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Dear Editors,

I hereby submit the article: “High frequency ultrasound for the analysis of growth restricted fetuses in natural killer cell depleted mast cell-deficient mice” to be considered for publication in the *Journal of Visualized Experiments.* The article was written in response to an invitation by Jaydev Upponi and has not been submitted elsewhere.

Our article explains in detail the usage of the VEVO 2100 Imaging System, an ultrasound device that enables us to follow–up fetal as well as placental and blood flow parameters throughout pregnancy. Our manuscript provides on the one hand insights into the biological functions of innate immune cells and their role in pregnancy diseases. One the other hand the detailed protocol allows the readers to use the Vevo 2100 Imaging System for their own research either pregnancy-related or non-related. We believe that this work is suitable for the scientific audience of the *Journal of Visualized Experiments.*

We thank you very much for your attention and look forward to your consideration.

Yours sincerely,

Prof. Ana Claudia Zenclussen

TITLE:

High Frequency Ultrasound for the Analysis of Fetal and Placental Development *In Vivo*

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KEYWORDS:

Fetal development, intrauterine growth restriction, placenta, mast cells, natural killer cells, ultrasound imaging, B-mode, color doppler mode, pulse-wave doppler mode

SUMMARY:

Here we describe the technique of high frequency ultrasound for *in vivo* analysis of fetuses in mice. This method allows the follow-up of fetuses and the analysis of placental parameters as well as maternal and fetal blood flow throughout pregnancy.

ABSTRACT:

Ultrasound imaging is a widespread method used to detect organ anomalies and tumors in human and animal tissues. The method is non-invasive, harmless, and painless, and the application is easy, fast, and can be done anywhere, even with mobile devices. During pregnancy, ultrasound imaging is standardly used to closely monitor fetal development. The technique is important to assess intrauterine growth restriction (IUGR), a pregnancy complication with short- and long-term health consequences for both the mother and fetus. Understanding the process of IUGR is indispensable for developing effective therapeutic strategies.

The ultrasound system used in this manuscript is an ultrasound device produced for the analysis of small animals and can be used in various research fields, including pregnancy research. Here we describe the usage of the system for *in vivo* analysis of fetuses from natural killer (NK) cell/mast cell (MC)-deficient mothers that give birth to growth-restricted pups. The protocol includes preparation of the system, handling of the mice before and during measurements, and the usage of the B-mode, color doppler mode, and pulse-wave doppler mode. Fetal size, placental size, and blood supply to the fetus were analyzed. We found reduced implantation sizes and

smaller placentas in NK/MC-deficient mice from mid-gestation onwards. In addition, MC/NK-deficiency was associated with absent and reversed end diastolic flow in the fetal *Arteria umbilicalis* (UmA) and an elevated resistance index. The methods described in the protocol can easily be used for related and non-related research topics.

INTRODUCTION:

Ultrasound is sound waves with frequencies above the audible range of the human ear, higher than about 20 kHz[1]. Animals like bats, wales, dolphins[2,3], mice[4], rats[5], and mouse lemurs[6] all use ultrasound for orientation or communication. Humans take advantage of ultrasound for several technical and medical applications. An ultrasound device is able to create the sound wave and distribute and represent the signal. If ultrasound encounters an obstacle, the sound is reflected, absorbed or can go through it. The application of ultrasound as an imaging method, called sonography, is used for the analysis of organic tissues in human or veterinary medicine like the heart (echocardiography)[7,8], lung[9], thyroid gland[10], kidneys[11], and urinary and reproductive tracts[12,13]; detecting gallstones[14] and tumors[15]; and evaluating perfusion of blood vessels or organs[16,17]. Ultrasound is a standard method in prenatal care during pregnancy, and fetal developmental disabilities or impairments can be recognized early. Specifically, the growth of a fetus is closely monitored at regular intervals to recognize a possible IUGR. Finally, fetal blood flow can be monitored, as this can point out growth restrictions[18–21].

A major advantage of ultrasound imaging compared to other methods like radiography is the sound's harmlessness of the tissues to be analyzed. This easy and fast method is non-invasive, painless, and can be used a number of times. The initial outlay of an ultrasound device is expensive; however, the consumable materials needed are cheap. The ultrasound system used in this manuscript is suitable for a range of animal models *(i.e.,* mice and fish) While for humans an ultrasound device requires a frequency of 3-15 mHz, a frequency of 15-70 mHz is required for mice.

The present manuscript describes a protocol for the use of B-mode, color doppler mode, and pulse-wave doppler mode. The description includes preparation of the mice as well as performance, data acquisition, and storage. This method has been successfully applied to different mouse strains at all gestational days and can be used to investigate fetal and placental development as well as maternal and fetal blood parameters. Here, all applications are explained based on our studies employing pregnant MC/NK-deficient and control mice.

PROTOCOL:

All methods described here have been approved by the "Landesverwaltungsamt Sachsen Anhalt: 42502-2-1296UniMD."

1. Experimental Procedure

1.1 Mate 6 to 8-week-old female MC-deficient C57BL/6J-Cpa3$^{Cre/+}$ (Cpa3$^{Cre/+}$) mice and MC-sufficient C57BL/6J-Cpa3$^{+/+}$ (colony controls; Cpa3$^{+/+}$) with BALB/c males.

1.2 Define the gestation day (gd) 0 after confirmation of the vaginal plug and treat the females immediately after plug confirmation.

Note: A plug is the sperm of the male in the vaginal orifice of the female.

1.2.1 Inject 250 µL of PBS intraperitoneally in control $Cpa3^{+/+}$ females.

1.2.2 Inject 250 µL of anti-CD122 (0.25 mg) intraperitoneally in MC-deficient $Cpa3^{Cre/+}$ females.

Note: An injection of 0.25 mg of anti-CD122 depletes peripheral NKs and uNKs in MC-deficient $Cpa3^{Cre/+}$ females as described previously[22].

1.3 Wait until gd5.

Note: At gd5, there is the earliest possibility for implantation analysis.

1.3.1 Proceed with steps 2-5 for the ultrasound analysis.

1.4 Perform the ultrasound imaging at gd5, 8, 10, 12, and 14.

2. Preparation of the Ultrasound System

2.1 Turn on the system (**Figure 1A**; main power on the back and computer standby at the left site), the heated platform (**Figure 1B**; at the control pad), and the gel warmer (**Figure 1C**).

Note: Ultrasound gel needs to warm up for approximately 0.5 h.

2.2 Ensure that the isoflurane unit is filling sufficiently (**Figure 1D**).

2.3 Open a **New Study** or **New Series** in an existing study in the browser. Fill in all the required information (owner, study name, series name, animal data) in the **Study Info** window. Click **Ok**.

2.4. After clicking **Ok**, ensure that the B-mode imaging window appears and the imaging in B-mode begins automatically.

3. Mouse Handling

3.1 Anesthetization of the mouse

3.1.1 Place the mouse in the knockdown box (**Figure 1E**), close the box, open the isoflurane tube to the knockdown box, and turn on the isoflurane (concentration 3.5%).

