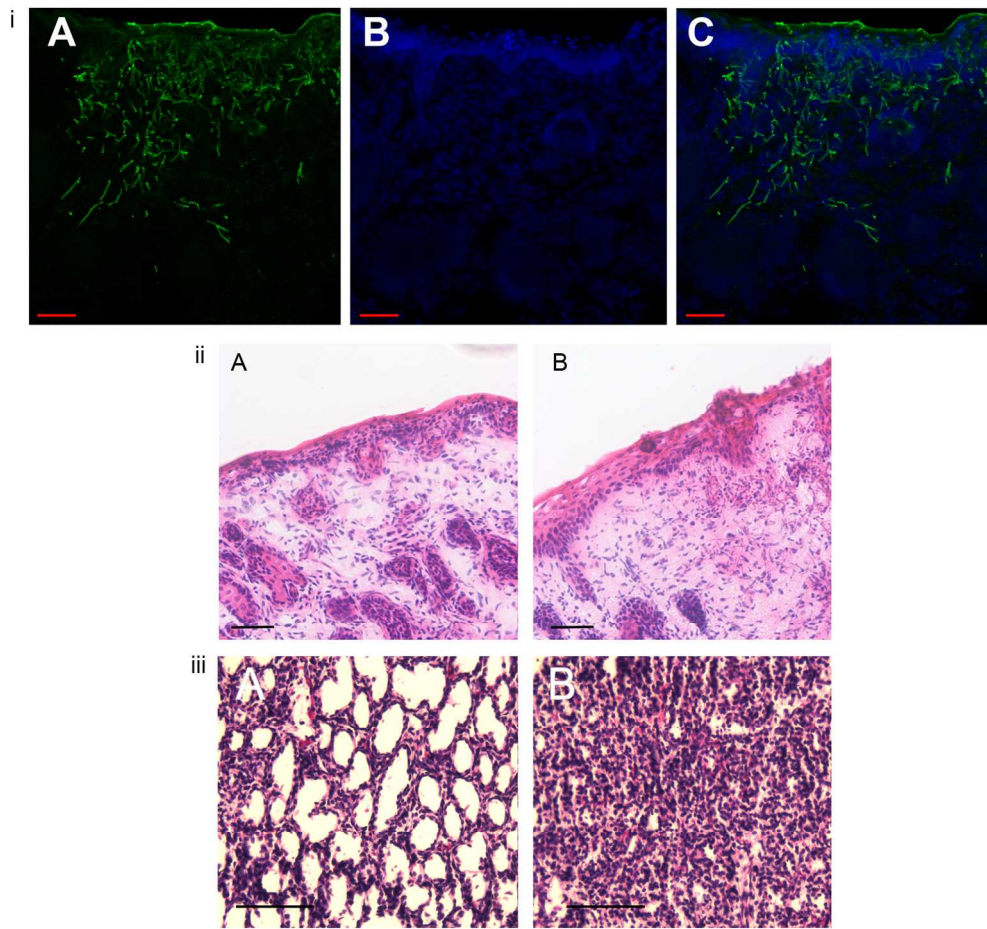


Figure 1: Representative images of histological analysis. Panel i, confocal images fetal skin from *C. albicans* infected fetus. Hyphae probed with *C. albicans*-specific primary antibody (green) with DAPI nuclear staining (blue). Panel ii, H&E images of fetal skin from control (A) and *C. albicans*-exposed (B) animals. Panel iii, H&E images of perfusion-fixed fetal lung from control (A) and *C. albicans*-exposed (B) animals.
147x138mm (300 x 300 DPI)

