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Feasibility of 2-D ultrasound shear wave elastography of fetal lungs in case of threatened preterm labor: a protocol study



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Manuscripts

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3 **Feasibility of 2-D ultrasound shear wave elastography of fetal lungs in case of**
4 **threatened preterm labor: a protocol study**
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3 **Contributors** : All the authors contributed to the conception and design of the study. NM,
4 SA, and RR provided the idea for the research or article, created the hypothesis and wrote the
5 original proposal. NM, CV, LP and RR significantly contributed to writing the paper. NM,
6 CV, GB and LP wrote this protocol paper. All authors read and approved the final manuscript.
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14

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ABSTRACT

Introduction

2-D ultrasound Shear Wave Elastography (SWE) could be considered as a new noninvasive tool for monitoring fetal lung development based on evaluation of mechanical properties during pregnancy. Interesting results are available concerning use of SWE on developing organs, especially on premature infants and animals models. The main objective in this study is to evaluate feasibility of 2-D SWE in human fetal lungs between 24 and 34 weeks of gestation (WG) and to modelize fetal lung-to-liver elastography ratio (LLE ratio). Secondary objective is to assess LLE ratio's variations between normal lung and lung surfactant-enriched after a corticosteroids course indicated for a threatened preterm labor (TPL).

Methods and analysis

A prospective case-control study will be performed between 24 and 34 weeks of gestation (WG), after the fetal period of organogenesis. Fetal lungs and liver will be explored by SWE into two groups: fetuses of women with an uncomplicated pregnancy will be considered as control group and fetuses of women with a TPL requiring administration of corticosteroids will be enrolled as cases. Corticosteroids generate an effect model interesting for assessment of LLE ratio variation in the cases. Primary judgment criterion is the value of Elasticity modulus (E).

We shall not attempt to define reference value of fetal lung elasticity or to correlate SWE value with respiratory function at birth. This protocol is designed for demonstrating applicability of the technique in fetal lungs and liver.

Ethics and dissemination

The study respects the ethical standards established in the declaration of Helsinki. Approval of the study protocol was obtained from the human ethical research committee (Comité de Protection des Personnes EST II, process number 15/494) and the French National Agency for Medicines and Health Products Safety (process number 2015-A01575-44). All participants will sign a statement of informed consent.

Trial Registration number: NCT02870608

Key words: Shear wave elastography, stiffness, ultrasound, lung, prenatal

Strengths and limitation of the study

- The study is designed to evaluate feasibility of 2-D ultrasound Shear Wave Elastography in human fetal lungs between 24 and 34 WG and to modelize fetal lung-to-liver elastography ratio (LLE ratio).
- This study will be the first one to demonstrate applicability of Shear Wave Elastography in human fetal lungs.
- Different factors will be taken into account to understand variability of measures: acquisition depth, number of measurements and representative values such as median or mean values.
- We shall not attempt to define reference value of fetal lung elasticity or to correlate SWE value with respiratory function at birth.

INTRODUCTION:

Background

Prenatal evaluation of fetal lung maturity (FLM) is still a challenge and different approaches have been reported with invasive biological tools or fetal imaging including ultrasounds and magnetic resonance imaging (MRI). FLM is mainly correlated with surfactant level and numerous tests have been developed to quantify this production: lecithin/sphingomyelin ratio (L/S), laminar body count, testing for Phosphatidyl glycerol with thin-layer chromatography, and estimation of the surfactant/albumin ratio using polarized light. All of these tests have been shown to have good sensitivity and negative predictive values; however, they all have a low positive predictive value and need invasive procedure^{1,2}. Thus, amniocentesis for FLM became obsolete³.

Lung and liver signal or echogenicity were often compared as ratio to assess FLM in several studies⁴⁻¹⁰. Non-invasive assessment of FLM has been first attempted using ultrasonography quantified by gray level histogram^{4,11,12}. These studies demonstrated sonographic changes associated with lung maturation but weak correlations between echogenicity and respiratory distress syndrome (RDS). Quantitative analysis with specific software has been secondly proposed to improve robustness. Fetal lung texture analysis with this method demonstrated a strong correlation with gestational age, biological test on amniotic fluid and risk of neonatal respiratory morbidity^{13, 14}. Additionally, several studies found a significant association between fetal lung-to-liver signal intensity ratio and estimated gestational age with fetal Magnetic Resonance Imaging (MRI). Actually, this technique is mainly used to assess fetal lung development and volume, especially in case of congenital diaphragmatic hernia or severe oligohydramnios. Nevertheless, the accuracy of both MRI and ultrasounds for estimating long-term respiratory outcomes remains limited¹⁵⁻¹⁹. These previous techniques propose prenatal evaluation of lung maturity based on imaging features, but prediction of the pulmonary function through prenatal biomechanical properties seems relevant. With this hypothesis, we advanced the concept of “prenatal functional imaging” and shear wave elastography (SWE) could be a promising tool.

SWE is a recent ultrasound technology, which enables to assess the stiffness of tissues in real time²⁰. In locally homogeneous and purely elastic medium, we can calculate Young's modulus (kPa) from the shear wave speed ($\text{m}\cdot\text{s}^{-1}$) by using SWE with the formula: $E= 3\rho\cdot c_{\text{sw}}^2$ (ρ being the medium density and c_{sw} the shear wave speed). The stiffness at any location of

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3 the ROI can be sampled using measurement tools to obtain a quantitative evaluation either in
4 terms of shear wave speed or Young's modulus. A low shear wave speed corresponds to a
5 soft tissue, while a shear wave high speed indicates a stiff tissue. Study of deep organs is
6 possible with SWE because this method does not require any compression- relaxation
7 sequence on the target organ. One of the advantages of SWE over other elastography methods
8 is that the generation of the mechanical impulse is operator-independent. SWE is expanding
9 its range nowadays by its promising role for examination of various organs (liver, thyroid)
10 and for aiding discrimination of lesion characteristic, especially breast or prostate tumors²¹⁻²⁴.

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There is to date no publication regarding its use on human fetuses. The Food and Drug Administration (FDA) has so far, not yet approved use of SWE for obstetric clinical applications because of the paucity of data in the literature. Although there is no report about apparent histological changes with shear wave, the absence of others bioeffects could not be discarded and further studies are recommended²⁵⁻²⁷. However, setting parameters respect mechanical and thermal indexes for soft-tissues and bone, which are necessary to allow an obstetric examination defined by the FDA^{28,29}.

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Interesting and reassuring results are available concerning use of SWE on developing organs, especially on premature infants and animals models. Principals organs explored are the liver, brain and lungs. Quarello et al demonstrated the possibility to use SWE in non-human primate fetuses and its feasibility to explore fetal organs. They found that elasticity values were related to organs and gestational age³⁰. Concerning fetal lungs elasticity, measurements of the proximal lungs seem to increase throughout the pregnancy. Concerning use of SWE on premature infants, results are more numerous and include exploration of the liver or the brain. Alison et al. have demonstrated feasibility and reproducibility of liver stiffness in preterm neonates with intra uterine growth restriction (IUGR) and gestational age at birth between 26-31 weeks of gestation WG³¹. Others studies reported contribution of SWE to the diagnosis of biliary atresia in neonates^{32,33}. Kim et al described variation of brain elasticity in different regions in healthy neonates born between 28 and 40 WG³⁴. Su et al quantitatively evaluated the effect of acoustic radiation force impulse imaging (ARFI) in neonatal brain development. The authors found that full-term neonates had significantly higher elasticity values than preterm neonates and there were no reported immediate adverse events³⁵. Others teams work on application of this technology to neonates as a supplementary tool to detect early ischemic brain injury³⁶.

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If we describe fetal thorax centered on a 4-chamber view of the fetal heart with SWE, the following observations can be made (figure 1):

- SWE is able to make distinction between fetal soft and stiff tissues. Fetal ribs appear red colored mapped whereas proximal lung appears blue colored.
- A homogeneous colored area can be obtained on the proximal lung with a color scale ranging from 0 to 48 kPa. A homogeneous ROI is important for the reliability of the results.
- SWE can differ according to the acquisition depth. Proximal lung appears with a blue homogenous distribution whereas distal lung appears with green homogenous distribution.

Objectives

The main objective of this pilot study is to evaluate feasibility of SWE in human fetal lungs between 24 and 34 WG and to modelize fetal lung-to-liver elastography ratio (LLE ratio) from the most reproducible regions of both target organs, between 24 and 34 WG. Secondary objective is to assess LLE ratio's variations between fetal lungs with different supposed biomechanical properties: normal lungs and lungs surfactant-enriched after a corticosteroids course indicated for a threatened preterm labor.

METHODS/DESIGN

Study design and setting

A prospective case-control study will be performed at the University Hospital of Besançon, (Besançon, France), Department of Obstetrics and Gynecology. Fetal lungs and liver will be explored by SWE between 24 and 34 WG in two groups: fetuses of women with an uncomplicated pregnancy will be considered as control group, and fetuses of women with a threatened preterm labor requiring administration of corticosteroids will be enrolled as cases. In this pilot study, corticosteroids generate an effect model interesting for assessment of LLE ratio variation in the cases. We take advantage of the action of corticosteroids to explore another medium with different supposed biomechanical properties. Indeed, corticosteroids accelerated development of type 2 pneumocytes leading to surfactant production. Similarly, there is a significant increase in saturated phosphatidylcholine content in fetal lung, modifying propagation of shear waves potentially³⁷.

Control group will be recruited during routine prenatal visits or ultrasounds according to the French ultrasounds schedule (ultrasound screening during the second trimester between 20-25 WG and the third trimester between 30-35 WG³⁸). Cases will be enrolled during their hospitalization for threatened preterm delivery (figure 2). The design, conduction and reporting of this study will follow the norms of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement³⁹.