3.1.2 When the mouse is anesthetized, lower (to concentration 1.5%) and redirect the isoflurane flow by opening the tube in the direction of the heating platform and close the flow to the

knockdown box.

Note: To reach sufficient anesthesia, wait an additional 10 s after the mouse is no longer moving.

3.1.3 Transfer the mouse quickly from the knockout box to the heating platform (**Figure 1F**) in a dorsal position, and gently position its nose in the anesthesia nose tube located on the top of the platform.

3.2 Fixation, depilation, and preparation of the mouse for measuring

3.2.1 Place eye protection cream in each eye of the mouse to prevent dry eyes.

3.2.2 Place one drop of electrode gel on each of the four copper areas on the heated platform (**Figure 1F**).

3.2.3 Tap the paws with surgical tape on the electrode gel-coated areas of the heating platform.

3.2.4 Check ECG [optimal value = 450-550 beats/min (BPM)] and respiratory physiology at all times.

Note: By using a rectal probe, body temperature measurement is possible, but not necessary.

3.2.5 Place depilatory cream at the abdomen of the mouse, rub the cream with a cotton swab and wait around 1 min. Remove the cream with a water-soaked compress. Repeat this step if not all hairs are gone.

3.2.6 Apply the pre-warmed ultrasound gel on the depilated skin.

4 Measurements and Acquisition of Images and Videos

4.1 Hold the transducer (**Figure 1G**) in the hand or clamp it in the holding device (**Figure 1H**; holding device is recommended).

4.2 Identify the bladder with the transducer and use it as reference point. Move the transducer to the left and right sites of the abdomen to trace implantations.

4.3 B-mode for visualization of anatomical structures in 2D grayscale image

4.3.1 Move the transducer or heating platform table where the mouse is fixated until the first implantation is visible on the screen at its largest size.

4.3.1.1 Select **Image Label** and enter a name, or **Frame Store** (storage without name) to store single frames, or **Cine Store** to store a cineloop for whole implantation measurements.

4.3.2 Move the transducer or table to bring the placenta to a position where blood flow in the UmA is visible. Store a single frame or cineloop (see step 4.3.1.1) for placental measurements.

Note: Placental measurements are possible from gd10 onwards.

4.3.3 Continue with all implantations using the same method.

4.4 Color doppler mode to visualize and determine the direction of blood flow

4.4.1 Press the **Color** button.

4.4.2 Move the Color Box (in this area, the signal is visible) to the required position by using the trackball. If necessary, change the size of the box by pressing **Update** and move the trackball (to the right side/upward = bigger; to the left side/downward = smaller). When the box has the right size, press **Select**.

4.4.3 Store single frames or cineloops as described in step 4.3.1.1.

4.5 Pulse-wave (PW) doppler mode to quantify blood flow through the vessels in the *Arteria uterina* (uterine artery, UA) and UmA

4.5.1 Locate the region of interest in the color doppler acquisition.

Note: The UA is located caudal to the bladder, and the UmA is located between the fetus and placenta.

4.5.2 Press **PW**, and a dashed line will appear. Move this line to the blood vessel of interest and adjust the angle of the line using the "Doppler Angle" knob in line with the blood flow. Press **Update**.

Note: The angle between the direction of the blood flow and the transducer must be consistent in all animals, especially when using angles of greater than 60° (here, 70° for UAs and 45° for UmAs were used).

4.5.3 Store a cineloop of the appearing doppler lines in the PW doppler acquisition window.

5. Reviewing and Finishing Data Acquisition and Saving a Series

5.1 To review data, press **Study Management.** Scroll to the thumbnail image of interest and double-click **Update**.

5.2 Press first **Study Management** then **Close** in the browser window to finish data acquisition and save a recorded series.

Note: After closing a series, it is not possible to store frames or cineloops in this series anymore.

6. Mouse Handling Following Acquisition of Data

6.1 Remove the gel from the anaesthetized animal with the help of dry compresses.

6.2 Remove the surgical tape carefully from the paws.

6.3 Close the isoflurane tube (concentration 0%).

6.4. Proceed with following ultrasound analysis at gd5, 8, 10, and 12.

6.4.1. Place the animal alone in a cage for a minimum of 5 min so it has time to wake up and orientate.

6.4.2. Place the mouse back in the original cage.

Note: Do not turn off the isoflurane before removing the gel and surgical tape, as mice wake up very quickly (around 20 s) after turning off the isoflurane.

6.5. Proceed with the following ultrasound analysis at gd14.

6.5.1. Sacrifice the female before it wakes up by cervical dislocation. Open the animal, remove the uterus, separate the fetuses and placentas, and measure fetal and placental weights.

7. Copying and Importing the Data

7.1 Mark one or more series by clicking **Export To** and choose the storage space to copy data onto a hard disk.

7.2 Open the software at a computer and click **Copy From** and select the study/series from the hard disk to import a study/series into the software.

7.3 Analyze the data with the software.

REPRESENTATIVE RESULTS:

Individual components of the ultrasound system used in this manuscript are shown in **Figure 1**. **Figure 2** shows representative ultrasound images acquired in B-mode at gd5, 8, 10, and 12 **(B)** and corresponding implantation area measurement results **(A)**, demonstrating a significant reduced implantation area of anti-CD122-treated $Cpa3^{Cre/+}$ mice from gd10 onwards.

Figure 3 shows single parts of an implantation (decidua basalis, placenta, embryo) acquired in B-mode (**Figure 3A**) and conduced placental measurement (area, thickness, diameter) (**Figure 3B**). Placental measurements resulted in a significantly reduced placental area (**Figure 3A**), thickness

(**Figure 3B**), and diameter (**Figure 3C**) in anti-CD122-treated $Cpa3^{Cre/+}$ mice compared to WTs at gd10 and gd12. In contrast, placental area and diameter were comparable between the groups at gd14, and thickness was significantly increased in anti-CD122-treated $Cpa3^{Cre/+}$ mice in comparison to WTs at gd14.

Figure 4 shows fetal and placental weight at gd14. Results revealed a significantly decreased fetal weight (**Figure 4A**), comparable placental weight (**Figure 4B**), and significantly decreased feto-placental index (FPI) (**Figure 4C**) in anti-CD122-treated $Cpa3^{Cre/+}$ mice compared to WTs. **Figure 5** shows a representative PW doppler image of the UA of a WT mouse (**Figure 5A**) and measurements of peak systolic velocity (PSV) (**Figure 5B**), end diastolic velocity (EDV) (**Figure 5C**), and the calculated resistance index (**Figure 5D**), whereby all values were comparable between the groups. **Figure 6** shows a representative color doppler image of a WT fetal UmA at gd14 (**Figure 6A**) and representative PW doppler images with normal, absent, or reversed end diastolic flow (**Figure 6B**) and measurements of PVS (**Figure 6C**), EDV (**Figure 6D**), systolic/diastolic ratio (**Figure 6E**)**,** and resistance index (**Figure 6F**). The resistance index of anti-CD122-treated $Cpa3^{Cre/+}$ mice was significantly increased compared to WT mice.

