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C-section of Preclinical Animal Model of chorioamnionitis Triggered by Group B Streptococcus (GBS)

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TITLE:

C-section of Preclinical Animal Model of Chorioamnionitis Triggered by Group B *Streptococcus* (GBS)

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KEYWORDS:

Attention deficit hyperactivity disorder, ADHD, autism spectrum disorder, ASD, C-section, chorioamnionitis, fetal infection, Group B *Streptococcus*, GBS, intra-uterine growth retardation, IUGR, maternal immune activation, maternal infection, neurobehavioral impairment, perinatal inflammation.

SUMMARY:

The goal of this protocol is to describe a preclinical animal model of Group B *Streptococcus* (GBS)-induced chorioamnionitis. The study is designed to investigate mechanistic processes, potential causal links with developmental impairments, and finally to develop translational anti-inflammatory placento- and neuro-protective treatments.

ABSTRACT:

Group B *Streptococcus* (GBS) is one of the most common bacteria isolated during human pregnancy. It is a leading cause of placental infection/inflammation, termed chorioamnionitis. Chorioamnionitis exposes the developing fetus to a high risk of organ injuries, perinatal morbidity, and mortality, as well as life-long neurobehavioral impairments and other non-neurological developmental issues. The two most frequent subtypes of GBS isolates from maternal and fetal tissues are serotypes Ia (13%–23%) and III (25%–53%). Our lab has developed and characterized a rat model of GBS-induced chorioamnionitis to study subsequent impacts on the central nervous system of the developing fetus and to understand underlying mechanistic aspects. This article presents the design as well as uses of the preclinical rat model, which closely reproduces the hallmark of GBS-induced chorioamnionitis in humans. This article aims to help

scientists reproduce the experimental design as well as to provide support through examples of troubleshooting. The present model may also contribute to potential discoveries through uncovering causes, mechanisms, and novel therapeutic avenues, which remain unsettled in many developmental impairments arising from chorioamnionitis. Furthermore, the use of this model may be extended to the studies of perinatal non-neurological common and severe morbidities affecting, for instance, the retina, bowel, lung, and kidney. The main interest of this research is in the field of GBS-induced fetal neurodevelopmental impairments such as cerebral palsy (CP), attention deficit hyperactivity disorder (ADHD), and autism spectrum disorder (ASD). The rationale supporting this model is presented in this article, followed by procedures and results.

INTRODUCTION:

Maternal immune activation (MIA) has been described as one of the most critical independent risk factors for premature birth, fetal death, and lifelong cognitive and behavioral impairments in the progeny[1–4]. Much of the existing preclinical research about the role of gestational inflammation on placental and developmental outcomes uses pathogen components, such as lipopolysaccharide (LPS) from *E. coli* and the synthetic analog of viral double-stranded RNA, polyinosinic: polycytidylic acid (Poly[I: C]), that mimic viral infections. However, even though Group B *Streptococcus* (GBS) is the most frequent cause of perinatal infection, few animal models have addressed its role in inflammatory mechanisms at play and the outcomes[5].

GBS is an encapsulated gram-positive coccus that colonizes the lower genital tract in approximately 15%–30% of pregnant women[6]. It leads to placental infection/inflammation, termed chorioamnionitis[7,8]. Of the ten GBS serotypes, the two most frequent serotypes Ia and III are major infectious determinants of injuries in maternofetal tissues[9,10]. GBS infection has been shown to lead to a higher inflammatory response in fetal blood and placental deficiency, which are highly suspected to be involved in multiple neurodevelopmental disorders such as cerebral palsy (CP), attention deficit hyperactivity disorder (ADHD), and autism spectrum disorder (ASD)[5,11].

Over the past ten years, we have developed a rat model of GBS-induced chorioamnionitis that leads to various developmental impairments in the offspring[12]. This preclinical model demonstrates the causal link between GBS-induced placental inflammation and a range of sex-specific neurodevelopmental impairments in the offspring[13–15]. The goal of this article is to provide readers with insight into the design of a preclinical rat model of end-gestational infection and resulting neuro-behavioral impairments in the offspring. The present protocol aims to mimic the clinical reality of GBS-induced chorioamnionitis.

Results from this preclinical model show that end-gestational intra-peritoneal (IP) inoculation (**Figure 1**) of GBS leads to (i) placental infection and inflammation, fulfilling the diagnostic criteria of chorioamnionitis[16]; (ii) a massive upregulation of IL-1β and downstream inflammatory molecules from the IL-1-pathway, within the placenta[12]; (iii) neurodevelopmental impairments in the offspring[12]; (iv) sex differences in immune responses and subsequent neurobehavioral impairments, such as female offspring presenting adult ADHD-like traits while male offspring present early-onset and long-lasting ASD-like traits; (v) distinct neurobehavioral outcomes in the

progeny depending on the GBS serotype used to induce chorioamnionitis[14,15]. In line with these findings, the main next steps utilizing this model will be to test, firstly, the role of androgen in GBS-induced chorioamnionitis and, secondly, the placental- and neuro-protective role of molecules targeting specific inflammatory pathways, in the hope to bring some of these molecules to the threshold of therapeutic clinical trials.

PROTOCOL:

All experiments were approved by the Research Institute of McGill University Health Centre (RI-MUHC). All experiments were performed according to the Canadian Council on Animal Care.

1. **Pregnant Lewis rats**

1.1. Obtain Lewis rats from commercial sources at gestational day (G)14. House them in an appropriate animal facility (RI-MUHC animal facility) in a controlled environment at 20–23 °C with a 12 h light/dark cycle, and access to water and food *ad libitum*[17].

1.2. Weigh dams daily to detect any illness behavior from G14 (i.e., the day of arrival) until G22 (i.e., the day of C-section)

2. **Bacterial growth**

2.1. On G18, prepare two sterile test tubes with 5 mL of sterile Brain Heart Infusion (BHI) broth. Take a small portion of frozen bacteria stock (β-hemolytic capsular serotype Ia in BHI and 15% glycerol[14]) from -80 °C and add it into 5 mL BHI tubes (**Figure 2**).

2.2. Place the tubes in the shaker (240 rpm) for 18 h at 37 °C.

2.3. On G19, prepare a 3% solution of GBS in sterile BHI broth by collecting 1.5 mL of the incubated solution into 48.5 mL sterile BHI broth.

2.4. Collect 1.5 mL of the 3% GBS plus BHI solution into a cuvette. Using a spectrophotometer, record the initial absorption as T_0 (optical density $(OD)_{600\ nm}$).

NOTE: A blank made with sterile BHI broth was used each time to balance the spectrophotometer.

2.5. Place the 3% solution in the incubator at 37 °C with 240 rpm shaking for approximately 2 h. Check the absorption every 20 min after 2 h until a measure of absorbance between 0.6 and 0.8 ($OD_{600\ nm}$) has been reached.

2.6. After reaching the desired absorbance, collect 20 mL of 3% GBS plus BHI solution and add it to a 50 mL tube.

2.7. Centrifuge (1792 x *g*) the samples at 4 °C for 13 min and wash the precipitated GBS twice with 20 mL of 0.9% sterile saline.

2.8. Suspend the precipitated GBS in 2 mL of 0.9% sterile saline. Keep this aliquot on ice until the time of injection.

2.9. Inject (intraperitoneally) the control group with 100 µL of sterile 0.9% saline and the GBS group with 100 µL β-hemolytic serotype Ia GBS suspended in sterile 0.9% saline.

NOTE: The injected dose was 10^8 colony forming units (CFUs) of GBS or saline (for control). Inoculation of 10^8 CFU has been well established as a model of human chorioamnionitis. Inoculation with a higher dose of GBS will likely cause dam mortality. Injecting less than the mentioned dose will not mimic the infection and inflammation.