Ethical considerations

The study respects the ethical standards established in the declaration of Helsinki⁴⁰. Investigators undertake to respect law no. 2004-806 of August 9,2004 relative to public health policy on clinical trials, made effective by implementing Decree 2006-477 of April 26,2006⁴¹. Approval of the study protocol was obtained from the human ethical research committee (Comité de Protection des Personnes EST II, process number **15/494**) and the French National Agency for Medicines and Health Products Safety (process number **2015-A01575-44**). The study is registered on clinical trial.gov with the following number: **NCT02870608**. All participants will sign a statement of informed consent and will be allowed to abandon the study at any time, with no negative consequences. All procedures of the study will be confidential. The study will involve collection and storage of ultrasound images and

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3 study participants will therefore be thoroughly informed. All variables (patient parameters,
4 data items, data elements) will be aggregated into electronic Case report Forms.
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6 As explained in the background, The Food and Drug Administration (FDA) has so far,
7 not yet approved use of SWE for obstetric clinical applications because of the paucity of data
8 in the literature. In this study, fetuses will be included from 24 to 34 WG, over the period of
9 organogenesis. In the patient information sheet, it is specified that previous studies explored
10 liver or brain of premature infants with SWE. Alison et al. studied liver stiffness in preterm
11 neonates with intra uterine growth restriction (IUGR) and gestational age at birth between 26-
12 31 WG³¹. Franchi-Abella et al evaluate feasibility and diagnostic accuracy of SWE for the
13 assessment of liver stiffness in 96 patients and 7 were preterm newborns between 30-35 WG
14⁴². Both studies did not report adverse outcomes and were approved by institutional review
15 board. Moreover, consent form mentions conclusions of an experimental study of the
16 biological effects associated with SWE in brains of neonatal mice. Results indicated that
17 using SWE doe not cause detectable histologic changes. Potential effects on intracellular
18 signaling pathways were detected when the SWE scanning lasted more than 30 min²⁵. Thus,
19 patients will be informed that our study is focused on fetuses with a gestational age similar to
20 preterm newborn included in the above-mentioned studies. Moreover, exploration with SWE
21 will concern only lung and liver with duration of less than 10 min.
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34 **Participants**

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37 Cases will be recruited during hospitalization for a threatened preterm labor requiring
38 administration of corticosteroids. The complementary inclusion criteria are: pregnant women
39 aged 18 years or more, singleton pregnancy with eutrophic fetus, signature of consent and
40 affiliation to health insurance scheme (figure 2). Patients will be stratified by gestational age
41 between 24 and 34 WG, with one class every WG (10 classes in total) and 5 patients per class.
42 After obtaining consent, a SWE exam will be performed before the first intramuscular
43 administration of corticosteroids “day 0” (betamethasone 12mg). A second SWE exam will be
44 performed within 24 h after the second intramuscular administration of corticosteroids “day
45 2”. A third SWE will be performed between the fifth and the seventh day following the first
46 administration of corticosteroids “day 5-7”, if the women are not early discharge at home.
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54 For the control group, women will be matched by gestational age and will be recruited
55 during their pregnancy monitoring. Information concerning the study will be given as early as
56 the second trimester by obstetrician or midwives during medical visits or ultrasounds. The
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3 complementary inclusion criteria are following: major pregnant women, uncomplicated
4 pregnancy between 24 and 34 WG, singleton pregnancy with eutrophic fetus, signature of
5 consent and affiliation to health insurance scheme (figure 2). If they are willing to participate,
6 a first SWE exam will be proposed at “day 0”, followed by a second one in two days “day 2”.
7 Patients who come back for an SWE exam performed outside current medical visit will
8 receive financial compensation for travel expenses. As well as cases, control patients will be
9 stratified by gestational age between 24 and 34 WG.
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13 Exclusion criteria common to both groups are: Fetal lung or liver pathologies,
14 inclusion in another medical study, patients under legal incapacity.
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19 **Variables and measuring technique**

- 20 • **Prenatal variables**

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22 Primary judgment criterion is the value of elasticity modulus (E) (Young’s modulus)
23 expressed in kilopascal (kPa). A Logic E9 system (General Electric Medical System,
24 Milwaukee, WI, USA) with CE certification CE LNE/G-MED [CE0459] will be used for this
25 study, equipped with abdominal convex probe 1-6 MHz (C1-6-D probe). To provide robust
26 shear wave and optimize its detection, Logiq E9 uses innovative techniques. Comb-push
27 Ultrasound SWE generates multiple shear wave sources into the region of interest (ROI) and
28 uses directional filtering to remove the interference. The TAST technique is able to robustly
29 track shear waves and correct for the sequential tracking delay⁴³. This technique has the merit
30 of rapid and solid reconstruction of a large elasticity map with the only single acquisition by
31 generating multiple shear waves from multiple unfocused push beams.
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42 The shear wave acquisition measurement protocol is saved in a specific preset that will
43 be used for every patient: Mode 2D: Harmonic imaging central depth = 6 cm, frequency = 6
44 MHz, Acoustic Power 100%; Mode elastography: Shear wave signal frequency ranges from
45 50 to 400 kHz, Acoustic power of the pushing beam: 100%, Tracking power: 100%, color
46 scale ranging from 0 to 48 kPa. Each measurement has to be performed on a homogeneous
47 area to be valid. Operators will perform 2 cycles of 3 elasticity measurements while
48 systematically repositioning the probe on each target organ. A cycle includes: 3
49 measurements on the proximal lung (anterior “P1”, medium “P2” and posterior portion “P3”),
50 3 measurements on the distal lung (anterior “D1”, medium “D2” and posterior portion “D3”)
51 and 3 measurements on 3 liver segments (IV, V, VI) (figure 3). Implementation of Shear
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3 Wave elastography displays 2D images of Young's Modulus in the target organ. In real time,
4 the elasticity appears color coded, where blue identifies deformable tissue and red indicates
5 rigid tissues. The stiffness at any location will be sampled using a round ROI of 5 mm.
6 Distance between the probe and the target organ will be collected. The average stiffness in the
7 ROI will be automatically recorded by the system in a worksheet. The investigators will
8 adjust the position of the ROI using the B-mode image for guidance and take care to obtain
9 the most homogeneous color coded ROI before stiffness estimation (figure 4). Technical
10 failure was defined as failure to obtain a homogeneous color map in more than 50% in the
11 sampling area. To test the inter-observer variability, a second observer will successively
12 perform measurements on 30 fetuses. All measurements will be carried out directly by
13 ultrasound on the target organ and the second operator will be blinded to the feasibility and
14 results obtained by the first one.
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24 Evaluation of fetal weight will be performed during each exam according to Hadlock
25 formula based on Cephalic circumference (CC), Abdominal circumference (AC) and femoral
26 length (FL): $\log_{10} EPF = 1,326 + 0,0107 PC + 0,0438 PA + 0,158 LF + 0,00326 (PA \times LF)$
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31 • **Postnatal variables**
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34 The following pharmacovigilance data will be collected at birth and 3 months later in
35 order to assess safety of the device:
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38 - At birth in both groups: term of birth, weight, neonatal transfer, Apgar Score and
39 evaluation of respiratory distress by Silverman Score ⁴⁵.
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41 - Three months after birth: medical history, health problems, respiratory diseases or
42 symptoms, liver disease or symptoms and number of hospitalization since birth.
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46 **Limitation of Bias**
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48 Use of sampling method with 5 patients per class of gestational age will limit
49 repartition bias between control and cases. Inclusion of any pregnant women attending
50 Besançon University maternity for the control group, and inclusion of all women with a
51 threatened preterm labor will limit risk of selection bias. Risk of loss will be limited in the
52 cases group between "day 0" and "day 2" because patients will remain hospitalized for
53 corticosteroids administration. Nevertheless, risk of loss is most important between "day 2"
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3 and “day 5-7” because of possible early discharge at home after the second administration of
4 corticosteroids. For ethical and medical reasons, cases early discharge at home cannot be
5 convened in “day 5-7”. Risk of loss will be limited in the control group because of a financial
6 compensation for travel expenses. To limit inter-observer variation, expert sonographers will
7 perform elastography exams.
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11 12 13 **Study size**

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15 Because of the paucity of data regarding SWE on human fetal lung, the sample size
16 calculation was based on measurements of fetal lungs in pregnant baboons and extrapolations
17 made by clinicians, with the following assumptions: average expected value of elasticity
18 coefficient for fetal lung will be 2 KPa at 24 WG and 4 KPa at 34 WG. We hypothesized a
19 linear increase of elasticity coefficient during pregnancy in the control group and a decrease
20 in cases exposed to corticosteroids. At “day 2”, we therefore expect in the cases a variation of
21 0.2 KPa (corresponding to one week of lung maturation during gestation). Forty-two patients
22 per group will be needed to have 90% power to statistically demonstrate such a difference
23 (0.2 versus 0.057) assuming an α risk of 0.05 and standard deviation of 0.2.
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33 **Proposed statistical analysis**

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36 • **Technical validation:**

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38 Feasibility will be evaluated by assessing the number of exams performed and the number
39 of exams with interpretable results. Different limiting factors that can affect the results will be
40 evaluated, especially reduced amniotic fluid, fetal position and maternal body mass index.
41 Intra and inter-observer reproducibility will be evaluated by means of the intra-class
42 correlation coefficient (ICC). Further, each sets of measurements for evaluation of inter- and
43 intra-observer variability will be compared by calculating the following parameters as
44 described by Bland and Altman and graphically presented as Bland-Altman scatter plots (46).
45 Only repeatable and reproducible values of each ROI will be considered to modelize LLE
46 ratio.
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55 • **Clinical evaluation**

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3 Evolution of fetal lung SWE, fetal liver SWE and lung-to-liver-SWE ratio will be
4 assessed by group and overall. Lung-to-liver-SWE Ratio will be initially defined as the value
5 of the lung elasticity divided by the value of the liver elasticity.
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8 SWE values before corticosteroids administration will be compared between cases and
9 control groups. If there is a statistically significant difference, a confusional bias will have to
10 be sought between fetal lung elasticity values and threatened preterm labor. All results will be
11 presented as “delta” variation between two measurements in order to limit “non-comparability
12 bias” between both groups. If corticosteroids affect fetal lung elasticity, “delta before/after”
13 will be more important in cases than controls.
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18 Analysis of mean differences between groups will be carried out using a Student’s test or
19 Wilcoxon test according to distribution of data for quantitative variables. Shapiro-Wilk test
20 will be used to determine if data set is well modeled by a normal distribution. The relationship
21 of quantitative variables to each other will be tested using Pearson’s or Spearman’s
22 correlation as appropriate.
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26 Qualitative variable will be expressed as frequencies and quantitative variables will be
27 displayed as the mean \pm SD if normally distributed or as the median \pm IQR (Inter Quartile
28 Range) if asymmetrically distributed. Qualitative variables will be analyzed using a χ^2 test or
29 Fisher test.
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33 For statistical analyses, the level of statistical significance will be set at 5% ($p < 0.05$).
34 Statistical analysis will be performed with statistical software SAS for Windows, version 9.4
35 and MedCalc software, version 15.
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DISCUSSION

SWE could be considered as a new noninvasive tool for monitoring fetal lung development based on evaluation of mechanical properties during pregnancy. However, standardization for measuring SWE in human fetal lung is necessary to demonstrate applicability of the technique, and to understand limiting factors. This pilot study will focus on technical performances before considering clinical applications. In this protocol, SWE will be used after fetal period of organogenesis and pharmacovigilance data will be collected at birth and 3 months later in order to assess safety of the device.