FIGURE LEGENDS:

Figure 1: The imaging system. Main control unit (A) with heating platform control pad (B), gel warmer (C), isoflurane control unit (D), knockdown box (E), heated platform with four copper areas (F; F.1), transducer (G), and transducer holding device (H).

Figure 2: Comparison of implantation areas at gd5, 8, 10, and 12. (A) Implantation areas from WT $Cpa3^{+/+}$ + PBS mice (mice n = 2-5, implantations n = 6-31 per day) and MC/NK-deficient $Cpa3^{Cre/+}$ + anti-CD122 mice (mice n = 3, implantations n = 8-16 per day) at gd5, 8, 10, and 12. Results are presented as individual values for each single implantation and mean. Statistical differences were obtained using an unpaired *t*-test (**$p < 0.01$, ***$p < 0.001$). (B) Representative ultrasound images from $Cpa3^{+/+}$ + PBS mice at gd5 (i), gd8 (ii), gd10 (iii), and gd12 (iv). gd, gestation day; WT, wild type; MC, mast cell; NK, natural killer cell. This figure is republished from a previous publication[23].

Figure 3: Placental measurements at gd10, 12, and 14. (A) Representative ultrasound image of a WT implantation at gd10 showing the decidua basalis, placenta, and embryo. (B) Representative ultrasound image of a WT implantation at gd12 showing placental thickness (thick) and placental diameter (dia). Placental area (C), placental thickness (D), and placental diameter (e) from WT $Cpa3^{+/+}$ + PBS mice (mice n = 3-5, placentas n = 12-22 per day) and MC/NK-deficient $Cpa3^{Cre/+}$ + anti-CD122 mice (mice n = 3-4, placentas n = 8-14 per day) at gd10, 12, and 14. Results are presented as individual values for each single placenta and mean. Statistical differences were obtained using an unpaired *t*-test (*$p < 0.05$, **$p < 0.01$). gd, gestation day; WT, wild type; thick, thickness; dia, diameter; MC, mast cell; NK, natural killer cell. This figure is republished from a previous publication[23].

Figure 4: Fetal and placental weight measurements and feto-placental index (FPI) at gd14. Fetal weights (A), placental weights (B), and FPIs (C) from progeny of WT $Cpa3^{+/+}$ + PBS mice (mice n =

4, fetus/placentas n = 35) and MC/NK-deficient $Cpa3^{Cre/+}$ + anti-CD122 mice (mice n = 3, fetus/placentas n = 28) at gd14. Results are presented as individual values and mean. Statistical differences were obtained using unpaired t-test (*p < 0.05, **p < 0.01). gd, gestation day; WT, wild type; MC, mast cell; NK, natural killer cell. This figure is republished from a previous publication[23].

Figure 5: Analysis of uterine artery velocities at gd10. (A) Representative pulse-wave doppler images from WT $Cpa3^{+/+}$ + PBS mice showing PSV and EDV. PSV (B), EDV (C), and resistance index (D) of uterine arteries from $Cpa3^{+/+}$ + PBS (n = 3) and $Cpa3^{Cre/+}$ + anti-CD122 (n = 3) mice at gd10 of pregnancy. Data are presented as mean with SEM. Statistical analysis was performed using the Mann–Whitney U test. gd, gestation day; WT, wild type; MC, mast cell; NK, natural killer cell; PSV, peak systolic velocity; EDV, end diastolic velocity. This figure is republished from a previous publication[23].

Figure 6: Analysis of umbilical artery velocities at gd14. (A) Representative Color Doppler image of a fetal UmA at gd 14. (B) Representative pulse-wave doppler images from $Cpa3^{+/+}$ + PBS (i) and $Cpa3^{Cre/+}$ + anti-CD122 (ii, iii) mice, showing normal end diastolic flow (i), absent end diastolic flow (ii), or reversed end diastolic flow (iii). PSV (C), EDV (D), systolic/diastolic ratio (E), and resistance index (F) of UmAs of fetuses from $Cpa3^{+/+}$ + PBS (mice n = 3, UmA measurements n = 7) and $Cpa3^{Cre/+}$ + anti-CD122 (mice n = 3, UmA measurements n = 10) mice at gd14. Data are presented as mean with SEM. Statistical analysis was performed using an unpaired *t*-test (*p < 0.05). UmA, umbilical artery; gd, gestation day; PSV, peak systolic velocity; EDV, end diastolic velocity. This figure is republished from a previous publication[23].

DISCUSSION:

Using our ultrasound system, we demonstrated fetal growth restriction in MC/NK-deficient mothers from gd10 on. Furthermore, at gd10 and 12, we observed reduced placental dimensions, and at gd14 the absence or reversion of end diastolic flow in the UmAs of some fetuses of uMC/uNK-deficient mice. This sign of poor vascularization was associated with a significant resistance index of the arteries indicating IUGR. Results confirm the important role of uMCs and uNKs in pregnancy and fetal well-being and in understanding the course of IUGR.

The protocol is applicable at every gestation day from gd5 onwards (after implantation). There are some critical steps in the protocol that must be taken into consideration. Firstly, hair removal must be done carefully. For example, excessive contact with the depilation cream may cause skin irritation. However, incomplete hair removal leads to signal interference visible as a shadow on the screen. Another reason for an insufficient signal (shadows or grainy pictures) can also be a too-low amount of gel placed between the mouse and ultrasound beam. In our experience, rather a high amount of gel (approximately 10 mL) is necessary for sufficient signal visibility. Second, 2D measurements can be somehow prone to inaccuracy. To minimize measurement differences between implantations, we advise the use of the largest available size when encircling the implantation. For precise placenta measurements, all implantations were positioned in a way in which UmA blood flow could be seen. Additionally, in order to minimize sources of mistakes, measurements should be always performed by the same operator. Third, for pulse-wave doppler

measurements, it is important to watch the angle between the direction of blood flow and the ultrasound beam. A too-high angle or different angles between the animals in a single experiment may lead to inaccurate velocity measurements. Attention should also be paid to the risk of repetitive narcotization of the females. To reduce this risk and stress for the mother, ultrasound measurements should be done no more than every second day.

The possibility to follow-up fetuses at relevant gestational days throughout pregnancy is a great advantage of the ultrasound technology. Contrary to sacrificing mice at different pregnancy stages, the technology enables us to perform accurate longitudinal analyses of individual pregnant mice. Despite this strength, there are some limitations of the system that should be considered. For example, fetuses may change positions during the course of pregnancy. Hence, it may be difficult to allocate certain data sets obtained at different times to individual fetuses. Additionally, sometimes it is not possible to monitor some fetuses at later gestation days, as i) their position can be difficult to reach with the beam, ii) fetuses may be too large to fit the screen, or iii) they may be hidden underneath the intestine. Depending on the mouse strain, whole implantation measurements are possible until gd12 or gd14. Later on, only single organs of the fetuses, including the heart, can be measured and recorded. The whole implantation itself is too large at later pregnancy stages to fit in the screen.