2.10. Make dilutions between 10^{-5} and 10^{-10} and plate the dilutions in triplicate on BHI agar plates. To rule out contamination, make two negative controls (without any addition of substance), one on a BHI agar plate and the other on a CHROMID Strepto B agar plate. Make two positive controls by plating the prepared bacteria on the BHI agar plate and the CHROMID Strepto B agar plate. Place all plates in the incubator overnight at 37 °C (**Figure 3**).

NOTE: CHROMID Strepto B agar plates are a selective medium for screening GBS on which the GBS colonies appear red.

3. **Injection technique**

3.1. On G19, remove the rat gently from the cage and place it onto a flat surface. Immobilize the rat by using a towel to cover the head and upper body. Lift the hind leg to allow easy access to the injection site.

NOTE: Make sure the appropriate anatomical area for injection is in the lower right quadrant of the abdomen to avoid puncture of organs such as the urinary bladder and cecum (**Figure 1**).

3.2. Use a U-100 insulin syringe with a 29 G ½ inch needle. Insert the needle bevel facing up towards the head at a 40–45° angle to the horizontal, as shown in **Figure 1**. Perform GBS injections once for each dam. Make sure to perform injections every 1 h to avoid a time effect between inoculated dams.

NOTE: Injections should be varied between the left and right sides on days when more than one injection per day is performed.

4. **Dose determination**

4.1. On G20, verify four controls (step 10.2) and count the bacterial colonies on each BHI agar plate.

4.2. Calculate the mean GBS colonies for each dilution factor (10^{-5} to 10^{-10}) to determine the exact injected dose of GBS

5. **C-section and tissue collection**

5.1. Perform C-sections on G22 (72 h post-injection) and perform subsequent surgeries with 1 h between dams according to each dam's inoculation time.

5.2. Anesthetize the dam in a euthanasia chamber with 2% isoflurane and 1.5% O_2, for general anesthesia.

5.3. Place the dam on a heating pad covered with an appropriate surgical dressing and apply the ophthalmic ointment to the eye to avoid drying.

5.4. Prepare the surgical area by removing hair from the lower abdominal area using a blade or scalpel.

5.5. Clean the surgical area with sterile gauze soaked with disinfectant.

5.6. Using a sterile scalpel and fine-tipped scissors, make a horizontal incision in the lower abdomen of the rat. Make a vertical incision on either side of the abdomen to reveal underlying organs.

5.7. Separate placenta samples from fetuses. Record the weights of fetuses, placentas, and the fetus/placenta ratio.

5.8. Using a sterile scalpel, cut the placenta into two halves.

5.8.1. Use 2-methylbutane to fast freeze one-half of the placenta and keep at -80 ˚C until needed for determination of protein levels using ELISA.

5.8.2. Fix the other half of the placenta in 4% buffered formaldehyde for *in situ* analysis by immunohistochemistry (IHC) to study the expression of GBS and polymorphonuclear cells (PMNs) in collected placentas.

5.9. Decapitate to collect blood from live fetuses and transfer the blood to Lithium Heparin Gel Separator tubes.

5.10. Centrifuge (18,928 x *g*) blood samples at 4 °C to separate the plasma and store the plasma samples at -80 °C until further analysis.

NOTE: The collected fetal blood plasma samples will be used for ELISA to check the protein levels of different cytokines in fetus blood.

5.11. Collect fetal tails to determine the sex of fetuses by amplification of a sequence within the SRY gene, using the following primers (forward primer: 5' - TAC AGC CTG AGG ACA TAT TA3'; reverse primer: 5' - GCA CTT TAA CCC TTC GAT GA -3') as described earlier[18].

5.12. Using a 5 mL 23 G needle, collect blood from the dam by cardiac puncture for use in ELISA to check and compare protein levels of different cytokines in the dam blood with those in fetal blood. Euthanize the dams by diaphragm puncture and decapitation method.

NOTE: Between animals, clean all the used instruments with sterile tissue and sterile saline. To perform neuropathological and behavioral studies in the progeny, dams gave birth naturally on G23. After euthanizing offspring on postnatal day (PN) 80, brains were collected for molecular and histological studies.

REPRESENTATIVE RESULTS:

IP inoculation of GBS resulted in placental infection

Gram and/or immunohistochemistry (IHC) (using polyclonal antibodies targeting GBS serotype Ia) staining showed that GBS infection reached the decidual compartment of the placenta. Infection also spread from the decidua to the labyrinth, chorionic plate, and in some instances, to fetuses leading to fetal death (5.8 ± 0.8 in GBS-exposed vs. 9.3 ± 0.6 pups in control (CTL) litters)[18]. Hence the litter size was decreased at birth among GBS-exposed fetuses compared to unexposed fetuses[18]. Intra-litter inconsistencies in terms of placental infection were observed. The GBS infection was self-limited in all dams as well as in newborns, but some dams presented GBS bacteremia/septicemia[15]. Those developing such dissemination of GBS infection also developed a higher pro-inflammatory cytokine response[13]. No positive GBS blood culture was detected in pups at P1[14]. Maternal behavior was not impacted by end-gestational infections. There was no mortality in GBS-exposed pups during the postnatal period.

The GBS-induced placental inflammatory response fulfilled the diagnostic criteria of human chorioamnionitis

Interleukin (IL-1β) and polymorphonuclear cells (PMNs) play a major role in anti-GBS host defense[15]. As expected, at 3, 6, 24, 48, and 72 h post-GBS infection, IL-1β titers in the blood of GBS-exposed dams were higher than in unexposed dams[18]. A similar IL-1 response was detected in fetal blood and placentas 72 h after injection[18]. At both 24 h and 48 h post-GBS inoculation, both maternal and fetal compartments of the placenta showed a significant increase of PMN and GBS infiltration compared to controls[15]. These findings were demonstrated through histology (**Figure 4 and Figure 5**)[15]. In summary, these results show that IP inoculation of GBS results in placental infection and induces an innate immune response, which is typical of chorioamnionitis.

Sex-specific innate immune response triggered by GBS

At 72 h post-GBS infection, PMN cell infiltration in the fetal compartment of the placenta was higher in males than in females. The PMN-chemoattractant, CINC-1, also showed a 1.6-fold increase in male placentas compared to litter-matched females. Similarly, there was a significant

interaction between sex and treatment for IL-1β detected in the placenta at 72 h post-GBS infection (**Figure 6**)[15]. Levels of IL-1β detected in fetal sera correlated positively with those found in maternal circulation (**Figure 7**)[15]. No sex effect was observed in other pro-inflammatory cytokines implicated in GBS-induced chorioamnionitis, including IL-18, IL-6, and TNF-α. Altogether, GBS-induced innate immune signaling through the IL-1-CINC-1-PMN axis presents a sexually dichotomous profile, with more prominent inflammation in males compared to females[12].

Brain injury and neurodevelopmental impairments in male versus female progeny in utero exposed to GBS infection

Enlargement of lateral ventricles of the brains from GBS Ia-exposed males (but not females) was shown through histology at P40[14]. GBS-exposed forebrains also showed a reduction in thickness of the corpus callosum and the external capsule[14]. Offspring's social behaviors at P40 were of interest to study as this is a feature of ASD-like behavior, which is suspected to be induced by a combination of genetic and environmental factors, including pathogen-induced maternal immune activation[14]. The total duration of social interactions was significantly decreased in GBS Ia-exposed males vs CTL males as well as GBS-exposed females[14]. In the same line male (but not female) offspring presented decreased pre-pulse inhibition (P35), ultrasonic vocalization (P7), and nest-seeking (P9) behaviors, which are other cardinal markers of ASD-like behavior in rodents[14]. In summary, these results show that gestational exposure to GBS Ia-induced chorioamnionitis plays a key role in the generation of sexually dichotomous, neurodevelopmental abnormalities comparable to human ASD. In contrast, more than males, female offspring with *in utero* exposure to GBS Ia-induced chorioamnionitis present hyperactivity and disinhibition after puberty and at the adult age, which is reminiscent of ADHD-like behavior in human adult females[19]. Intra-uterine growth retardation (IUGR), which persisted beyond the adult age, the dysmyelinated white matter of the corpus callosum adjacent to the primary motor cortices, and cerebral-palsy-like motor impairment occurred in newborn pups with *in utero* exposure to GBS III[16]. Interestingly, the severity of the IUGR of males exposed *in utero* to GBS III chorioamnionitis correlated with the intensity of their motor impairments assessed by decreased total distance traveled at P25 measured by the Open field test[16]. The severity of the decrease in thicknesses of the primary motor (M1) cortices correlated with the decreased density of microglial cells in the corpus callosum of GBS III exposed male rats, but not female rats[16].