With the study design, we shall not attempt to define reference value of fetal lung elasticity during pregnancy or to correlate SWE value with respiratory function at birth. The main objective is to evaluate feasibility and reproducibility of SWE in human fetal lung between 24 and 34 WG and to modelize a new LLE ratio. Indeed, each target organ will be explored through three measurements to define the most relevant and reproducible regions with the lowest intra and inter-observer variability. Use of LLE ratio is interesting because it allows comparison between two tissues in which the liver is taken as a reference organ. Several studies indicate that liver signal or echogenicity remain constant through gestation suggestive of a regular evolution of the tissue characteristics^{47,48}. Thus, we will compare evolution of SWE values between two organs subjected to the same technical variations, especially for depth. Both lung and liver tissue density increase during pregnancy and we expect a linear increase of SWE values of these organs. In case of variations of lung SWE values after a course of corticosteroids, LLE ratio should be modified. If we suppose that liver elasticity will be constant between two separate exams of 48h, LLE ratio will decrease if corticosteroids lead to attenuation of shear wave speed. Conversely, if lung elasticity increases after a course of corticosteroids, LLE ratio will also increase.

One advantage of this protocol is to evaluate SWE in three tissues with different supposed biomechanical properties: normal lung, lung with increase of surfactant synthesis after corticosteroids course, and liver. Corticosteroids generate an effect model interesting for assessment of LLE's ratio variation in the cases because of a significant increase of phosphatidylcholine content in fetal lungs. This effect is mediated by an activation of choline-phosphate cytidyltransferase³⁷. Given the placental transfer and time to induce cellular mechanism of transcription, an artificial delay of 24h seems to be necessary to produce surfactant in pneumocytes type 2. The benefit of corticosteroid administration is greatest at 2–

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3 7 days after the initial dose ^{49,50}. Two approaches can be proposed after a complete course of
4 corticosteroids. On the one hand, SWE of fetal lung could increase because corticosteroids
5 accelerate FLM, and we could expect an advanced ripening by one WG. On the other hand,
6 increase in phospholipids content in fetal lung after corticosteroids could increase viscosity
7 and lead to dispersion of shear wave speed and attenuation.
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12 Different factors will be taken into account in this study to understand variability of
13 measures: acquisition depth, number of measurements and representative values such as
14 median or mean values. Shear wave velocity can be different according to the acquisition
15 depth and this parameter is gradually underestimated with increasing depth. Effect is greatest
16 in stiff tissues but little effect is seen in soft target ⁵¹⁻⁵³. This phenomenon might be explained
17 by a damping of the acoustic push pulse that generates the shear waves by both increased
18 attenuation and increased stiffness of the target ⁵⁴. Thus, we expected low variation between
19 proximal and distal lung because they may be considered as soft tissues. There are also
20 debates about the acquisition number during SWE and the designers of the device did not
21 provided advices. The number of measurements (NMs) reported in the recent literature on
22 various organs is inconsistent (commonly seen are 3, 4 and 5 NMs) ⁵⁵⁻⁵⁶. Quarello et al
23 reported 3 NMs on fetal lungs in pregnant baboons and ICC for and inter observer variability
24 were better for an average value of three measurements ⁵⁷. For Alison et al, 9 NMs were
25 performed on liver in preterm neonates with intra uterine growth restriction (3 measurements
26 from three different liver segments). Measurements showed high reproducibility on average
27 values (ICC = 0.94–0.98 for intra-operator, 0.86 for inter-operator) ³¹. One objective of our
28 protocol is to determine the NM's necessary to evaluate fetal lung SWE with a good inter and
29 intra-observer reproducibility. Thus, each value will be analyzed to determine if mean lung
30 and liver SWE can be achieved through only 1 valid measurement or more, and if its
31 performance is equivalent to 2 or 3 valid measurements.
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46 To sum up, we hope that the results of this study will contribute to clarify applicability
47 of SWE on human fetal lung between 24 and 34WG. SWE could be a new tool for reliable
48 prediction of lung development through biomechanical properties. This study will be the first
49 one to propose a protocol of measurement underlying limiting factors.
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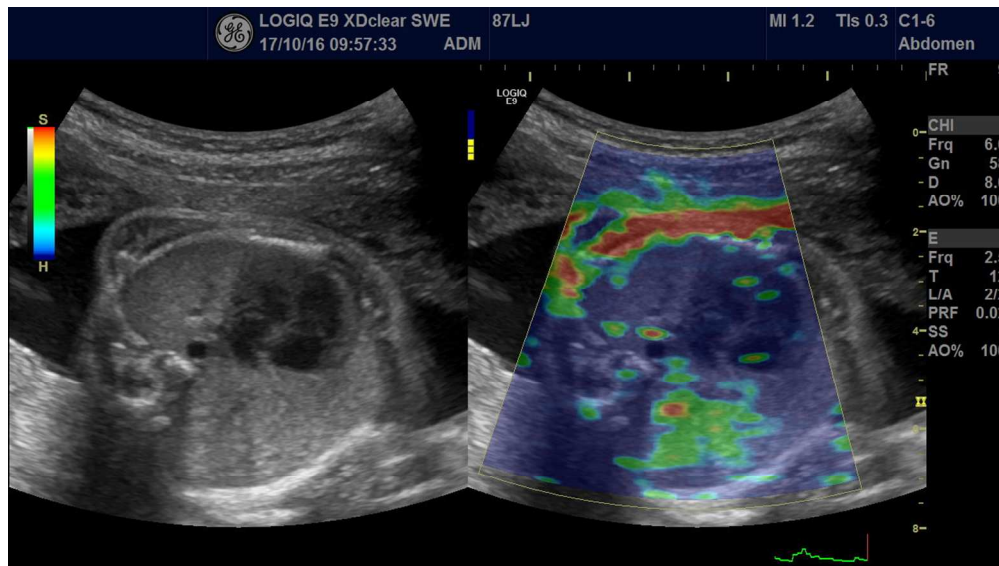
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3 **Figure 1:** SWE on fetal thorax centered on a 4-chamber view of the fetal heart.
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5 **Figure 2:** Design of the study.
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8 **Figure 3:** Measurement sites with SWE: 3 measurements on the proximal lung (anterior “P1”,
9 medium “P2” and posterior portion “P3”), 3 measurements on the distal lung (anterior “D1”,
10 medium “D2” and posterior portion “D3”) and 3 measurements on 3 liver segments (IV, V,
11 VI) according to Couinaud classification.
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15 **Figure 4:** SWE on proximal fetal lung. Image (a) is degraded by artifact on the left side of the
16 ROI due to rib shadowing. By moving the probe and the ROI to another location, a more
17 homogeneous image (b) is obtained.
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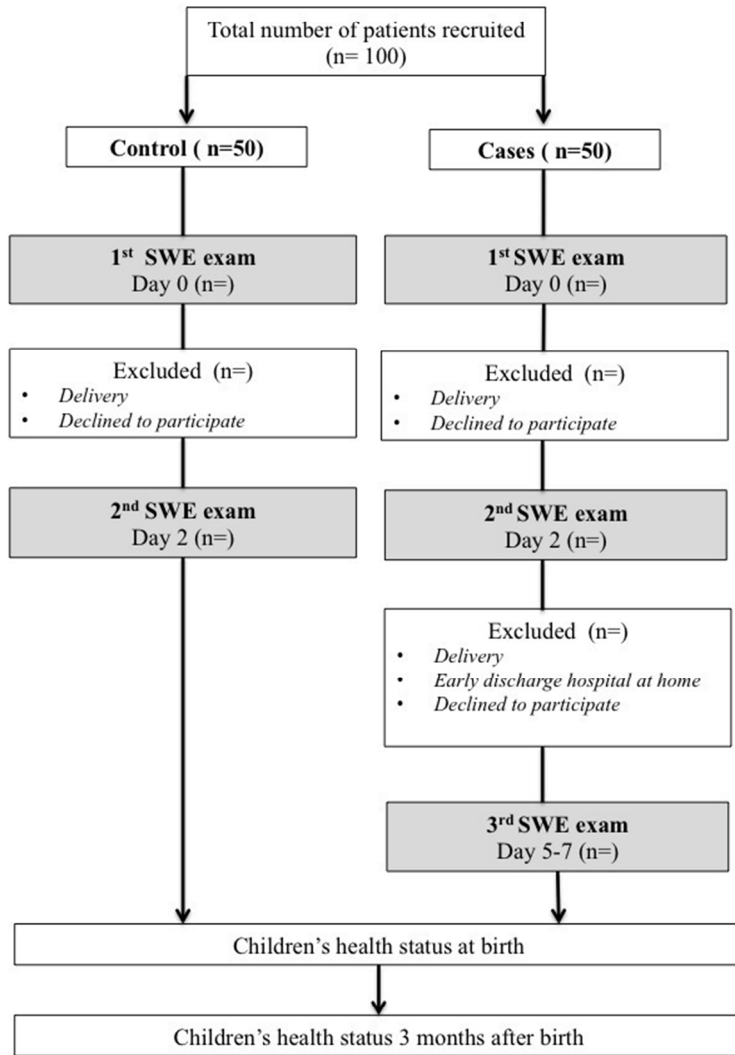


2-D ultrasound SWE on fetal thorax centered on a 4-chamber view of the fetal heart.