To the best of our knowledge, ultrasound imaging is (together with magnetic resonance imaging and computer tomography) the only available method to analyze the indicated parameters during pregnancy without sacrificing several animals at different gestational days. This is especially true for doppler imaging, which is the only method able to accurately evaluate blood flow and direction (red = flow in the direction of the ultrasound beam; blue = flow in the opposite direction of the ultrasound beam). During pulse-wave doppler imaging, the ultrasound beam sends out several pulses that are returned by the tissue and provide velocity information about blood flow[24].

As ultrasound itself seems to be harmless for the mother and fetus, ultrasound imaging is perfectly suited for pregnancy research. Nevertheless, the methods described in this manuscript can be applied to numerous other research areas, as well; for example, the system also allows for 3D measurements, visualization and quantification of tissue movement over time, visualization of blood flow in tumors, detection of biomarkers on the cell surface, blood pressure measurements, and ultrasound-guided injections.

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DISCLOSURES:

The authors have nothing to disclose.

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Fig. 1

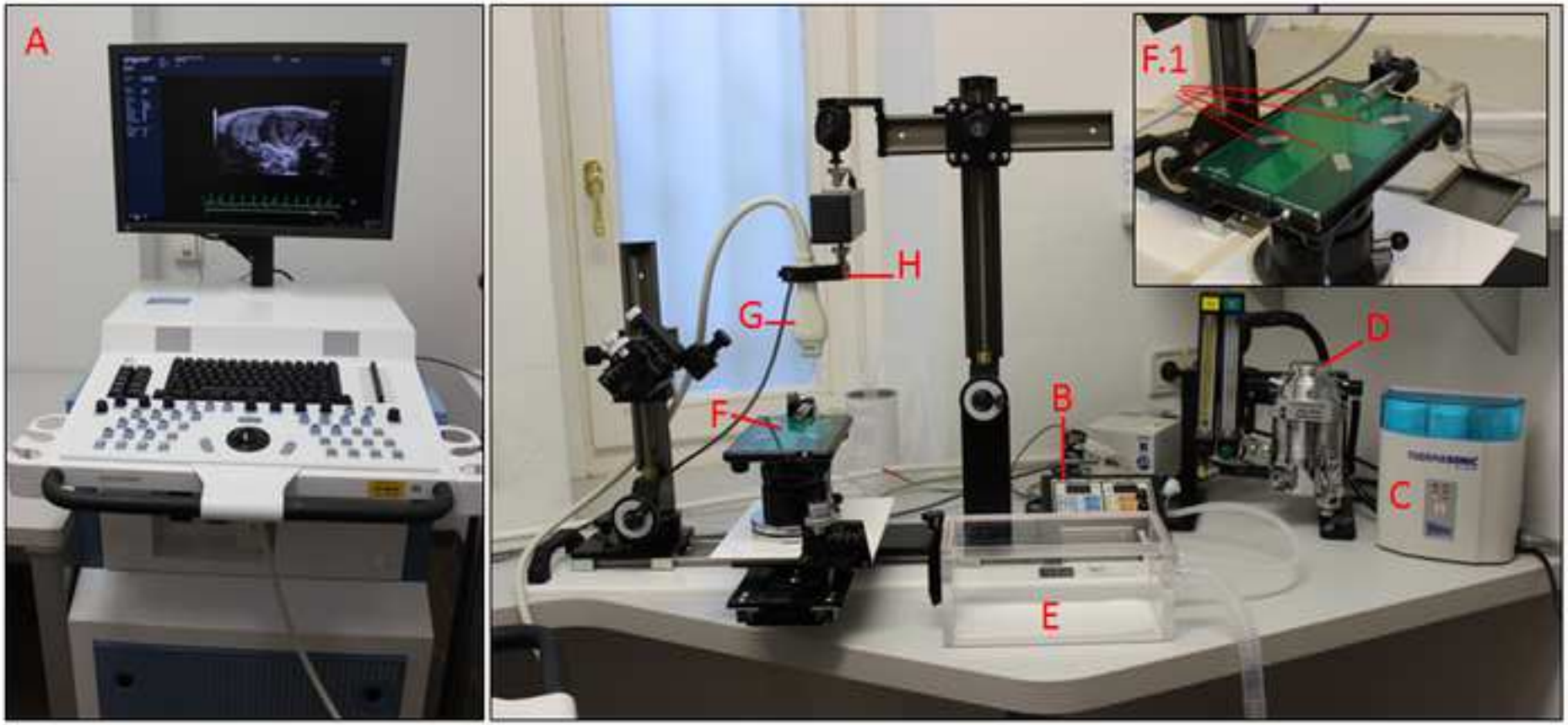
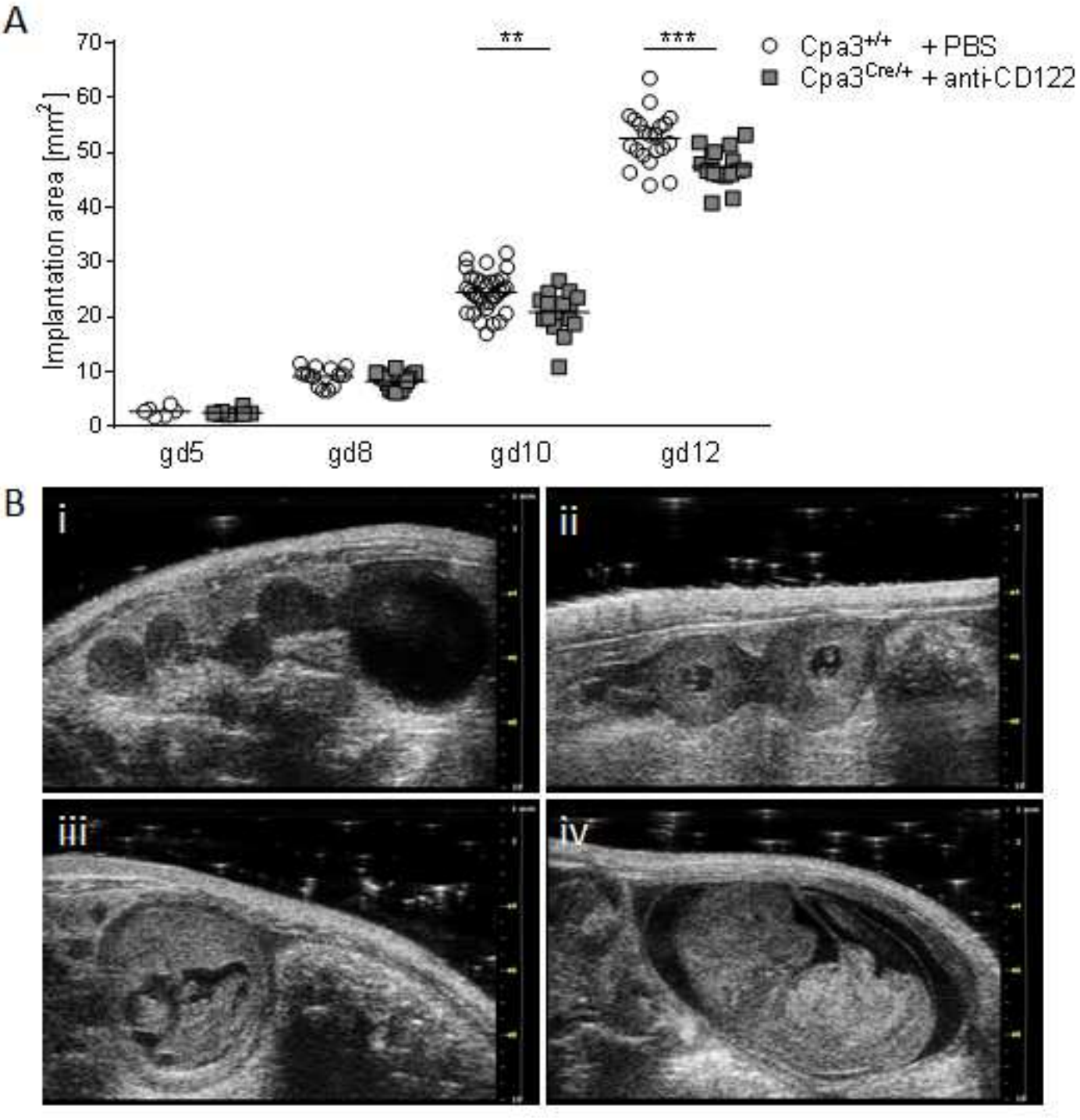