FIGURE LEGENDS:

Figure 1: Intraperitoneal inoculation. Level and location of needle insertion for appropriate GBS IP inoculations of dams.

Figure 2: Processing GBS Bacteria. Materials used for GBS inoculation on G18.

Figure 3: Plating GBS. Serial dilutions of GBS and CHROMID Strepto B agar plates for GBS screening.

Figure 4: PMN cell infiltration within the placenta. PMN cell infiltration in the placenta following GBS infection. Representative images of PMN infiltration in the decidua (a), junctional zone (b), labyrinth (c), and amnion (d) at 72 h post-inoculation. Black triangles show infiltrated PMN cells. Reprinted with permission from reference[15].

Figure 5: GBS infection of the placenta. Representative images of immunohistochemical detection of GBS Ia infiltrates in placentas from GBS-infected versus non-infected dams. Reprinted with permission from reference[15].

Figure 6: Concentration of IL-1β in placentas at 48 h and 72 h. A linear mixed model was used to compare GBS-infected placentas with CTL placentas, with Sidak's multiple comparisons when the interaction between sex and treatment was significant. **P < 0.01, ***P < 0.001. Number (n) of placentas in the CTL group is n = 5 (48 h) and n = 5 (72 h) per sex and that in the GBS group is n = 4 (48 h) and n = 6 (72 h) per sex. One male (M) and one female (F) placenta were used per litter for analysis. Reprinted with permission from reference[15].

Figure 7: Correlation between levels of IL-1β in fetal sera and maternal sera at 72 h post-GBS inoculation. The within-subject correlation between the fetal and maternal levels of IL-1β was analyzed by linear regression. Reprinted with permission from reference[15].

DISCUSSION:

Critical steps in the protocol

Several steps of the protocol are critical and require some quality controls. For instance, there is a risk of contamination of the GBS stock by other pathogens. This can be rapidly identified using the appropriate technique of GBS microbial identification such as colony aspect on BHI agar (e.g., size, shape, color), plating in duplicate the β-hemolytic GBS dose on Columbia blood agar with 5% sheep blood medium and on CHROMID Strepto B agar, a selective chromogenic medium for the screening of GBS.

Another issue is that some dams are asymptomatic carriers of GBS. Hence, some accidental infections might theoretically occur in non-GBS inoculated control dams. However, in our hands, GBS staining in the placenta of control animals never detected GBS-induced chorioamnionitis. If this unlikely event were to happen, it would not significantly affect the experimental design we propose. Due to this reason, it was not deemed necessary to use pathogen-free animals.

Protecting infected dams and their offspring from undue stress is important. Any stress added to the infection can induce noxious effects on the experimental subjects. Hence, great attention must be paid to appropriate husbandry, reassuring way of handling animals, protection against background noises, respect of a very quiet environment around delivery and birth, avoidance of mixing animal species in the same room, and other classic measures of prevention of stress routinely used in animal facilities.

Modifications and troubleshooting of the method

Several variations of the above-described protocol might be considered. Lewis rats were used in this protocol. The maternal behavior of rats, compared to mice, is more tolerant to health issues of the progeny, which favored the quality of care of the offspring and resilience of the dam to health issues in the progeny[20]. However, it might be useful to adapt our experimental design to other rodents such as mice, and to other strains of rats, not only to study interspecies consistency but also to get access to some experimental techniques such as transgenic animals which are more available and cost-effective in mice compared to rats.

To establish the difference between infectious and/or inflammatory effects of GBS-induced chorioamnionitis, inactivated GBS Ia was used instead of alive bacteria[12,18]. The technique of GBS inactivation has been described earlier[12]. In another experimental design of GBS inactivation, infected dams were treated with the antibiotic (ampicillin), which is relevant to the treatment used at the bedside in human chorioamnionitis. Also, the ampicillin treatment induced a transient surge of pro-inflammatory response (cytokines release, and PMN infiltration, within the placenta)[13]. These variations around our original model open novel avenues of research, which remain to be explored to better understand physiopathology and develop a new therapeutic intervention in GBS-induced chorioamnionitis.

Another issue encountered was an over-mortality of infected dams observed sporadically. In our experience, this was sometimes due to an inadequate technique of IP injection (see steps 3.1 and 3.2) affecting vital abdominal organs, or due to direct injection within the vascular bloodstream. Alternatively, some mutations were observed over time in our stock of the GBS Ia strain, creating a drift toward a more aggressive and life-threatening strain. This can be easily suspected in some instances by observing a more rapid *in vitro* proliferation of the bacteria.

Limitations and significance of the method with respect to existing/alternative methods

There are limitations in this preclinical model of GBS-induced chorioamnionitis. Firstly, it does not accurately reproduce ascending chorioamnionitis (i.e., from the vaginal cavity) caused by GBS in humans. In studies by Randis et al. and Nobel et al., authors describe a novel model of ascending GBS infection in pregnant mice[21,22]. Such a model offers many advantages, including that it closely mimics human colonization by GBS, minimizes invasive manipulation of dams, and avoids preterm delivery or fetal loss[21,22]. Rats have more pregnancies than humans; this creates differences in the exposure of the fetus to bacteria based on closeness to the lower genital tract. Secondly, bacteria are inoculated into the peritoneal cavity in this model. This protocol does not replicate most human chorioamnionitis, which is primarily within the uterus, even if the hematogenous route of placental contamination is also described for instance from urinary infection[23]. Other preclinical models of GBS-induced chorioamnionitis used the intra-vaginal route of inoculation. However, this was associated with intra- and inter-litter variance of GBS load between placentas/fetuses as it depended on location within the uterine cavity. Intra-uterine injections can sometimes produce abscesses due to a focal injection area, making chorioamnionitis unlikely to occur, thereby not accurately mimicking human chorioamnionitis. Furthermore, since the target of injection is so small, a lack of litter reproducibility is likely. Thirdly, other differences exist between rodents and humans. These include pH of the vagina, hormonal cycling, vaginal microbiota, and bacterial adherence between the murine vaginal

epithelium and the human vaginal mucosa[21], and in the immune response[24,25]. Regardless, the main objective in designing this model was to mimic GBS-induced chorioamnionitis. This methodology is similar to that of many other investigators who have used IP injections of poly(I:C), LPS, and other infectious components of microorganisms to mimic MIA[1,26–31]. Fourthly, another limitation in the GBS rat model is that placental morphology is different between humans and rodents; for example, the maternofetal interdigitation is villous in humans but not in rats. This may therefore result in species-specific interactions between pathogens and placental cells[32]. An attractive model is the guinea pig due to similarities to human pregnancy, including comparable progesterone levels, placental structure, sensitivity to pathogens, and length of gestation[33]. Fifthly, while current studies focus on GBS serotype Ia and III, other serotypes of GBS are also implicated in infection during pregnancy, particularly serotype V. Finally, another limitation of this work is that dams experiencing GBS infection might develop changes in their maternal behaviors toward their pups. As a result of the infection, GBS-positive dams experience reduced weight gain which is indicative of sickness. The use of foster mothers might be proposed but it also induces biases.