108x60mm (300 x 300 DPI)

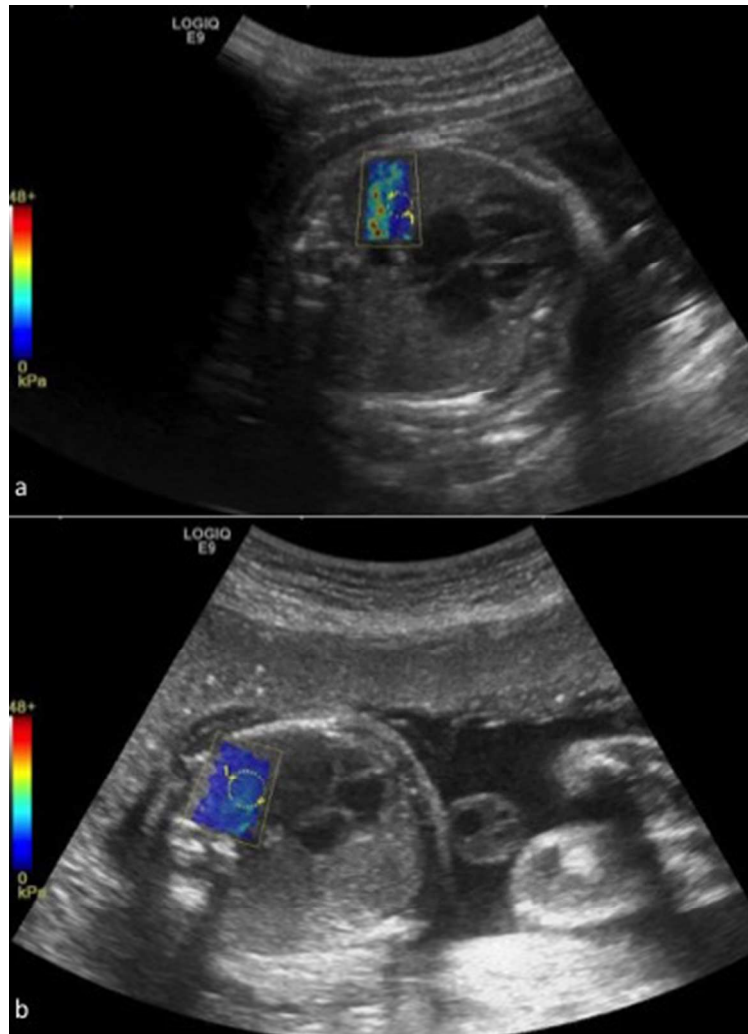
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Design of the study.

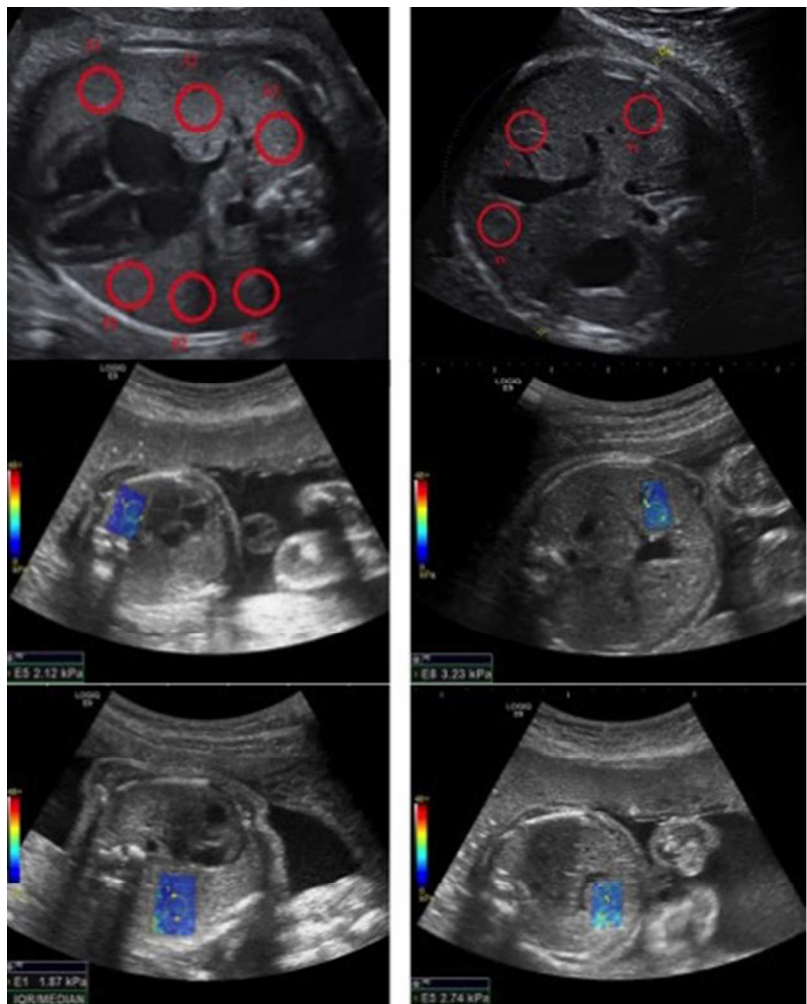
60x81mm (300 x 300 DPI)



SWE on proximal fetal lung. Image (a) is degraded by artifact on the left side of the ROI due to rib shadowing. By moving the probe and the ROI to another location, a more homogeneous image (b) is obtained.

31x43mm (300 x 300 DPI)

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Measurement sites with SWE: 3 measurements on the proximal lung (anterior "P1", medium "P2" and posterior portion "P3"), 3 measurements on the distal lung (anterior "D1", medium "D2" and posterior portion "D3") and 3 measurements on 3 liver segments (IV, V, VI).

33x42mm (300 x 300 DPI)



STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of *case-control studies*

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	3
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	5,6
Methods			
Study design	4	Present key elements of study design early in the paper	8
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8
Participants	6	(a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	8,9
		(b) For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	9,10
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	9,10,11
Bias	9	Describe any efforts to address potential sources of bias	11
Study size	10	Explain how the study size was arrived at	11
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	11,12
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	12
		(b) Describe any methods used to examine subgroups and interactions	12
		(c) Explain how missing data were addressed	12
		(d) If applicable, explain how matching of cases and controls was addressed	12
		(e) Describe any sensitivity analyses	12
Results			12

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8,9
		(b) Give reasons for non-participation at each stage	10
		(c) Consider use of a flow diagram	10
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8,9
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(b) Report category boundaries when continuous variables were categorized	
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14
Generalisability	21	Discuss the generalisability (external validity) of the study results	14
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Feasibility of 2-D ultrasound shear wave elastography of fetal lungs in case of threatened preterm labor: a study protocol



Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2017-018130.R1
Article Type:	Protocol
Date Submitted by the Author:	09-Aug-2017
Complete List of Authors:	<p>MOTTET, Nicolas; University Hospital of Besançon, Department of Obstetrics and Gynecology ; Nanomedecine Laboratory, INSERM EA4662, University of Franche-Comte</p> <p>Aubry, Sébastien ; University Hospital of Besançon, Department of Radiology; University of Franche-Comte, Nanomedecine laboratory, INSERM EA4662</p> <p>Vidal, Chrystelle; University Hospital of Besançon, Centre d'investigation Clinique-Innovation Technologique 1431, INSERM</p> <p>Boiteux, Guillaume; University Hospital of Besançon, Centre d'investigation Clinique-Innovation Technologique 1431, INSERM</p> <p>Metz, Jean-Patrick; University Hospital of Besançon, Department of Obstetrics and Gynecology</p> <p>Riethmuller, Didier ; University Hospital of Besançon, Department of Obstetrics and Gynecology</p> <p>Pazart, Lionel; University Hospital of Besançon, Inserm Centre Investigation Clinique-Innovation Technologique 1431, INSERM; Inserm, CIC1431</p> <p>Ramanah, Rajeev; University Hospital of Besançon; University of Franche-Comte, Department of Obstetrics and Gynecology; Nanomedecine Laboratory, INSERM EA4662, University of Franche-Comte</p>
Primary Subject Heading:	Radiology and imaging
Secondary Subject Heading:	Obstetrics and gynaecology, Paediatrics
Keywords:	Ultrasonography < OBSTETRICS, Prenatal diagnosis < OBSTETRICS, Fetal medicine < OBSTETRICS, shear wave elastography, lung

SCHOLARONE™
Manuscripts

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3 **Feasibility of 2-D ultrasound shear wave elastography of fetal lungs in case of**
4 **threatened preterm labor: a study protocol**
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8 Nicolas Mottet ^{1,2}, Sébastien Aubry ^{2,3}, Chrystelle Vidal ⁴, Guillaume Boiteux ⁴, Jean-Patrick
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3 **Contributors** : All the authors contributed to the conception and design of the study. NM,
4 SA, and RR provided the idea for the research or article, created the hypothesis and wrote the
5 original proposal. NM, CV, LP and RR significantly contributed to writing the paper. NM,
6 CV, GB and LP wrote this protocol paper. All authors read and approved the final manuscript.
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11 API/2015/60
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13 **Competing interests** None declared.
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16 Patient consent obtained.
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20 **Ethics approval** The Human Research Ethics Committee (Comité de Protection des
21 Personnes EST II, process number **15/494**)
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ABSTRACT

Introduction

2-D ultrasound Shear Wave Elastography (SWE) could be considered as a new noninvasive tool for monitoring fetal lung development based on evaluation of mechanical properties during pregnancy. Interesting results are available concerning use of SWE on developing organs, especially on premature infants and animals models. The main objective in this study is to evaluate feasibility of 2-D SWE in human fetal lungs between 24 and 34 weeks of gestation (WG). Secondary objective is to modelize fetal lung-to-liver elastography ratio (LLE ratio) and to assess variations between normal lung and lung surfactant-enriched after a corticosteroids course indicated for a threatened preterm labor (TPL).