Fig. 2



Figure

Click here to access/download;Figure;Fig 3.tif

Fig. 3

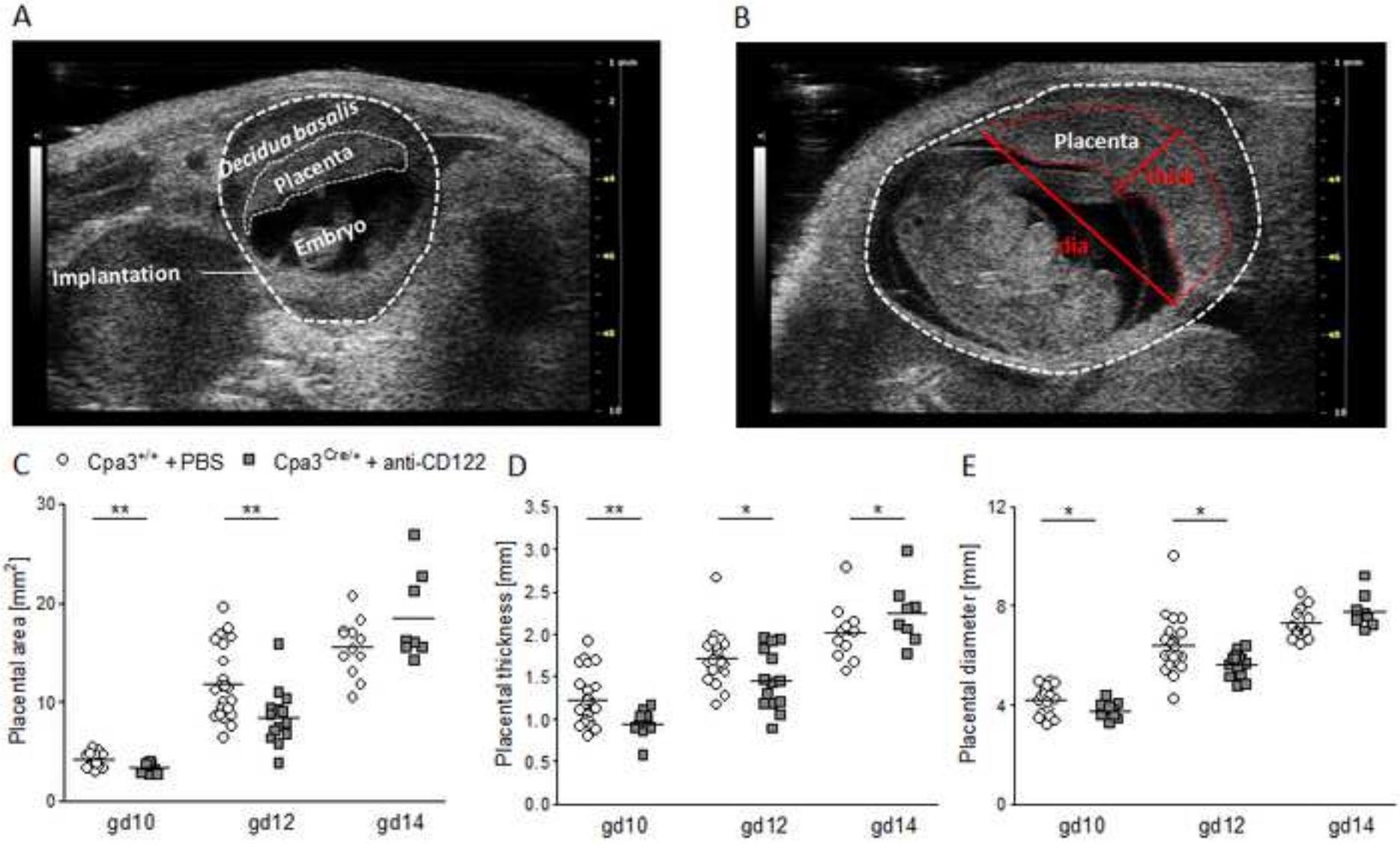


Fig. 4