Importance and potential applications of the method in specific research areas

There is a lack of studies in the literature addressing the nature of the infectious/inflammatory mechanisms, which underpin GBS-induced chorioamnionitis and their roles in developmental impairments. In humans, the hallmark of acute chorioamnionitis is a massive infiltration of PMNs[34]. PMNs play an important role in the production of IL-1β, which triggers the production of chemokines that further drive infiltration of PMNs in GBS-infected organs[35]. The maternal IL-1β response observed upon GBS infection likely resulted in the recruitment of PMN cells within the decidua and labyrinth, possibly originating from both maternal and fetal blood. Thus, determining the role of IL-1 is an important avenue of research in GBS-induced chorioamnionitis, which might be tested using a GBS model.

Using the established end-gestational GBS model developed in our lab, it was determined that there was an *in utero* sex-specific innate immune response. The exaggerated male innate immune response observed may be connected to a sex-specific anti-inflammatory response. Clinical studies have shown that there is a higher concentration of IL-1 receptor antagonist (IL-1Ra) in female amniotic fluid compared to males[36,37]. Since the androgen receptor (AR) is present in all innate immune cells involved in chorioamnionitis, these conclusions could be a result of the influence of sex hormones on the production of IL-1 by immune cells[38,39]. Thus, determining the role of testosterone on the GBS-induced placental and fetal response would be of interest. Another avenue of further research would be to induce the IL-1 blockade by IL-1Ra to uncover the role that IL-1 plays in GBS-induced maternal immune activation.

The perinatal period is extremely important for brain development and thus the dysregulation of cytokines during this sensitive period can result in CP, learning impairments, ADHD, ASD, and other developmental impairments[40,41]. Using the model of GBS-induced chorioamnionitis, the progeny displayed sexually dichotomous behavioral impairments: ASD-like, cerebral palsy-like traits, forebrain white matter tract alterations in males versus ADHD-like behavior in females. This is in keeping with the male sex bias, which is documented in multiple human perinatal

morbidities, including ASD, CP, and ADHD. In conclusion, these results provide further clarity into the inflammatory processes that contribute to the skewed sex ratio of neurobehavioral impairments after placental infections.

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DISCLOSURE:

The authors have no financial conflicts of interest.

REFERENCES:

1. Hui, C. W. et al. Prenatal immune challenge in mice leads to partly sex-dependent behavioral, microglial, and molecular abnormalities associated with schizophrenia. *Frontiers in Molecular Neuroscience*. **11**, 13 (2018).
2. Costa, A. et al. Activation of the NLRP3 inflammasome by group B streptococci. *Journal of Immunology.* **188** (4), 1953–1960 (2012).
3. Gupta, R. et al. RNA and beta-hemolysin of group B Streptococcus induce interleukin-1beta (IL-1beta) by activating NLRP3 inflammasomes in mouse macrophages. *Journal of Biological Chemistry*. **289** (20), 13701–13705 (2014).
4. Henneke, P. et al. Lipoproteins are critical TLR2 activating toxins in group B streptococcal sepsis. *Journal of Immunology*. **180** (9), 6149–6158 (2008).
5. Nelson, K. B., Chang, T. Is cerebral palsy preventable? *Current Opinion in Neurology*. **21** (2), 129–135 (2008).
6. Larsen, J. W., Sever, J. L. Group B Streptococcus and pregnancy: a review. *American Journal of Obstetrics and Gynecology*. **198** (4), 440–448; discussion 448–450 (2008).
7. Patras, K. A., Nizet, V. Group B Streptococcal maternal colonization and neonatal disease: molecular mechanisms and preventative approaches. *Frontiers in Pediatrics*. **6**, 27 (2018).
8. Tita, A. T., Andrews, W. W. Diagnosis and management of clinical chorioamnionitis. *Clinics in Perinatology*. **37** (2), 339–354 (2010).
9. Teatero, S. et al. Serotype distribution, population structure, and antimicrobial resistance of Group B Streptococcus strains recovered from colonized pregnant women. *Journal of Clinical Microbiology*. **55** (2), 412–422 (2017).
10. Lu, B. et al. Microbiological and clinical characteristics of Group B Streptococcus isolatescausing materno-neonatal infections: high prevalence of CC17/PI-1 and PI-2b sublineage in neonatal infections. *Journal of Medical Microbiology*. **67** (11), 1551–1559 (2018).
11. Limperopoulos, C. et al. Positive screening for autism in ex-preterm infants: prevalence and risk factors. *Pediatrics*. **121** (4), 758–765 (2008).

12. Bergeron, J. D. et al. White matter injury and autistic-like behavior predominantly affecting male rat offspring exposed to group B streptococcal maternal inflammation. *Developmental Neuroscience*. **35** (6), 504–515 (2013).
13. Giraud, A. et al. Ampicillin treatment increases placental Interleukin-1 beta concentration and polymorphonuclear infiltration in Group B Streptococcus-induced chorioamnionitis: A preclinical study. *Neonatology*. **117** (3), 369–373 (2020).
14. Allard, M. J. et al. A sexually dichotomous, autistic-like phenotype is induced by Group B Streptococcus maternofetal immune activation. *Autism Research*. **10** (2), 233–245 (2017).
15. Allard, M. J., Giraud, A., Segura, M., Sebire, G. Sex-specific maternofetal innate immune responses triggered by group B Streptococci. *Scientific Reports*. **9** (1), 8587 (2019).
16. Allard, M. J., Brochu, M. E., Bergeron, J. D., Segura, M., Sebire, G. Causal role of group B Streptococcus-induced acute chorioamnionitis in intrauterine growth retardation and cerebral palsy-like impairments. *Journal of Developmental Origins of Health and Disease*. **10** (5), 595–602 (2019).
17. Girard, S., Tremblay, L., Lepage, M., Sebire, G. IL-1 receptor antagonist protects against placental and neurodevelopmental defects induced by maternal inflammation. *Journal of Immunology*. **184** (7), 3997–4005 (2010).
18. Bergeron, J. et al. Activation of the IL-1beta/CXCL1/MMP-10 axis in chorioamnionitis induced by inactivated Group B Streptococcus. *Placenta*. **47**, 116–123 (2016).
19. Allard, M. J., Brochu, M. E., Bergeron, J. D., Sebire, G. Hyperactive behavior in female rats in utero-exposed to group B Streptococcus-induced inflammation. *International Journal of Developmental Neuroscience*. **69**, 17–22 (2018).
20. Shuster, K. A. et al. Naturally occurring disseminated group B streptococcus infections in postnatal rats. *Comparative Medicine*. **63** (1), 55–61 (2013).
21. Randis, T. M. et al. Group B Streptococcus beta-hemolysin/cytolysin breaches maternal-fetal barriers to cause preterm birth and intrauterine fetal demise in vivo. *The Journal of Infectious Diseases*. **210** (2), 265–273 (2014).
22. Noble, K. et al. Group B Streptococcus cpsE is required for Serotype V capsule production and aids in biofilm formation and ascending infection of the reproductive tract during pregnancy. *ACS Infectious Diseases*. **7** (9), 2686–2696 (2021).
23. Kim, C. J. et al. Acute chorioamnionitis and funisitis: definition, pathologic features, and clinical significance. *American Journal of Obstetrics and Gynecology*. **213** (4 Suppl), S29–52 (2015).
24. Becker, K. J. Strain-related differences in the immune response: Relevance to human stroke. *Translational Stroke Research*. **7** (4), 303–312 (2016).
25. Mestas, J., Hughes, C. C. Of mice and not men: differences between mouse and human immunology. *Journal of Immunology*. **172** (5), 2731–2738 (2004).
26. Fernandez de Cossio, L., Guzman, A., van der Veldt, S., Luheshi, G. N. Prenatal infection leads to ASD-like behavior and altered synaptic pruning in the mouse offspring. *Brain, Behavior, and Immunity*. **63**, 88–98 (2017).
27. Shi, L., Fatemi, S. H., Sidwell, R. W., Patterson, P. H. Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. *The Journal of Neuroscience*. **23** (1), 297–302 (2003).