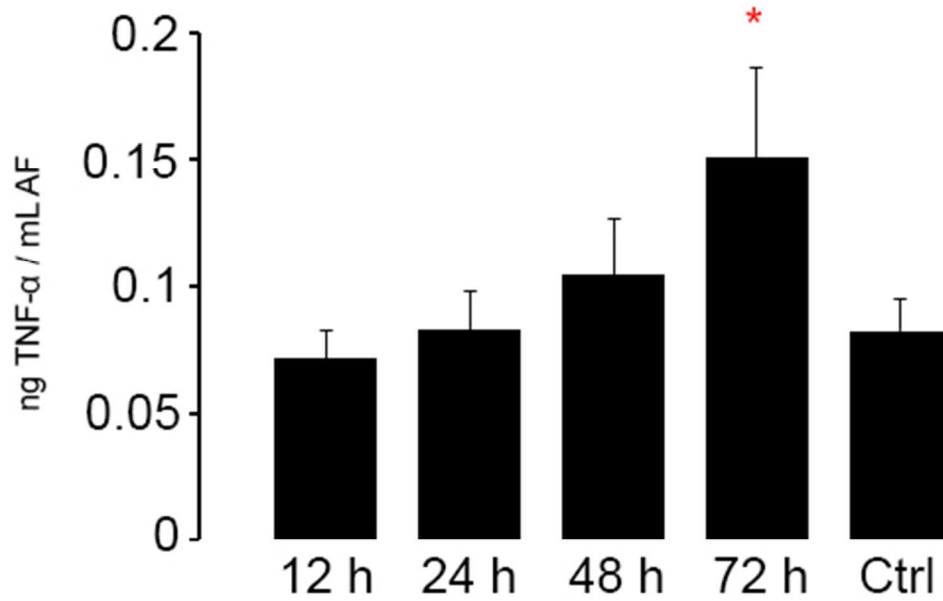


Figure 2: Mean concentration of TNF- α in amniotic fluid (ng/mL). Error bars represent 1 SD. h, hours post-C. albicans exposure; Ctrl, control. *, $p < 0.05$, vs. control.
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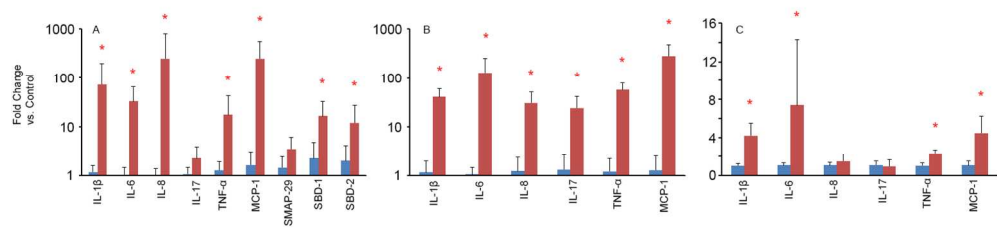


Figure 3: Mean fold change in cytokine / chemokine transcript expression in *C. albicans*-exposed tissues (red bars), relative to control tissues (blue bars). A, fetal lung; B, fetal liver; and C, fetal myocardium. Error bars represent 1 SD. *, $p < 0.05$, vs. control.
131x31mm (300 x 300 DPI)

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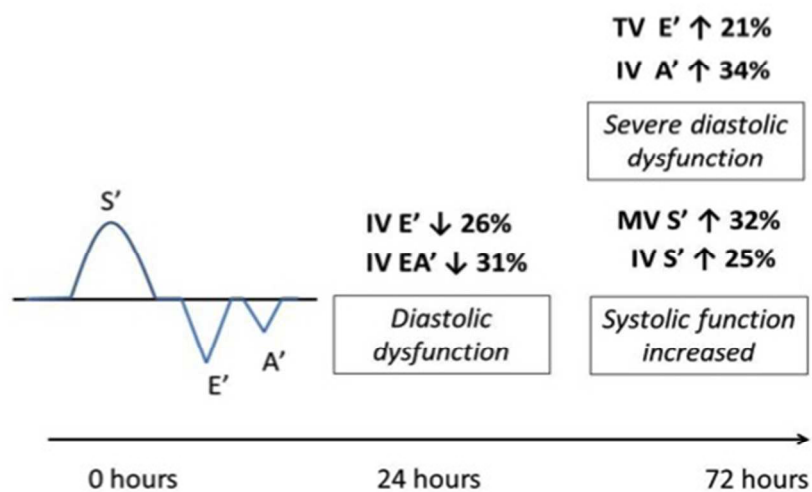


Figure 4: Diagram representing normal PW-TDI wave form representing mechanical events in cardiac cycle and summarizing changes seen in peak annular Systolic (S'), Early Diastolic (E') and Late Diastolic (E'/A') Ratio at Left annulus (MV), Right annulus (TV) and Interventricular Septum (IV) in fetal sheep at 24 hours and 72 hours after intra-amniotic injection of *C. albicans*.
220x124mm (72 x 72 DPI)

Review