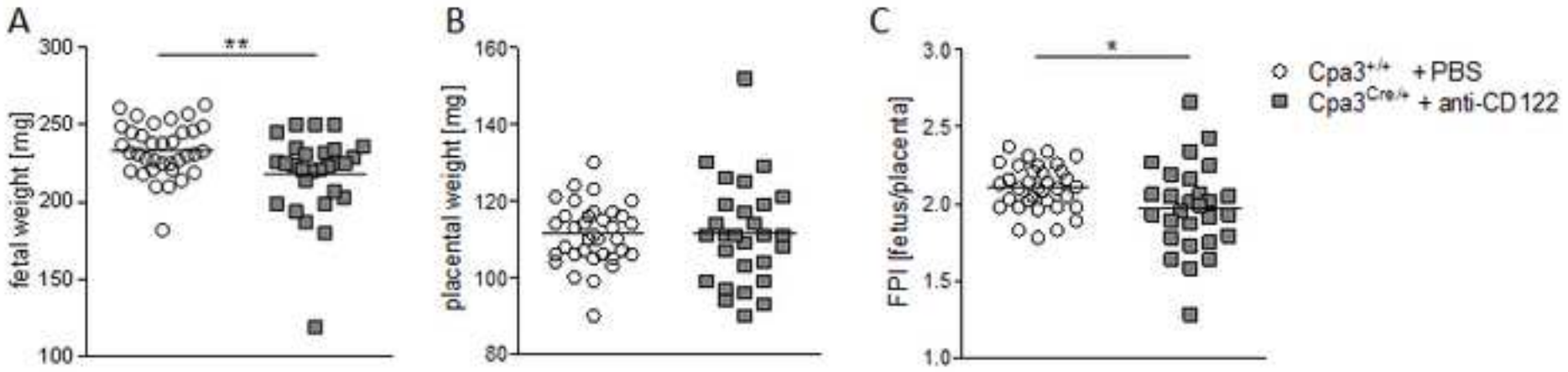


Fig. 5

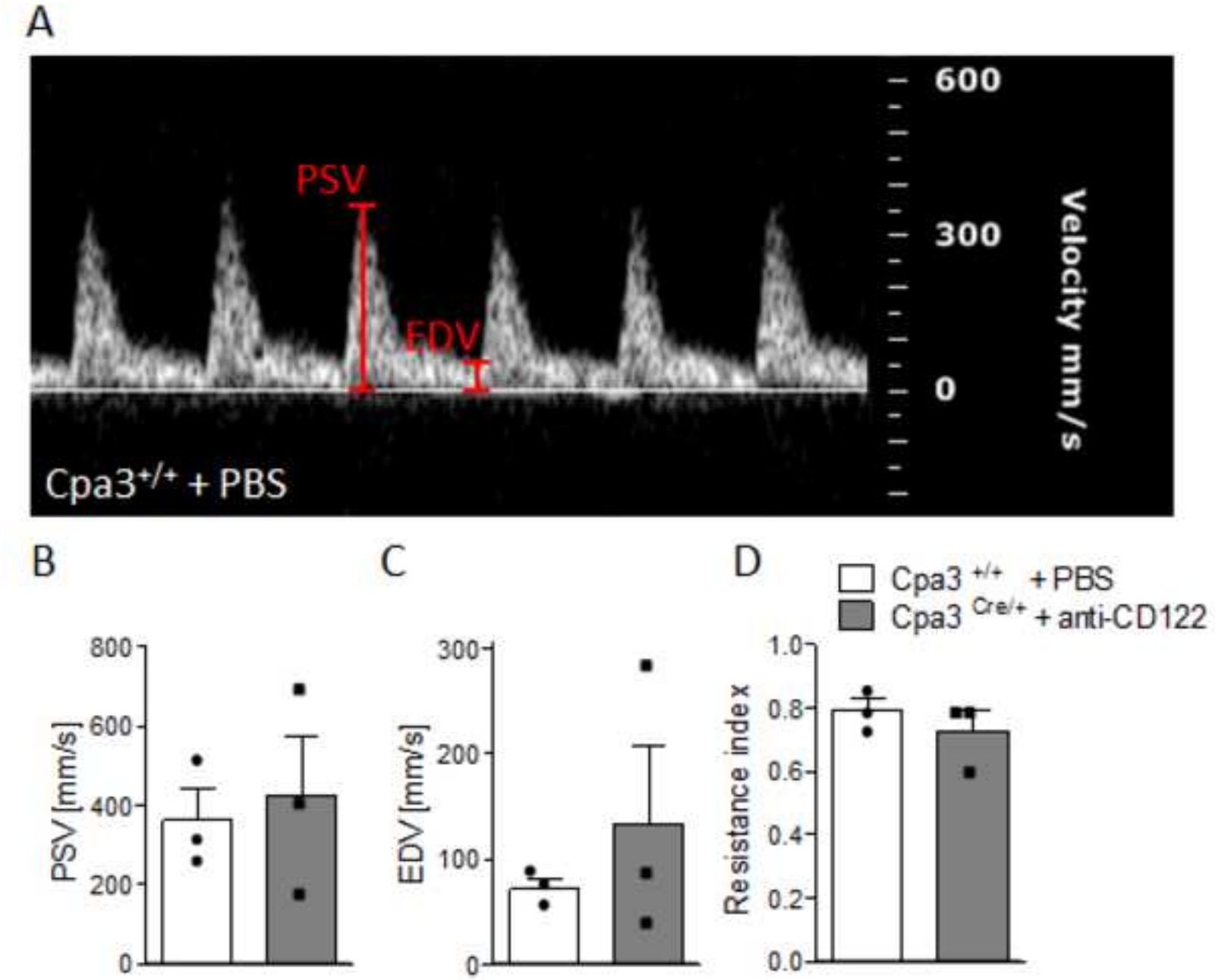
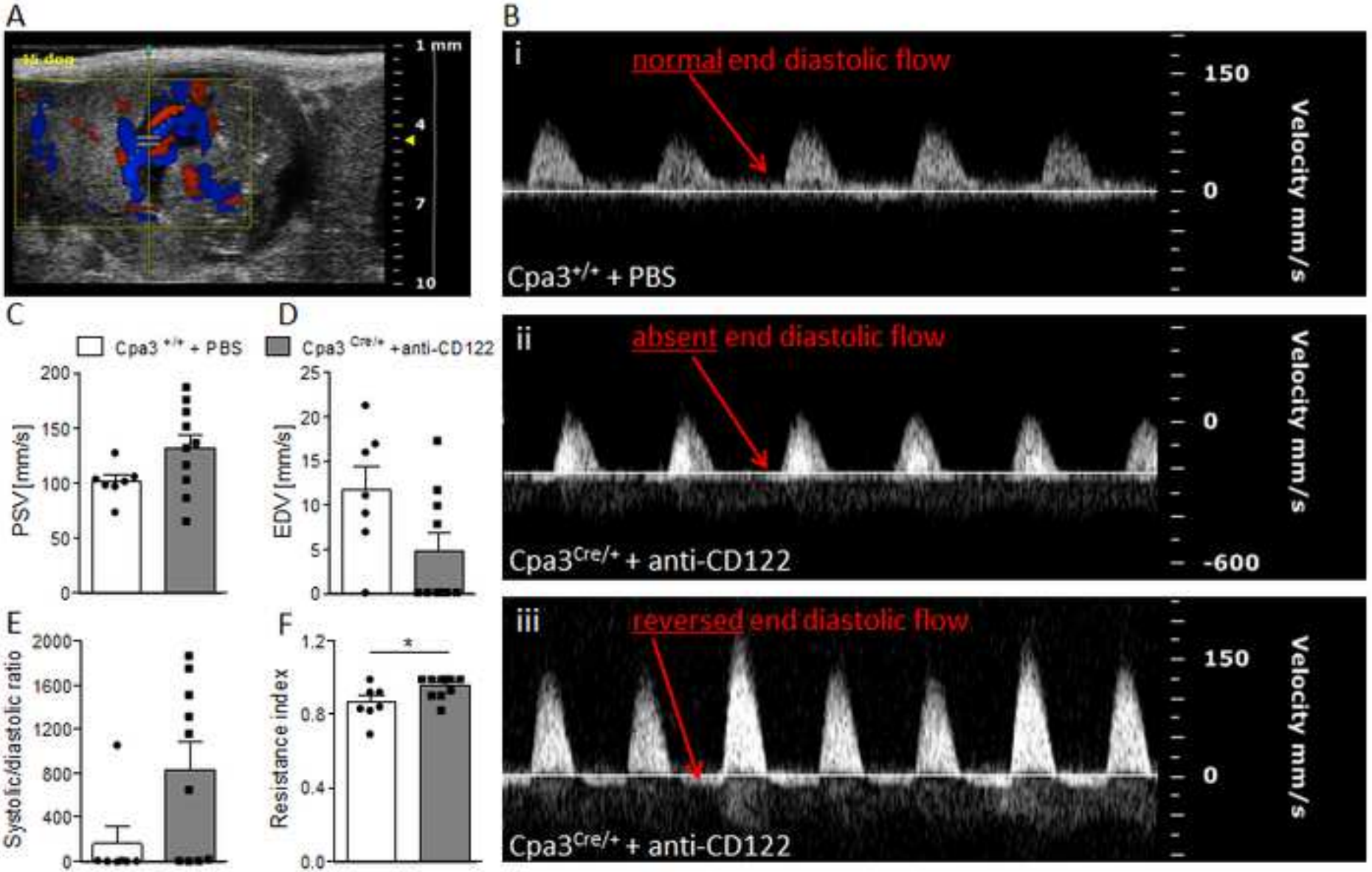