Methods and analysis

A prospective case-control study will be performed between 24 and 34 WG. Fetal lungs and liver will be explored by SWE into two groups: fetuses of women with an uncomplicated pregnancy (control group) and fetuses of women with a TPL requiring administration of corticosteroids (cases group). LLE Ratio will be defined as the value of the lung elasticity divided by the value of the liver elasticity.

Primary judgment criterion is the value of elasticity modulus expressed in kilopascal. Lungs and liver will be explored through three measurements to define the most reproducible regions with the lowest intra and inter-observer variability. Feasibility will be evaluated by assessing the number of exams performed and the number of exams with interpretable results. Intra and inter-observer reproducibility will be evaluated by means of the intra-class correlation coefficient.

Ethics and dissemination

Approval of the study protocol was obtained from the human ethical research committee (Comité de Protection des Personnes EST II, process number 15/494) and the French National Agency for Medicines and Health Products Safety (process number 2015-A01575-44). All participants will sign a statement of informed consent.

Trial Registration number: NCT02870608

Key words: Shear wave elastography, stiffness, ultrasound, lung, prenatal

Strengths and limitation of the study

- The study is designed to evaluate feasibility of 2-D ultrasound Shear Wave Elastography in human fetal lungs between 24 and 34 WG and to modelize fetal lung-to-liver elastography ratio (LLE ratio).
- This study will be the first one to demonstrate applicability of Shear Wave Elastography in human fetal lungs.
- Different factors will be taken into account to understand variability of measures: acquisition depth, number of measurements and representative values such as median or mean values.
- We shall not attempt to define reference value of fetal lung elasticity or to correlate SWE value with respiratory function at birth.

INTRODUCTION:

Background

Prenatal evaluation of fetal lung maturity (FLM) is still a challenge and different approaches have been reported with invasive biological tools or fetal imaging including ultrasounds and magnetic resonance imaging (MRI). FLM is mainly correlated with surfactant level and numerous tests have been developed to quantify this production: lecithin/sphingomyelin ratio (L/S), laminar body count, testing for Phosphatidyl glycerol with thin-layer chromatography, and estimation of the surfactant/albumin ratio using polarized light. All of these tests have been shown to have good sensitivity and negative predictive values; however, they all have a low positive predictive value and need invasive procedure^{1,2}. Thus, amniocentesis for FLM became obsolete³.

Lung and liver signal or echogenicity were often compared as ratio to assess FLM in several studies⁴⁻¹⁰. Non-invasive assessment of FLM has been first attempted using ultrasonography quantified by gray level histogram^{4,11,12}. These studies demonstrated sonographic changes associated with lung maturation but weak correlations between echogenicity and respiratory distress syndrome (RDS). Quantitative analysis with specific software has been secondly proposed to improve robustness. Fetal lung texture analysis with this method demonstrated a strong correlation with gestational age, biological test on amniotic fluid and risk of neonatal respiratory morbidity^{13, 14}. Additionally, several studies found a significant association between fetal lung-to-liver signal intensity ratio and estimated gestational age with fetal Magnetic Resonance Imaging (MRI). Actually, this technique is mainly used to assess fetal lung development and volume, especially in case of congenital diaphragmatic hernia or severe oligohydramnios. Nevertheless, the accuracy of both MRI and ultrasounds for estimating long-term respiratory outcomes remains limited¹⁵⁻¹⁹. These previous technique propose prenatal evaluation of lung maturity based on imaging features, but prediction of the pulmonary function through prenatal biomechanical properties seems relevant. With this hypothesis, we advanced the concept of “prenatal functional imaging” and shear wave elastography (SWE) could be a promising tool.

SWE is a recent ultrasound technology using acoustic radiation force imaging (ARFI), which enables to assess the stiffness of tissues in real time through a color quantitative elastogram²⁰. SWE assesses tissue elasticity (E), which is the tendency of tissue to resist deformation with an applied force. In locally homogeneous and purely elastic medium, we

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3 can calculate Young's modulus (kPa) from the shear wave speed ($\text{m}\cdot\text{s}^{-1}$) with the formula: $E=$
4 $3\rho\cdot c_{\text{sw}}^2$ (ρ being the medium density and c_{sw} the shear wave speed). The stiffness at any
5 location of the ROI can be sampled using measurement tools to obtain a quantitative
6 evaluation either in terms of shear wave speed or Young's modulus. A low shear wave speed
7 corresponds to a soft tissue, while a shear wave high speed indicates a stiff tissue. Study of
8 deep organs is possible with SWE because this method does not require any compression-
9 relaxation sequence on the target organ. One of the advantages of SWE over other
10 elastography methods is that the generation of the mechanical impulse is operator-
11 independent. SWE is expanding its range nowadays by its promising role for examination of
12 various organs (liver, thyroid) and for aiding discrimination of lesion characteristic, especially
13 breast or prostate tumors²¹⁻²⁴.

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22 There is to date no publication regarding its use on human fetuses. The Food and Drug
23 Administration (FDA) has so far, not yet approved use of SWE for obstetric clinical
24 applications because of the paucity of data in the literature. Although there is no report about
25 apparent histological changes with shear wave, the absence of others bioeffects could not be
26 discarded and further studies are recommended²⁵. However, setting parameters respect
27 mechanical and thermal indexes for soft-tissues and bone, which are necessary to allow an
28 obstetric examination defined by the FDA²⁶. Thus, there is a debate concerning safety of SWE
29 in fetal medicine. Herman et al studied the models and regulatory considerations for transient
30 temperature rise during ARFI, and found that any transient increase in temperature caused by
31 pulse bursts might still be within the safe limits determined by the (FDA)²⁷. Others authors
32 showed that transducer heating was below 1°C for the current clinical applications of ARFI.
33 According to experimental studies that simulate the heating of soft tissue during ARFI, they
34 demonstrate that ARFI on soft tissue is safe, provided that thermal index must be monitored
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22 Interesting and reassuring results are available concerning use of SWE on developing
23 organs, especially on premature infants and animals models. Principals organs explored are
24 the liver, brain and lungs. Quarello et al demonstrated the possibility to use SWE in non-
25 human primate fetuses and its feasibility to explore fetal organs. They found that elasticity
26 values were related to organs and gestational age³⁰. Concerning fetal lungs elasticity,
27 measurements of the proximal lungs seem to increase throughout the pregnancy. Concerning
28 use of SWE on premature infants, results are more numerous and include exploration of the
29 liver or the brain. Alison et al. have demonstrated feasibility and reproducibility of liver

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3 stiffness in preterm neonates with intra uterine growth restriction (IUGR) and gestational age
4 at birth between 26-31 weeks of gestation (WG)³¹. Others studies reported contribution of
5 SWE to the diagnosis of biliary atresia in neonates^{32,33}. Kim et al described variation of brain
6 elasticity in different regions in healthy neonates born between 28 and 40 WG³⁴. Su et al
7 quantitatively evaluated the effect of acoustic radiation force impulse imaging (ARFI) in
8 neonatal brain development. The authors found that full-term neonates had significantly
9 higher elasticity values than preterm neonates and there were no reported immediate adverse
10 events³⁵. Others teams work on application of this technology to neonates as a supplementary
11 tool to detect early ischemic brain injury³⁶.

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18 If we describe fetal thorax centered on a 4-chamber view of the fetal heart with SWE,
19 the following observations can be made (figure 1):

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23 - SWE is able to make distinction between fetal soft and stiff tissues. Fetal ribs
24 appear red colored mapped whereas proximal lung appears blue colored.
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26 - A homogeneous colored area can be obtained on the proximal lung with a
27 color scale ranging from 0 to 48 kPa. A homogeneous ROI is important for
28 the reliability of the results.
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30 - SWE can differ according to the acquisition depth. Proximal lung appears
31 with a blue homogenous distribution whereas distal lung appears with green
32 homogenous distribution.
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38 Objectives

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40 The main objective in this study is to evaluate feasibility of 2-D SWE in human fetal
41 lungs between 24 and 34 weeks of gestation (WG). Secondary objective is to modelize fetal
42 lung-to-liver elastography ratio (LLE ratio) and to assess variations between normal lung and
43 lung surfactant-enriched after a corticosteroids course indicated for a threatened preterm labor
44 (TPL).
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METHODS/DESIGN

Study design and setting

A prospective case-control study will be performed at the University Hospital of Besançon, (Besançon, France), Department of Obstetrics and Gynecology. Fetal lungs and liver will be explored by SWE between 24 and 34 WG in two groups: fetuses of women with an uncomplicated pregnancy will be considered as control group, and fetuses of women with a threatened preterm labor requiring administration of corticosteroids will be enrolled as cases. In this pilot study, corticosteroids generate an effect model interesting for assessment of LLE ratio variation in the cases. We take advantage of the action of corticosteroids to explore another medium with different supposed biomechanical properties. Indeed, corticosteroids accelerated development of type 2 pneumocytes leading to surfactant production. Similarly, there is a significant increase in saturated phosphatidylcholine content in fetal lung, modifying propagation of shear waves potentially³⁷.

Control group will be recruited during routine prenatal visits or ultrasounds according to the French ultrasounds schedule (ultrasound screening during the second trimester between 20-25 WG and the third trimester between 30-35 WG³⁸). Cases will be enrolled during their hospitalization for threatened preterm delivery (figure 2). The design, conduction and reporting of this study will follow the norms of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement³⁹.

Ethical considerations

The study respects the ethical standards established in the declaration of Helsinki⁴⁰. Investigators undertake to respect law no. 2004-806 of August 9,2004 relative to public health policy on clinical trials, made effective by implementing Decree 2006-477 of April 26,2006⁴¹. Approval of the study protocol was obtained from the human ethical research committee (Comité de Protection des Personnes EST II, process number **15/494**) and the French National Agency for Medicines and Health Products Safety (process number **2015-A01575-44**). The study is registered on clinical trial.gov with the following number: **NCT02870608**. All participants will sign a statement of informed consent and will be allowed to abandon the study at any time, with no negative consequences. All procedures of the study will be confidential. The study will involve collection and storage of ultrasound images and

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3 study participants will therefore be thoroughly informed. All variables (patient parameters,
4 data items, data elements) will be aggregated into electronic Case report Forms.