28. Boksa, P. Effects of prenatal infection on brain development and behavior: a review of findings from animal models. *Brain, Behavior, and Immunity*. **24** (6), 881–897 (2010).
29. Girard, S., Kadhim, H., Beaudet, N., Sarret, P., Sebire, G. Developmental motor deficits induced by combined fetal exposure to lipopolysaccharide and early neonatal hypoxia/ischemia: a novel animal model for cerebral palsy in very premature infants. *Neuroscience*. **158** (2), 673–682 (2009).
30. Meyer, U., Feldon, J. To poly(I:C) or not to poly(I:C): advancing preclinical schizophrenia research through the use of prenatal immune activation models. *Neuropharmacology*. **62** (3), 1308–1321 (2012).
31. Lammert, C. R., Lukens, J. R. Modeling autism-related disorders in mice with Maternal Immune Activation (MIA). *Methods. Journal of Molecular Biology*. **1960**, 227–236 (2019).
32. Gundling, W. E., Jr., Wildman, D. E. A review of inter- and intraspecific variation in the eutherian placenta. *Philosophical Transactions of the Royal Society B*. **370** (1663), 20140072 (2015).
33. Harrell, M. I. et al. Exploring the pregnant guinea pig as a model for Group B Streptococcus intrauterine infection. *The Journal of Infectious Diseases*. **2** (2) (2017).
34. Redline, R. W. Classification of placental lesions. *American Journal of Obstetrics and Gynecology*. **213** (4 Suppl), S21–28 (2015).
35. Erez, O. et al. Differential expression pattern of genes encoding for anti-microbial peptides in the fetal membranes of patients with spontaneous preterm labor and intact membranes and those with preterm prelabor rupture of the membranes. *Journal of Maternal-Fetal and Neonatal Medicine*. **22** (12), 1103–1115 (2009).
36. Burns, C., Hall, S. T., Smith, R., Blackwell, C. Cytokine levels in late pregnancy: Are female infants better protected against inflammation? *Frontiers in Immunology*. **6**, 318 (2015).
37. Elsmen, E., Ley, D., Cilio, C. M., Hansen-Pupp, I., Hellstrom-Westas, L. Umbilical cord levels of interleukin-1 receptor antagonist and neonatal outcome. *Biology of the Neonate*. **89** (4), 220–226 (2006).
38. Chuang, K. H. et al. Neutropenia with impaired host defense against microbial infection in mice lacking androgen receptor. *Journal of Experimental Medicine*. **206** (5), 1181–1199 (2009).
39 Mantalaris, A. et al. Localization of androgen receptor expression in human bone marrow. *The Journal of Pathology*. **193** (3), 361–366 (2001).
40. Rasmussen, J. M. et al. Maternal Interleukin-6 concentration during pregnancy is associated with variation in frontolimbic white matter and cognitive development in early life. *Neuroimage*. **185**, 825–835 (2019).
41. Dozmorov, M. G. et al. Associations between maternal cytokine levels during gestation and measures of child cognitive abilities and executive functioning. *Brain, Behavior, and Immunity*. **70**, 390–397 (2018).

Figure

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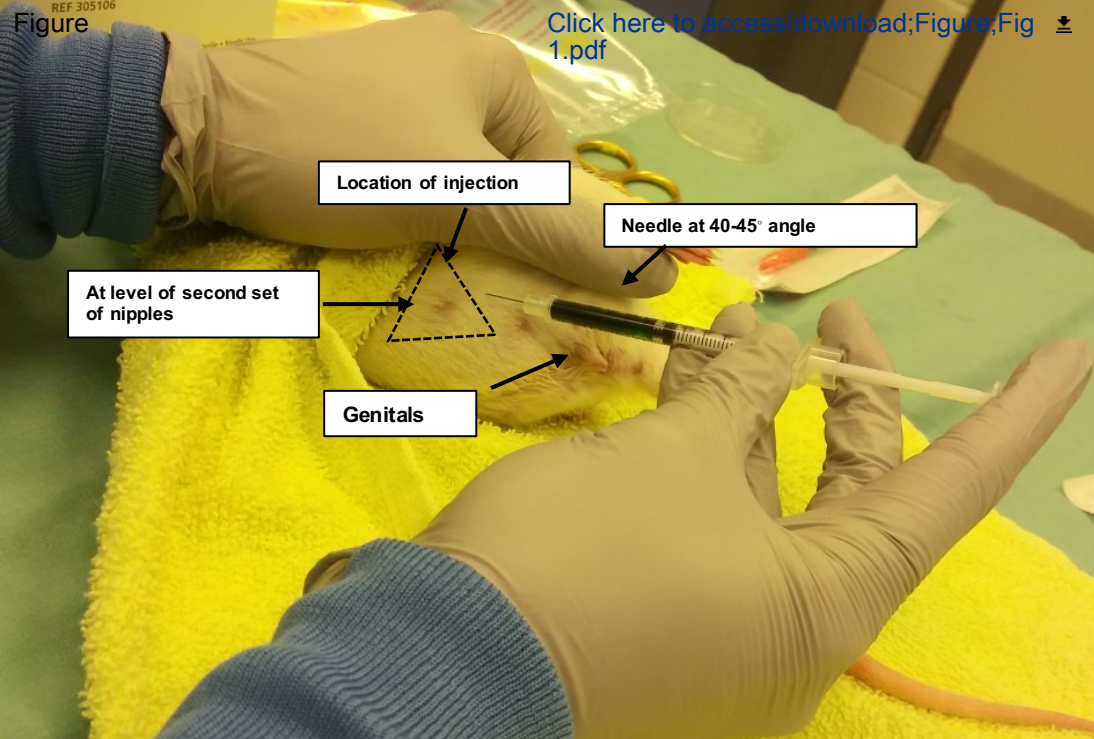

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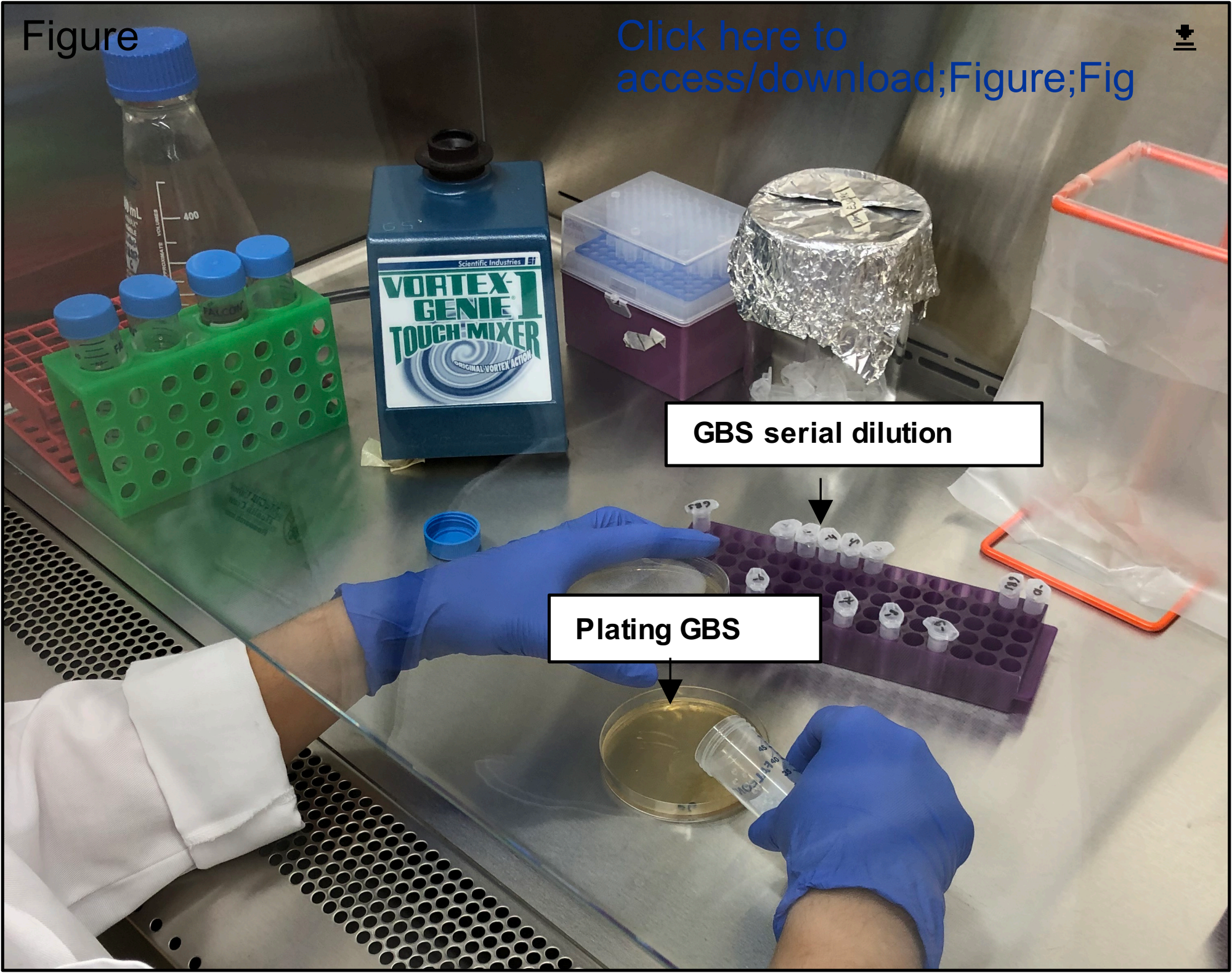