Fig. 6



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
LEAF anti-Maus CD122 (IL-2Rβ)	BioLegend	123204	Klon TM-β1; 500 µg
Vevo 2100 System	FujiFilm VisualSonics Inc.		Transducer MS550D-0421
Vevo LAB Software	FujiFilm VisualSonics Inc.		
Isoflurane	Baxter	PZN: 6497131	
Electrode gel	Parker	12_8	
Surgical tape	3M Transpore	1527-1	
Eye cream	Bayer	PZN: 1578675	
Cotton tipped applicators	Raucotupf	11969	100 pieces
Depilatory cream	Reckitt Benckiser	2077626	
Compresses	Nobamed Paul Danz AG	856110	10 x 10 cm
Ultrasound gel	Gello GmbH	246000	

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Author(s): Nicole Meyer, Thomas Schiller, Ana Claudia Zenclussen

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CORRESPONDING AUTHOR:

Name:	Ana Claudia Zenclussen
Department:	Otto-von-Guericke University
Institution:	Experimental Obstetrics and Gynecology
Article Title:	High frequency ultrasound for the analysis of growth restricted fetuses in natural killer cell depleted mast cell-deficient mice
Signature:	
Date:	June 8, 2018

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Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Author's response: As recommended we proofread the manuscript.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Author's response: The link to the editorial policy of Frontiers Immunology that allows re-prints was uploaded via a doc file to the Editorial Manager account of JOVE. A specific citation appears in the figure legend.

3. Please revise the title to be more concise.

Author's response: We have changed the title into: High frequency ultrasound for the analysis of fetal and placental development in vivo"

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

5. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Author's response: The revised version of the manuscript includes SI abbreviations for all units as well as a space between numbers and units.

6. 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 and Reagents. For example: Vevo 2100, FujiFilm VisualSonics Inc., MS550D-0421, etc.

Author's response: We thank the editor for this remark and removed all commercial language from the manuscript and used generic terms instead.

7. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of "Vevo 2100 Imaging System" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

Author's response: See comment number 6.

8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Author's response: The protocol text was revised and all personal pronouns were removed.

9. Please revise the protocol to contain only action items that direct the reader to do something (e.g.,

"Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.

Author's response: We thank the editor for pointing out this issue that was revised as recommended. Now every step of the protocol is written in imperative tense and additional information can be found in the "Note"-parts.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

For example:

1.5: Please describe how this step is actually done.

3.1.2: Please specify how proper anesthetization is confirmed.

Author's response: We added more details to our protocol steps to be sure that viewers can replicate the protocol easily.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The protocol submitted by Meyer et al presents a detailed description of high frequency ultrasound in mouse fetuses. The protocol is well written and easy to follow, with adequate details for a reader to be able to perform a similar analysis. The images presented with the manuscript greatly aid in understanding the experimental setup, as well as the data acquisition and analysis. Overall this is an excellent protocol. However, there are a few minor concerns that could be addressed to further strengthen the manuscript.

Minor Concerns:

The authors mention in the discussion that a major strength of the protocol is that it may be applied serially throughout a pregnancy in order to follow the same fetuses through in utero development. Although the protocol is relatively non invasive for the animals, they still need to be anaesthetised and have their fur fully removed. This causes a stress on the animal, particularly with temperature regulation. Could the authors comment on any post-op support that is given to the animal to minimise this stress? Also, how does repeated anaesthesia and hair removal affect the physiology of the pregnancy in mice?

Author's response: We thank the reviewer for this helpful comment. Indeed, the anesthesia causes stress to the animal. To reduce the stress level of the mice to a minimum we let the animal alone in a cage for a minimum of 5 min after the measurement. The animal has time to wake up and to orientate before we place it back in the original cage where it is housed together with other mice. Mice wake up very quickly after the isoflurane was turned off. It takes just around 20 s. Another point to reduce the stress is to do

the ultrasound measurements not every day but not more than every second day (here gd5, 8, 10, 12, 14). We have now mentioned these important points in the protocol.
We do not think that the hair removal affects the physiology of pregnancy in mice. For the used depilatory cream there is no hint that hair removal should not be performed during human pregnancy. We cannot be sure that the repeated anesthesia does not affect pregnancy parameters in mice. But if so, it should have the same effect in the control group.

It may be useful for readers who may be unfamiliar with the Vevo 2100 system to add some details about the sensitivity and reliability of the instrument. For example, approximately what magnitude of uterine artery velocities can be reliably resolved between groups? Also, approximately how many animals would need to be studied in order to find a statistically significant difference in various measurements?
Author's response:
The UA velocity depends on different conditions, for example if the animal is pregnant or not pregnant, on the specific gestation day as well as the mouse model. In the literature you find several publications analyzing UA velocities at different stages or under different conditions (examples: PMID:16603699, 26811058, 25968580, 23986360, 29319186). To proof the reliability of the instrument it is conceivable to sacrifice a few animals at gestation days of interest and analyze specific parameters e.g. placental weight to compare these results with the data obtained by using the Vevo 2100 System. How many animals are needed for a study depends on the individual experimental design. For analyzing fetuses or placentas possibly fewer animals are needed because mice have high litter sizes.

The authors also mention in the discussion that rather a high amount of ultrasound gel is needed to acquire high quality images. Please provide an approximate volume of gel needed per mouse.
Author's response: We used an amount of around 10 ml gel per mouse and included this information in the discussion part.

Reviewer #2:
Manuscript Summary: This manuscript aims to describe the technique and utility of ultrasound during murine fetal gestational to obtain longitudinal fetal growth patterns and placental development. This will provide less invasive options for fetal monitoring and research.

Major Concerns: No major concerns; well composed and easy to interpret

Minor Concerns:
- Needs minor language editing for better flow
Author's response: We thank the reviewer for this comment and proofread the manuscript as recommended.

- Introduction should be more concise
Author's response: Thank you! We have now changed the introduction for more conciseness.

- Ultrasound for mice is not "harmless" as anesthesia is required (carries small risks, pain of injection, etc)

Author's response: We agree with the reviewer and mentioned that there is a risk for the animal due to the isoflurane narcotization. To reduce the stress for the mouse because of the narcotization ultrasound measurements should be done not more often than every second day.

- "below the bladder" is confusing - use anatomical direction (caudal/cephalad)

Author's response: We thank the reviewer for this valuable suggestion and changed to the correct anatomical directions.