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6 As explained in the background, The Food and Drug Administration (FDA) has so far,
7 not yet approved use of SWE for obstetric clinical applications because of the paucity of data
8 in the literature. In this study, fetuses will be included from 24 to 34 WG, over the period of
9 organogenesis. In the patient information sheet, it is specified that previous studies explored
10 liver or brain of premature infants with SWE. Alison et al. studied liver stiffness in preterm
11 neonates with intra uterine growth restriction (IUGR) and gestational age at birth between 26-
12 31 WG³¹. Franchi-Abella et al evaluate feasibility and diagnostic accuracy of SWE for the
13 assessment of liver stiffness in 96 patients and 7 were preterm newborns between 30-35 WG
14⁴². Both studies did not report adverse outcomes and were approved by institutional review
15 board. Moreover, consent form mentions conclusions of an experimental study of the
16 biological effects associated with SWE in brains of neonatal mice. Results indicated that
17 using SWE doe not cause detectable histologic changes. Potential effects on intracellular
18 signaling pathways were detected when the SWE scanning lasted more than 30 min²⁵. Thus,
19 patients will be informed that our study is focused on fetuses with a gestational age similar to
20 preterm newborn included in the above-mentioned studies. Moreover, exploration with SWE
21 will concern only lung and liver with duration of less than 10 min in respect of biophysical
22 safety indexes: Thermal index $Ti \leq 0.7$ and Mechanical index $Mi < 1,9$.

33 34 35 36 **Participants**

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38 Cases will be recruited during hospitalization for a threatened preterm labor requiring
39 administration of corticosteroids. The complementary inclusion criteria are: pregnant women
40 aged 18 years or more, singleton pregnancy with eutrophic fetus, signature of consent and
41 affiliation to health insurance scheme (figure 2). Patients will be stratified by gestational age
42 between 24 and 34 WG, with one class every WG (10 classes in total) and 5 patients per class.
43 After obtaining consent, a SWE exam will be performed before the first intramuscular
44 administration of corticosteroids “day 0” (betamethasone 12mg). A second SWE exam will be
45 performed within 24 h after the second intramuscular administration of corticosteroids “day
46 2”. A third SWE will be performed between the fifth and the seventh day following the first
47 administration of corticosteroids “day 5-7”, if the women are not early discharge at home.

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49 For the control group, women will be matched by gestational age and will be recruited
50 during their pregnancy monitoring. Information concerning the study will be given as early as
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3 the second trimester by obstetrician or midwives during medical visits or ultrasounds. The
4 complementary inclusion criteria are following: major pregnant women, uncomplicated
5 pregnancy between 24 and 34 WG, singleton pregnancy with eutrophic fetus, signature of
6 consent and affiliation to health insurance scheme. If they are willing to participate, a first
7 SWE exam will be proposed at “day 0”, followed by a second one in two days “day 2”.
8 Patients who come back for an SWE exam performed outside current medical visit will
9 receive financial compensation for travel expenses. As well as cases, control patients will be
10 stratified by gestational age between 24 and 34 WG.

11 Exclusion criteria common to both groups are: Fetal lung or liver pathologies,
12 inclusion in another medical study, patients under legal incapacity.

20 21 **Variables and measuring technique**

- 22 • **Prenatal variables**

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25 Primary judgment criterion is the value of Young’s modulus (E) (elasticity modulus)
26 expressed in kilopascal (kPa). A Logic E9 system (General Electric Medical System,
27 Milwaukee, WI, USA) with CE certification CE LNE/G-MED [CE0459] will be used for this
28 study, equipped with abdominal convex probe 1-6 MHz (C1-6-D probe). This technique
29 includes a real-time visualization of a color quantitative elastogram coupled to a B-mode
30 image. To provide robust shear wave and optimize its detection, Logiq E9 uses innovative
31 techniques. Comb-push Ultrasound SWE generates multiple shear wave sources into the
32 region of interest (ROI) and uses directional filtering to remove the interference. The TAST
33 technique is able to robustly track shear waves and correct for the sequential tracking delay⁴³.
34 This technique has the merit of rapid and solid reconstruction of a large elasticity map
35 (elastogram) with the only single acquisition by generating multiple shear waves from
36 multiple unfocused push beams.

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38 The shear wave acquisition measurement protocol is saved in a specific preset that will be
39 used for every patient: 2D-Mode: Harmonic imaging central depth = 6 cm, frequency = 6
40 MHz, Acoustic Power 100%; Elastography-Mode: Shear wave signal frequency ranges from
41 50 to 400 kHz, Acoustic power of the pushing beam: 100%, Tracking power: 100%, color
42 scale ranging from 0 to 48 kPa. Biophysical safety indices: Thermal index $T_i \leq 0.7$ and
43 Mechanical index $M_i < 1,9$ (table 1).

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Fetal lungs will be voluntarily approached laterally to systematically obtain proximal

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3 and distal lungs regarding the distance to the probe. Each measurement has to be performed
4 on a homogeneous elastogram to be valid. Operators will perform 2 cycles of 9 Young's
5 modulus measurements while systematically repositioning the probe on each target organ.
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8 A cycle includes: 3 measurements on the proximal lung (anterior "P1", medium "P2" and
9 posterior portion "P3"), 3 measurements on the distal lung (anterior "D1", medium "D2" and
10 posterior portion "D3") and 3 measurements on 3 liver segments (IV, V, VI) (figure 3).
11 Implementation of Shear Wave elastography displays 2D images of Young's Modulus in the
12 target organ. In real time, the elasticity appears color coded, where blue identifies deformable
13 tissue and red indicates rigid tissues. The Young's modulus value at any location will be
14 sampled using a round ROI of 5 mm. Distance between the probe and the target organ will be
15 collected. The average elasticity in the ROI will be automatically recorded by the system in a
16 worksheet. The investigators will adjust the position of the ROI using the B-mode image for
17 guidance and take care to obtain the most homogeneous color coded ROI before Young's
18 modulus estimation (figure 4). Technical failure was defined as failure to obtain a
19 homogeneous elastogram in more than 50% in the sampling area. To test the inter-observer
20 variability, a second observer will successively perform measurements on 30 fetuses. All
21 measurements will be carried out directly by ultrasound on the target organ. The second
22 observer will perform measurements just after the first one and will be blinded to the
23 feasibility and results obtained by the first one.
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35 Estimation of Fetal Weight Estimation (EFW) will be performed during each exam
36 according to Hadlock formula based on Cephalic circumference (CC), Abdominal
37 circumference (AC) and femoral length (FL): $\log_{10} \text{ EFW} = 1,326 + 0,0107 \text{ PC} + 0,0438 \text{ PA}$
38 $+ 0,158 \text{ LF} + 0,00326 (\text{PA} \times \text{LF})$ ⁴⁴.
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42 • **Postnatal variables**

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45 The following pharmacovigilance data will be collected at birth and 3 months later in
46 order to assess safety of the device:
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48 - At birth in both groups: term of birth, weight, neonatal transfer, Apgar Score and
49 evaluation of respiratory distress by Silverman Score ⁴⁵.
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51 - Three months after birth: medical history, health problems, respiratory diseases or
52 symptoms, liver disease or symptoms and number of hospitalization since birth.
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57 **Limitation of Bias**
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3 Use of sampling method with 5 patients per class of gestational age will limit
4 repartition bias between control and cases. Inclusion of any pregnant women attending
5 Besançon University maternity for the control group, and inclusion of all women with a
6 threatened preterm labor will limit risk of selection bias. Risk of loss will be limited in the
7 cases group between “day 0” and “day 2” because patients will remain hospitalized for
8 corticosteroids administration. Nevertheless, risk of loss is most important between “day 2”
9 and “day 5-7” because of possible early discharge at home after the second administration of
10 corticosteroids. For ethical and medical reasons, cases early discharge at home cannot be
11 convened in “day 5-7”. Risk of loss will be limited in the control group because of a financial
12 compensation for travel expenses. To limit inter-observer variation, expert sonographers will
13 perform elastography exams.
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22 **Study size**

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24 Because of the paucity of data regarding SWE on human fetal lung, the sample size
25 calculation was based on measurements of fetal lungs in pregnant baboons and extrapolations
26 made by clinicians, with the following assumptions: average expected value of elasticity
27 coefficient for fetal lung will be 2 KPa at 24 WG and 4 KPa at 34 WG. We hypothesized a
28 linear increase of elasticity coefficient during pregnancy in the control group and a decrease
29 in cases exposed to corticosteroids. At “day 2”, we therefore expect in the cases a variation of
30 0.2 KPa (corresponding to one week of lung maturation during gestation). Forty-two patients
31 per group will be needed to have 90% power to statistically demonstrate such a difference
32 (0.2 versus 0.057) assuming an α risk of 0.05 and standard deviation of 0.2.
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43 **Proposed statistical analysis**

- 44 • **Technical validation:**

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46 Feasibility will be evaluated by assessing the number of exams performed and the number
47 of exams with interpretable results. All the variables and data that will be stored for each
48 SWE exam are summarized in table 1. Intra and inter-observer reproducibility will be
49 evaluated by the intra-class correlation coefficient (ICC) with 95% IC. Intra-observer
50 reproducibility will be calculated using the two cycles of measure, inter observer
51 reproducibility between the operators will be calculated for means of the two cycles of
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measures if intra-observer is high⁴⁶. Only repeatable and reproducible values of each ROI will be considered to modelize LLE ratio.

Table 1: Variables and data stored for each Shear Wave Elastography Exam.
ROI (Region of interest)

Maternal	Fetal	Technical
<ul style="list-style-type: none"> ▪ Age (years) ▪ Weeks of gestation ▪ Body mass Index (kg/m²) ▪ Subcutaneous adipose tissue thickness (cm) 	<ul style="list-style-type: none"> ▪ Presentation: <ul style="list-style-type: none"> - Cephalic - Breech - Transverse ▪ Weight (grams) ▪ Placenta: <ul style="list-style-type: none"> - Position: anterior, posterior, lateral, fundal - Thickness (cm) ▪ Amniotic fluid index (cm) 	<ul style="list-style-type: none"> ▪ Young's modulus (kPa) in 9 ROI: <ul style="list-style-type: none"> - Proximal lung: P1, P2, P3 - Distal lung: D1, D2, D3 - Liver: IV, V, VI ▪ Distance between probe and target ROI for each measurement (cm) ▪ Biophysical safety indices: <ul style="list-style-type: none"> - Mechanical index (Mi) - Thermal index (Ti)

- **Clinical evaluation**

Evolution of fetal lung SWE, fetal liver SWE and lung-to-liver-SWE ratio will be assessed by group and overall. Lung-to-liver-SWE Ratio will be initially defined as the value of the lung elasticity divided by the value of the liver elasticity.