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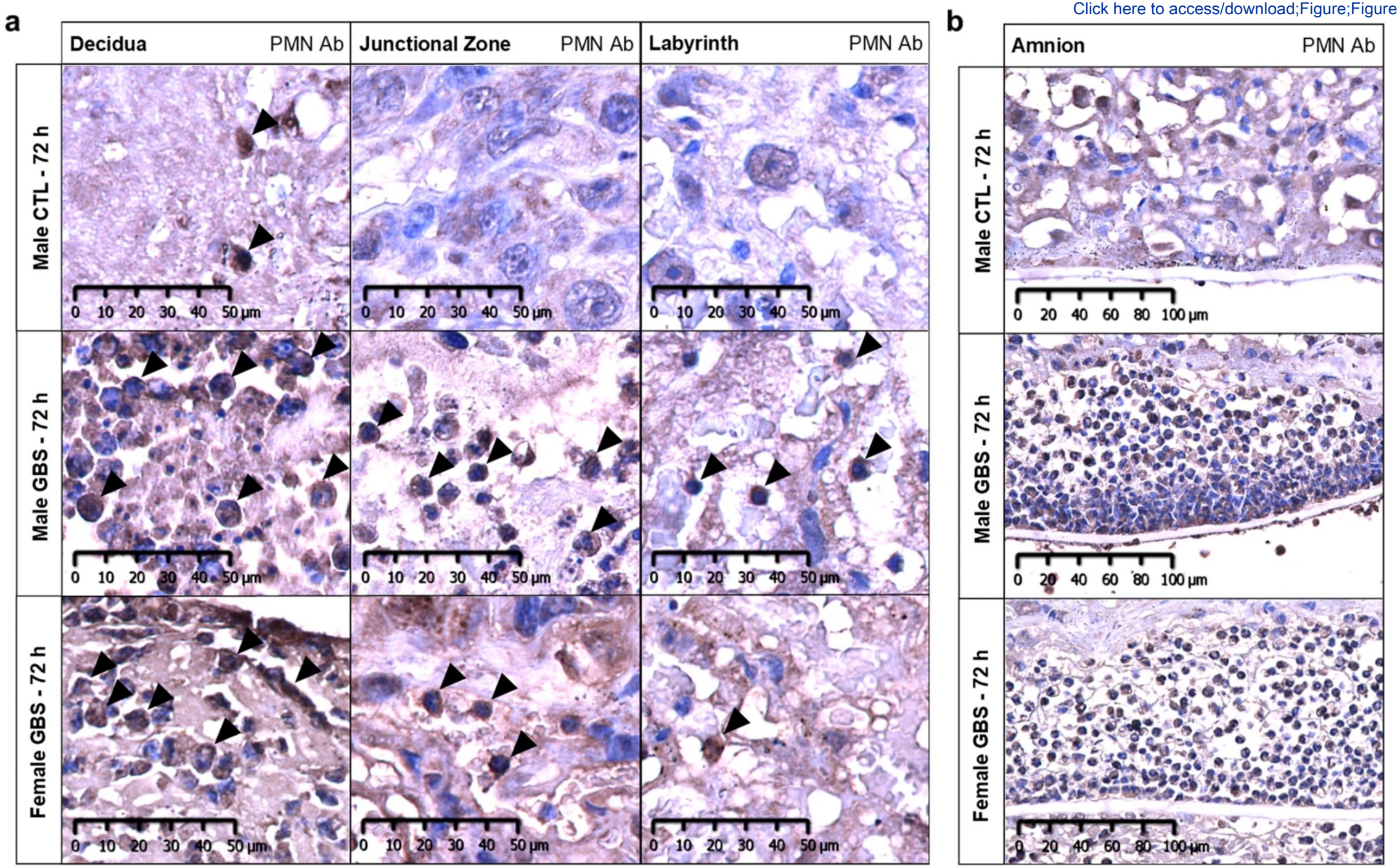

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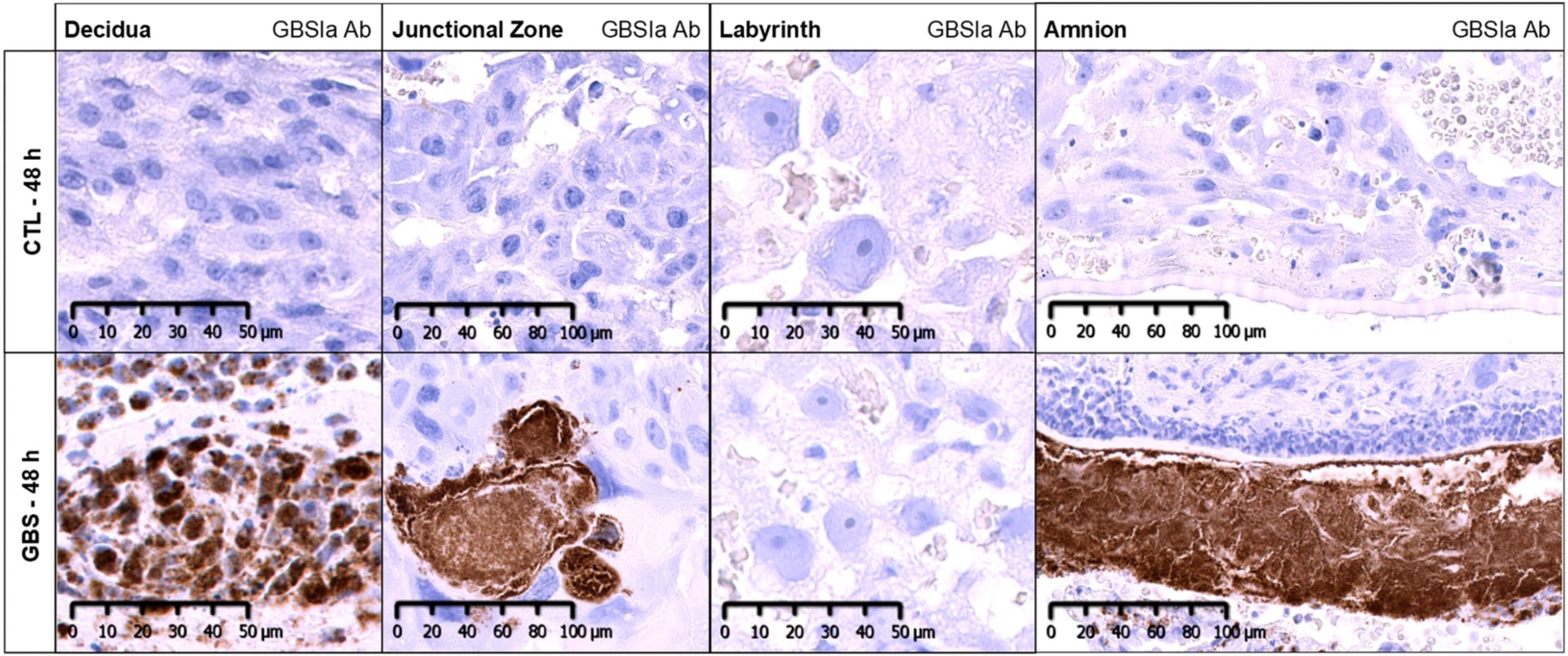