- How do you ensure the correct "transplacental" cross-section - if it is not midline, the placenta will be measured too small (or if the plane is not perpendicular, it will be measured too large)

Author's response: Because of the high amount of fetuses within the belly and the limited space it is not easy to present the correct transplantal cross-section of all placentas and it takes a certain time. Nevertheless, f*or precise placenta measurements all implantations were positioned in the same way. Frames or cineloops were stored when the UmA blood flow could be seen. This position could not always be reached for all placentas, so that not every placenta from each animal may be measured but as many as technically possible.*

- Authors should note that MRI and CT have been used on pregnant mice, so ultrasound is not the only method

Author's response: We thank the reviewer for this comment, agree and added the information to the manuscript.

Reviewer #3:

Manuscript Summary:

This manuscript describes how the Vevo 2100 high resolution ultrasound imaging device works and can be used to study intrauterine growth restriction (IUGR), now called fetal growth restriction (FGR). The authors provide step by step procedural details to fire up the equipment and to prepare mice for this protocol. There is one major issue here. The manufacture of the device and other investigators have already described exactly the same details for assessing fetal blood flow, placental and fetal development, organogenesis, and tumors. The use of mice deficient in NK cells and mast cells may be an additional focus, although the rationale for NK and mast cell deficiency is not provided.

Author's response: We thank you for this remark. Based on our previous publications (doi: 10.1038/srep45106, doi: 10.3389/fimmu.2017.01913. eCollection 2017.) we were invited from JOVE to write this manuscript about the ultrasound technique. As the journal focus on methods rather than on the background of the study itself, we didn't provide the rationale for NK and MC-deficiency. By citation of our previous studies we give the reader the possibility to understand the rationale behind the use of this mouse model. The only important point to know for this paper is that NK/MC-deficient mice give birth to growth restricted pups. This is written in the text.

We agree with the reviewer in the point that protocols from the manufacture exist. The text presented here has been proofed and accepted by the company before submission.

Major Concerns:

1. The authors write in introduction that the method is cost-effective. Although the device is extremely useful, this comment may not be a correct one. The equipment costs close to $300,000 with minimum accessories. Adding specialized probes for different applications will balloon up the price to $400,000 or more. Finding an easily accessible space in the animal care facility is another issue. Since the device is likely to be used by multiple investigators, is there one technical operator for the groups to guarantee reproducibility?

Author's response: We thank the reviewer for this comment and agree. Although consumable materials that are needed for the technique are cheap, the machine and accessories are very expensive. We are sorry for the incorrect statement and changed it within the introduction part.
Concerning your comment regarding finding space in the animal facility or multiple investigator usage: Of course the acquisition of an ultrasound device it not possible or useful for every laboratory. Every group should decide if there is the space, if they have the money and if it fits with the experimental designs. The protocol written in the present publication should help investigators that already have such a machine to improve its use or it may help other researchers to evaluate whether the described method may be the optimal one for answering their scientific question. The sentence that says the measurements should be done by the same operator is just a hint not a requirement in order to minimize mistakes.

2. It would have helped a reader if the authors distinguished the use of the device for FGR and FGR/preeclampsia. How can the FGR be uniquely studied under two scenarios?

Author's response: We thank the reviewer for this question. We didn't try to distinguish for FGR and FGR/preeclampsia with the use of the device. This was done with other methods in our previous study. We used high frequency ultrasound imaging to ascertain the in utero growth of fetuses and placentas from NK/MC -deficient mothers that give birth to growth-restricted pups and to determine the time point at which IUGR starts. We also employed Doppler measurements to document blood supply to the fetus in females that were deficient for NK cells and MCs.

3. Will anti-CD122 antibody treatment of wild-type mice show any effects on fetal growth as observed by The Vevo 2100 device (Figure 2)?

Author's response: The anti-CD122 antibody treatment of WT mice has no effect on the weight of fetuses at birth (doi: 10.1038/srep45106). The anti-CD122 antibody treatment of MC-deficient mice, as shown here, affects fetal growth from gd10 onwards (Fig. 2A) and leads to a significant lower fetal weight at gd14 (Fig. 4A) gd18, and also at birth (doi: 10.1038/srep45106).

4. In Figure 3, how come the placental area in anti-CD122 mice is normalized or higher on gd 14? Are these fetuses of the same size at birth? Such concerns also apply to other figures.

Author's response: We thank the reviewer for the interest in our experiments. As the placenta reach the full development around gd16 in mice, it is possible that they catch up their growth. At gd14 fetal weight is lower in MC/NK-deficient mice in contrast to controls (Fig. 4A) whereas placental weight is comparable between the groups (Fig. 4B). At birth fetuses of anti-CD122-treated mothers have a comparable weight

to fetuses of PBS-treated WT mothers. Fetuses of anti-CD122-treated MC-deficient mothers show an significant lower birth weight compared to fetuses of PBS-treated WT as well as anti-CD122-treated WT mothers (doi: 10.1038/srep45106).

5. Several references from Drs. Adamson's and Karumanchi's labs are not included.
Author's response: We are aware that Adamsons and Karumanchis labs have many relevant and very interesting publications about preeclampsia, hypertensive disorders, and fetal weigh. Nevertheless, for this manuscript, none of them was relevant.

Minor Concerns:
1. Please provide some conceptual explanations of their finding, such as the exact pathways that are dysregulated during pregnancy by NK and Mast cell deficiency.
Author's response: We understand that the reviewer is interested in specific pathways and the background of our study. Nevertheless, JoVE is a methods-based journal. To the best of our knowledge, an exact explanation of the background would not be suitable for the journal. In the "Transcript template" it is written for specific parts:
- "Abstract: The abstract should focus on the method being presented rather than the results of a specific experiment."
- "Discussion: The Discussion section of the article should be focused on the protocol and not the representative results. "
Please see our previous publication (doi: 10.1038/srep45106) for the exact pathways that are dysregulated during pregnancy by NK/MC-deficiency if you are interested in.
Nevertheless, if the Reviewer may be interested to read our original paper published in Scientific Report where these issues are addressed.

Dear Dr Meyer,

Thank you for your message. The article titled "Simultaneous Ablation of Uterine Natural Killer Cells and Uterine Mast Cells in Mice Leads to Poor Vascularization and Abnormal Doppler Measurements That Compromise Fetal Well-being" (Front. Immunol., 08 January 2018 | https://doi.org/10.3389/fimmu.2017.01913) is an open-access article distributed under the terms of the Creative Common Attribution ("CC BY") licence (please refer to the Copyright section of the article page). Authors retain the copyright of their work and the content is free to distribute and use, given that the original source is properly acknowledged and cited, subject to any copyright notices concerning any third-party content. You can find more information about the Frontiers policy on copyright at http://www.frontiersin.org/Copyright.aspx and at the bottom of the article webpage (please see link above) in the Copyright paragraph. Attribution of Frontiers as the original publisher is required. Please note that re-publishing should comply with any copyright notices on third-party graphics and with any need to obtain permission to reproduce those.

Best regards,
Nikolaos Chatziandreou
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