SWE values before corticosteroids administration will be compared between cases and control groups. If there is a statistically significant difference, a confusional bias will have to be sought between fetal lung elasticity values and threatened preterm labor. All results will be presented as “delta” variation between two measurements in order to limit “non-comparability bias” between both groups. If corticosteroids affect fetal lung elasticity, “delta before/after” will be more important in cases than controls.

Analysis of mean differences between groups will be carried out using a Student's test or Wilcoxon test according to distribution of data for quantitative variables. Shapiro-Wilk test

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3 will be used to determine if data set is well modeled by a normal distribution. The relationship
4 of quantitative variables to each other will be tested using Pearson's or Spearman's
5 correlation as appropriate.
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8 Qualitative variable will be expressed as frequencies and quantitative variables will be
9 displayed as the mean \pm SD if normally distributed or as the median \pm IQR (Inter Quartile
10 Range) if asymmetrically distributed. Qualitative variables will be analyzed using a χ^2 test or
11 Fisher test.
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14 For statistical analyses, the level of statistical significance will be set at 5% ($p < 0.05$).
15 Statistical analysis will be performed with statistical software SAS for Windows, version 9.4
16 and MedCalc software, version 15.
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DISCUSSION

2D-SWE could be considered as a new noninvasive tool for monitoring fetal lung development based on evaluation of mechanical properties during pregnancy. However, standardization for measuring SWE in human fetal lung is necessary to demonstrate applicability of the technique, and to understand limiting factors. This pilot study will focus on technical performances before considering clinical applications. In this protocol, SWE will be used after fetal period of organogenesis and pharmacovigilance data will be collected at birth and 3 months later in order to assess safety of the device.

With the study design, we shall not attempt to define reference value of fetal lung elasticity during pregnancy or to correlate SWE value with respiratory function at birth. The main objective is to evaluate feasibility and reproducibility of 2D-SWE in human fetal lung between 24 and 34 WG and to modelize a new LLE ratio. Indeed, each target organ will be explored through three measurements to define the most relevant and reproducible regions with the lowest intra and inter-observer variability. Use of LLE ratio is interesting because it allows comparison between two tissues in which the liver is taken as a reference organ. Several studies indicate that liver signal or echogenicity remain constant through gestation suggestive of a regular evolution of the tissue characteristics^{47,48}. Thus, we will compare evolution of SWE values between two organs subjected to the same technical variations, especially for depth. Both lung and liver tissue density increase during pregnancy and we expect a linear increase of SWE values of these organs. In case of variations of lung SWE values after a course of corticosteroids, LLE ratio should be modified. If we suppose that liver elasticity will be constant between two separate exams of 48h, LLE ratio will decrease if corticosteroids lead to attenuation of shear wave speed. Conversely, if lung elasticity increases after a course of corticosteroids, LLE ratio will also increase.

One advantage of this protocol is to evaluate SWE in three tissues with different supposed biomechanical properties: normal lung, lung with increase of surfactant synthesis after corticosteroids course, and liver. Corticosteroids generate an effect model interesting for assessment of LLE's ratio variation in the cases because of a significant increase of phosphatidylcholine content in fetal lungs. This effect is mediated by an activation of choline-phosphate cytidyltransferase³⁷. Given the placental transfer and time to induce cellular mechanism of transcription, an artificial delay of 24h seems to be necessary to produce surfactant in pneumocytes type 2. The benefit of corticosteroid administration is greatest at 2–

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3 7 days after the initial dose^{49,50}. Two approaches can be proposed after a complete course of
4 corticosteroids. On the one hand, SWE of fetal lung could increase because corticosteroids
5 accelerate FLM, and we could expect an advanced ripening by one WG. On the other hand,
6 increase in phospholipids content in fetal lung after corticosteroids could increase viscosity
7 and lead to dispersion of shear wave speed and attenuation.
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11 Different factors will be taken into account in this study to understand variability of
12 measures: acquisition depth, number of measurements and representative values such as
13 median or mean values. Shear wave velocity can be different according to the acquisition
14 depth and this parameter is gradually underestimated with increasing depth. Effect is greatest
15 in stiff tissues but little effect is seen in soft target⁵¹⁻⁵³. This phenomenon might be explained
16 by a damping of the acoustic push pulse that generates the shear waves by both increased
17 attenuation and increased stiffness of the target⁵⁴. Thus, we expected low variation between
18 proximal and distal lung because they may be considered as soft tissues. There are also
19 debates about the acquisition number during SWE and the designers of the device did not
20 provided advices. The number of measurements (NMs) reported in the recent literature on
21 various organs is inconsistent (commonly seen are 3, 4 and 5 NMs)⁵⁵⁻⁵⁶. Quarello et al
22 reported 3 NMs on fetal lungs in pregnant baboons and ICC for and inter observer variability
23 were better for an average value of three measurements⁵⁷. For Alison et al, 9 NMs were
24 performed on liver in preterm neonates with intra uterine growth restriction (3 measurements
25 from three different liver segments). Measurements showed high reproducibility on average
26 values (ICC = 0.94–0.98 for intra-operator, 0.86 for inter-operator)³¹. One objective of our
27 protocol is to determine the NM's necessary to evaluate fetal lung SWE with a good inter and
28 intra-observer reproducibility. Thus, each value will be analyzed to determine if mean lung
29 and liver SWE can be achieved through only 1 valid measurement or more, and if its
30 performance is equivalent to 2 or 3 valid measurements.
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46 To sum up, we hope that the results of this study will contribute to clarify applicability
47 of SWE on human fetal lung between 24 and 34WG. SWE could be a new tool for reliable
48 prediction of lung development through biomechanical properties. This study will be the first
49 one to propose a protocol of measurement underlying limiting factors.
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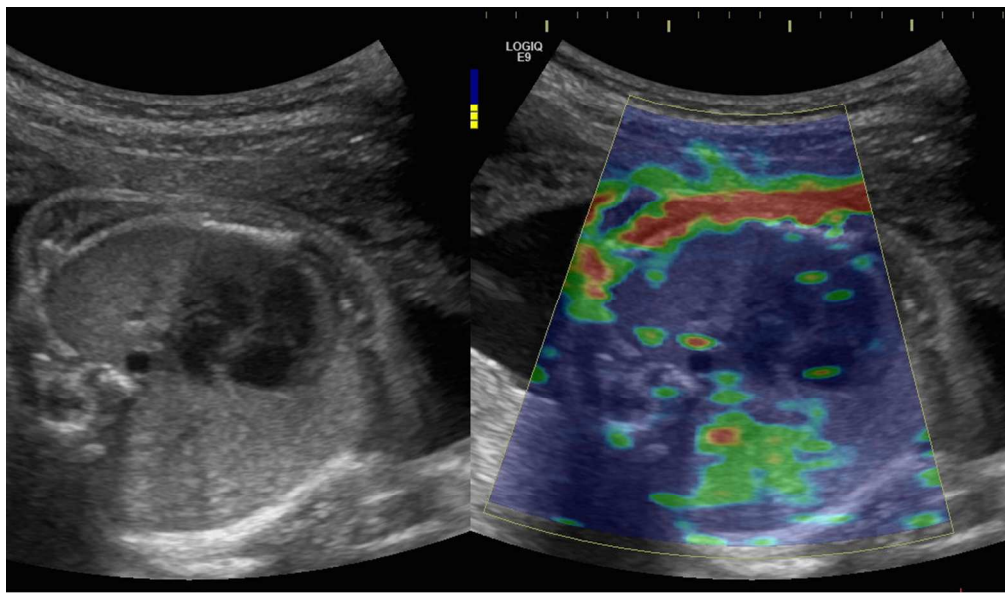
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3 **Figure 1:** Elastogram on fetal thorax centered on a 4-chamber view of the fetal heart, using
4 an abdominal convex probe 1-6 MHz (C1-6-D probe). Elasticity appears color-coded: blue
5 identifies deformable tissue and red indicates rigid tissues (kPa).
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9 **Figure 2:** Design of the study.
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12 **Figure 3:** Measurement sites with 2D comb-push SWE using an abdominal convex probe 1-6
13 MHz (C1-6-D probe). Color scale ranging from 0 to 48 kPa. Sufficient color maps covering
14 more than 50% of the sampling area are obtained considered as a technical success. Regions
15 of interest (ROI) are placed on homogeneous elastograms: 3 measurements on the proximal
16 lung (anterior “P1”, medium “P2” and posterior portion “P3”), 3 measurements on the distal
17 lung (anterior “D1”, medium “D2” and posterior portion “D3”) and 3 measurements on 3
18 liver segments (IV, V, VI). Operator will perform 2 cycles of 9 Young’s modulus
19 measurements while systematically repositioning the probe and each target ROI.
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27 **Figure 4:** 2D comb-push SWE on proximal fetal lung. Color scale ranging from 0 to 48 kPa.
28 On image (a), elastogram is not homogeneous and is degraded by artifact on the left side due
29 to rib shadowing. By moving the probe to another location, a more homogeneous elastogram
30 (b) is obtained allowing measurement.
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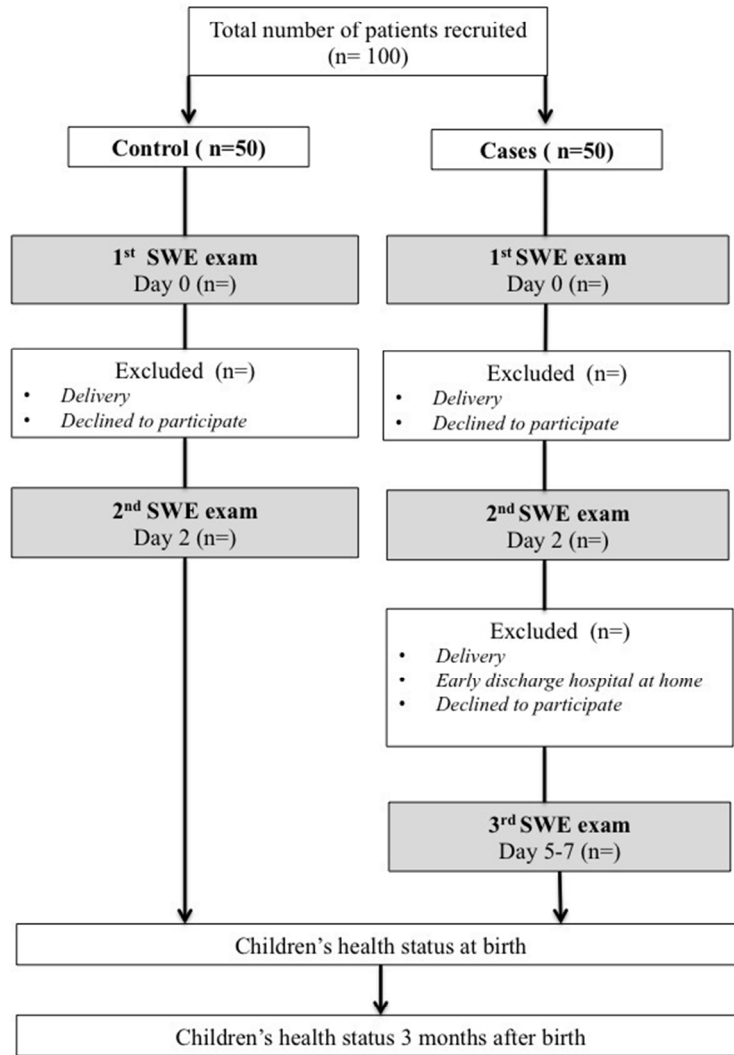
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Elastogram on fetal thorax centered on a 4-chamber view of the fetal heart, using an abdominal convex probe 1-6 MHz (C1-6-D probe). Elasticity appears color-coded: blue identifies deformable tissue and red indicates rigid tissues (kPa).