Figure

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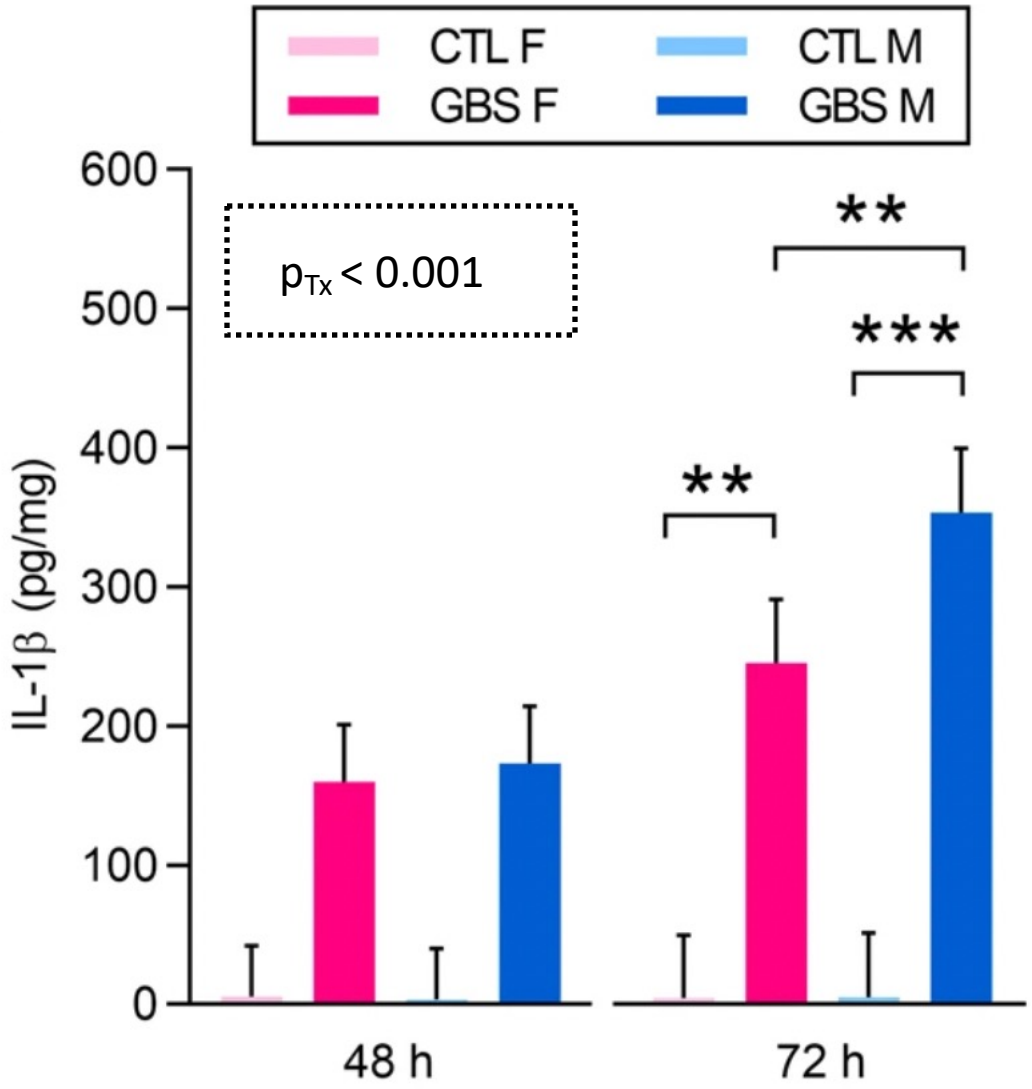

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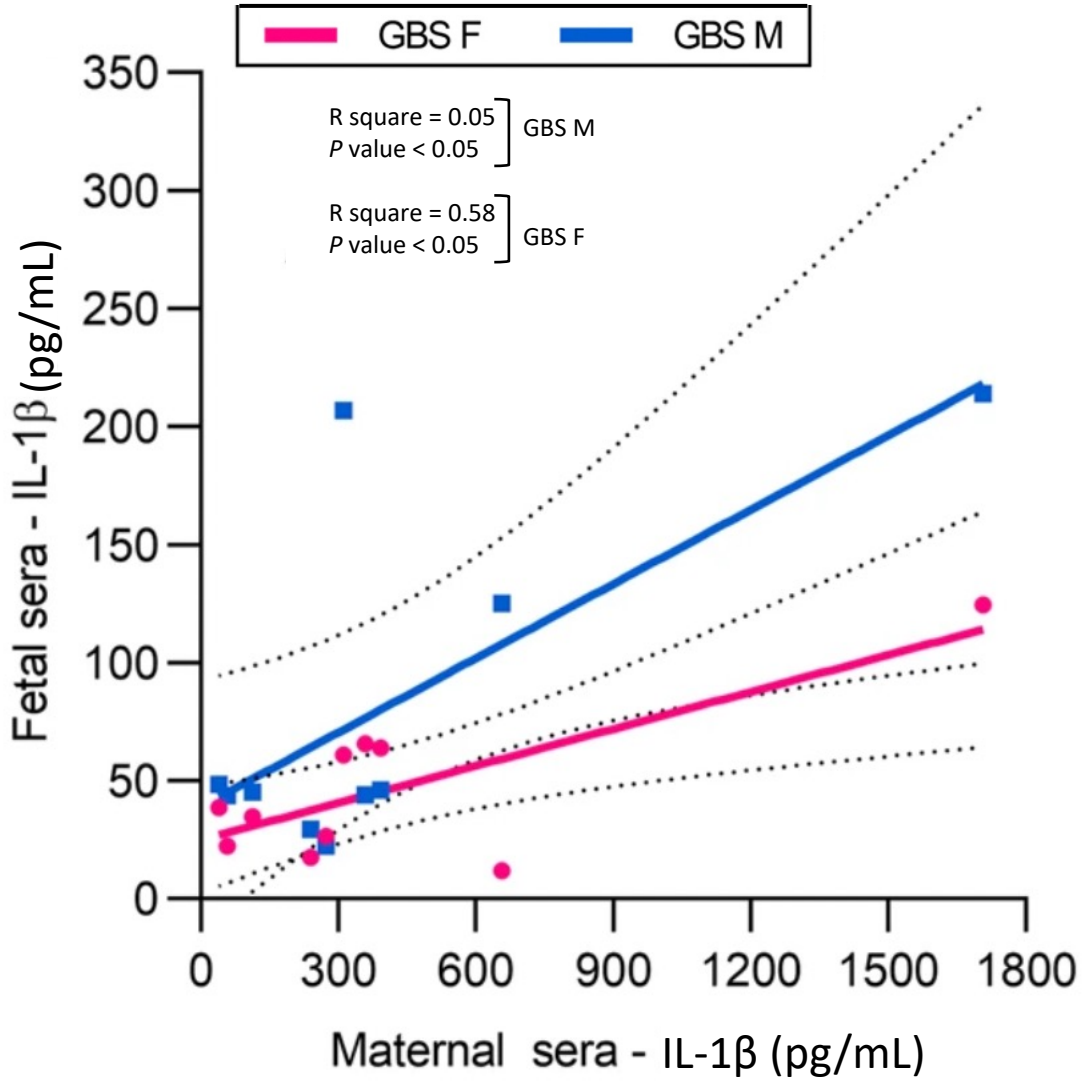

October 18th, 2021

Dear Editor,

Please find attached a manuscript entitled "**C-section of preclinical Animal Model of chorioamnionitis Triggered by Group B Streptococcus (GBS**)".

Thanks for taking the time to read this manuscript. I would thank you and the reviewers for all the useful feedback and comments.

With full of agreement with you, we provided the requested information in the text.

Here are detailed points explaining all the comments from the Editor and the r reviewers.

All authors have read and approved this submission. All authors disclose no source of conflict of interest.

Thank you very much in advance for the attention you will pay to our work.

Sincerely,

Taghreed Ayash, PhD
McGill University, Canada

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   - The requested information was provided

2. Please clarify the corresponding author for this study as the names are different in the Editorial Manager and the main manuscript.
   - The requested information was provided

3. Please revise the text within protocol steps 4.5, 4.9, 4.10, and lines 260-263 and lines 304-329 to avoid plagiarized text.
   - The requested information was provided

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
   - The requested information was provided

5. Please format in-text journal references to appear as numbered superscripts after the appropriate statement(s). Also, please remove citations from the abstract.
   - The requested information was provided

6. Since JoVE is a methods journal, please revise the Introduction to include all the following:
a) The advantages over alternative techniques with applicable references to previous studies
b) A description of the context of the technique in the wider body of literature
c) Information to help readers to determine whether the method is appropriate for their application.

   - The authors appreciate these comments, however, much of the requested information has been provided in the 'modifications and troubleshooting of the method' and 'limitation of the methods and significance of the method with respect to existing/alternative methods' sections. In order to avoid repetition, authors do not wish to include this information in the introduction.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials (e.g., Criterion, BD Biosciences, falcon, Becton Dickinson, CHROMID Strepto B, BioMerieux, Lithium Heparin Gel Separator Tubes, Becton Dickinson, Sigma Aldrich, Thermo Scientific).

   - The requested information was provided

8. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm). Please mention the centrifugation temperature throughout.

- The requested information was provided

9. Please abbreviate milliliter as mL throughout the manuscript.
-The requested information was provided

10. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). Any text that cannot be written in the imperative tense (e.g., provide extraneous details, optional steps, or recommendations) may be added as a “Note.”

- The requested information was provided

11. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Readers of all levels of experience and expertise should be able to follow your protocol. Alternatively, add references to published material specifying how to perform the protocol action.
Line 110: Please abbreviate ip at the first-time use.

- The requested information was provided

Line 112, 113: Please move these lines to Acknowledgements.

- The requested information was provided

Line 123: Please specify the needle gauge and length

- The requested information was provided

Step 4: Please specify if sterile conditions are used during the surgery.
- The requested information was provided

Line 164: Please specify the instrument used to make the incision and how big is the incision.
- The requested information was provided

Line 165: Please specify how the depth of anesthesia is confirmed and specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

- The requested information was provided

Line 173: Please specify the primer used for PCR.

- The requested information was provided, see line 213

Line 184: Please specify the euthanasia method.
- In full agreement we added few sentences for the requested information see line 208

Line 224: Please correct the typo: “TNF-□”

- The requested information was provided

12. Please insert single-line spacing between individual steps and sub-steps in the Protocol. Please highlight no more than 3 pages of the protocol (including headings and spacings) that identify all the essential action steps to be filmed as a video. Please highlight complete sentences in the protocol and ensure that the highlighted steps form a cohesive narrative with a logical flow. Also it should be in line with the Title of the manuscript.
- The requested information was provided

13. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text.

-The requested information was provided

14. Figure 6, 7: Please use JoVE format for numeric labels. For example, use 0.001 instead of .001, 0.50 instead of .50, etc.
15. Figure 7: please remove the label ‘g’ from the figure.
16. Please upload each figure individually in the Editorial Manager. Please ensure a high resolution for each figure.
17. Please number your bibliography references in the order of their appearance in the manuscript text. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate Journal names

- The requested information was provided

Reviewers' comments:

Reviewer #1:

Manuscript summary

1.Line 111: please include how many CFU were used to induce the chorioamnionitis. It would be important as well to address possible effects found on lower and higher amount of inoculated GBS.

- The requested information was provided

2.Authors have accurately stated the method limitations, appointing differences between rodents and humans. However, immunological differences were not addressed. Authors should at least cite them as they did with other variances.

- The requested information was provided, see line 403

Reviewer #2:

Manuscript summary:

The manuscript by Ayash et al., entitled, "Preclinical Animal Model of chorioamnionitis Triggered by Group B Streptococcus" is a methods paper which outlines the utility of a Lewis rat model of GBS intraperitoneal injection and invasive infection during pregnancy. The manuscript

is well-written and outlines important experimental procedures for this model. There are a few concerns that detract from the enthusiasm this Reviewer has for the manuscript.

Major critiques:
Intraperitoneal injection of the rat does not adequately mimic ascending vaginal infection, which is likely the more common route of infection by GBS (which frequently colonizes the rectovaginal cavities). There have been several manuscripts recently using a pregnant mouse model of ascending GBS vaginal infection which the authors do not mention (see below). It would be helpful to discuss the advantages of the rat model and the limitations with respect to these models. For example, genetic and immunological tools are likely more prevalent with the mouse models than the rat models.
Randis TM, Gelber SE, Hooven TA, Abellar RG, Akabas LH, Lewis EL, Walker LB, Byland LM, Nizet V, Ratner AJ. Group B Streptococcus β-hemolysin/cytolysin breaches maternal-fetal barriers to cause preterm birth and intrauterine fetal demise in vivo. J Infect Dis. 2014 Jul 15;210(2):265-73. doi: 10.1093/infdis/jiu067.
Noble K, Lu J, Guevara MA, Doster RS, Chambers SA, Rogers LM, Moore RE, Spicer SK, Eastman AJ, Francis JD, Manning SD, Rajagopal L, Aronoff DM, Townsend SD, Gaddy JA. Group B Streptococcus cpsE Is Required for Serotype V Capsule Production and Aids in Biofilm Formation and Ascending Infection of the Reproductive Tract during Pregnancy. ACS Infect Dis. 2021 Sep 10;7(9):2686-2696. doi: 10.1021/acsinfecdis.1c00182.

- The requested information was provided, see line 385
  "In studies by Randis et al and Nobel et al., authors describe a novel model of ascending GBS infection in pregnant mice…"

Harrell MI, Burnside K, Whidbey C, Vornhagen J, Adams Waldorf KM, Rajagopal L. Exploring the Pregnant Guinea Pig as a Model for Group B Streptococcus Intrauterine Infection. J Infect Dis Med. 2017 Sep;2(2):109. doi: 10.4172/2576-1420.1000109.

- The requested information was provided, see line 412
  "An attractive model is the guinea pig due to similarities to human pregnancy, including comparable progesterone levels, placental structure, sensitivity to pathogens and length of gestation.."

Why did the authors choose intraperitoneal inoculation instead of intrauterine?

- The requested information was provided (p18).

Minor critiques:

Line 189: Please capitalize the abbreviation "ip" and write the entire word before using the abbreviation.

- The requested information was provided

Line 224 (and throughout the manuscript): The abbreviation for tumor necrosis factor alpha has not formatted correctly (there is a box present for the latin character).

- The requested information was provided

In the introduction, the authors mention the importance of their model for studying FIRS, but they do not discuss how FIRS has been assessed in this model. It would be helpful to include that.

- In full agreement we provided the requested information, see line 69 “GBS infection has been shown to lead to a higher inflammatory response in fetal blood and placental..”

Sex-specific maternofetal innate immune responses triggered by group B Streptococci

Author: Marie-Julie Allard et al
Publication: Scientific Reports
Publisher: Springer Nature
Date: Jun 13, 2019