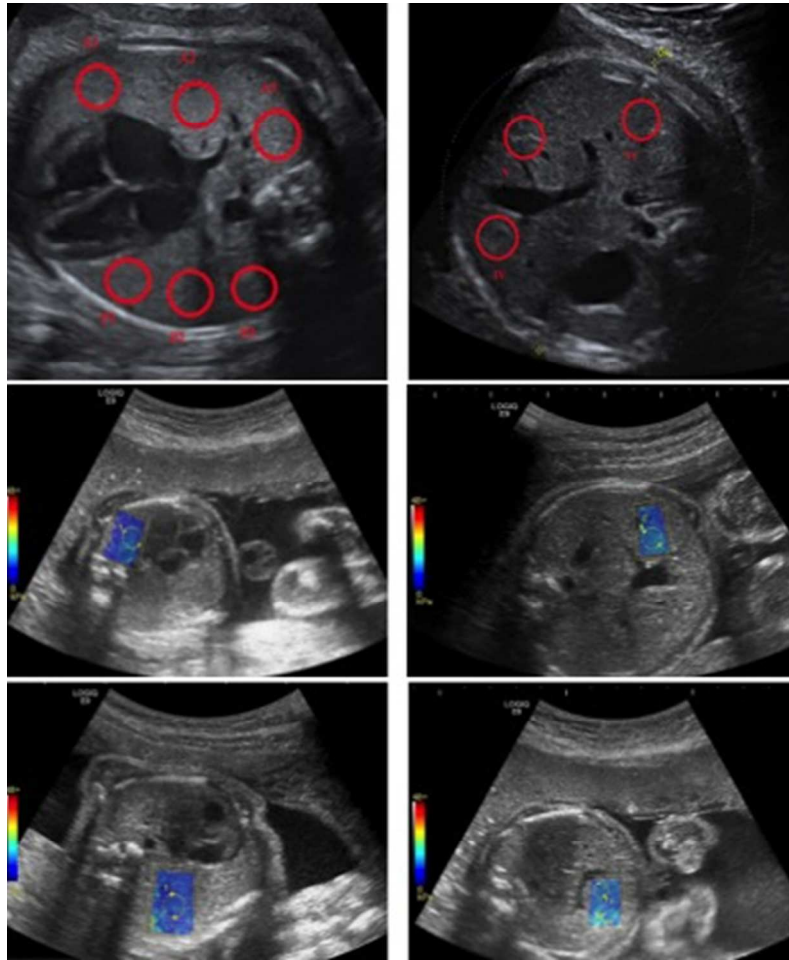
88x51mm (300 x 300 DPI)

Review only



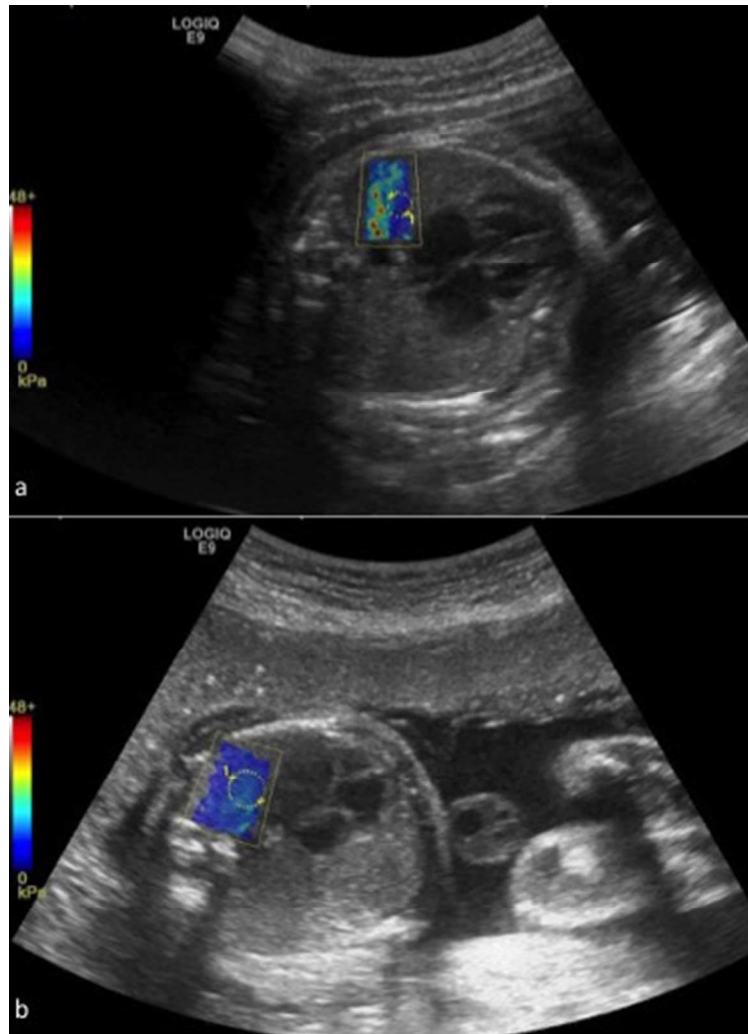
Design of the study.

60x81mm (300 x 300 DPI)



Measurement sites with 2D comb-push SWE using an abdominal convex probe 1-6 MHz (C1-6-D probe). Color scale ranging from 0 to 48 kPa. Sufficient color maps covering more than 50% of the sampling area are obtained considered as a technical success. Regions of interest (ROI) are placed on homogeneous elastograms: 3 measurements on the proximal lung (anterior "P1", medium "P2" and posterior portion "P3"), 3 measurements on the distal lung (anterior "D1", medium "D2" and posterior portion "D3") and 3 measurements on 3 liver segments (IV, V, VI). Operator will perform 2 cycles of 9 Young's modulus measurements while systematically repositioning the probe and each target ROI.

33x40mm (300 x 300 DPI)



2D comb-push SWE on proximal fetal lung. Color scale ranging from 0 to 48 kPa. On image (a), elastogram is not homogeneous and is degraded by artifact on the left side due to rib shadowing. By moving the probe to another location, a more homogeneous elastogram (b) is obtained allowing measurement.

31x43mm (300 x 300 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of case-control studies

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(a) Indicate the study’s design with a commonly used term in the title or the abstract	3
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	5,6
Methods			
Study design	4	Present key elements of study design early in the paper	8
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8
Participants	6	(a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	8,9
		(b) For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	9,10
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	9,10,11
Bias	9	Describe any efforts to address potential sources of bias	11
Study size	10	Explain how the study size was arrived at	11
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	11,12
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	12
		(b) Describe any methods used to examine subgroups and interactions	12
		(c) Explain how missing data were addressed	12
		(d) If applicable, explain how matching of cases and controls was addressed	12
		(e) Describe any sensitivity analyses	12
Results			12

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8,9
		(b) Give reasons for non-participation at each stage	10
		(c) Consider use of a flow diagram	10
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8,9
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(b) Report category boundaries when continuous variables were categorized	
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14
Generalisability	21	Discuss the generalisability (external validity) of the study results	14
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Feasibility of 2-D ultrasound shear wave elastography of fetal lungs in case of threatened preterm labor: a study protocol



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Primary Subject Heading:	Radiology and imaging
Secondary Subject Heading:	Obstetrics and gynaecology, Paediatrics
Keywords:	shear wave elastography, lung, fetal, elasticity

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Manuscripts

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3 **Feasibility of 2-D ultrasound shear wave elastography of fetal lungs in case of**
4 **threatened preterm labor: a study protocol**
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8 Nicolas Mottet ^{1,2}, Sébastien Aubry ^{2,3}, Chrystelle Vidal ⁴, Guillaume Boiteux ⁴, Jean-Patrick
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3 **Contributors** : All the authors contributed to the conception and design of the study. NM,
4 SA, and RR provided the idea for the research or article, created the hypothesis and wrote the
5 original proposal. NM, CV, LP and RR significantly contributed to writing the paper. NM,
6 CV, GB and LP wrote this protocol paper. All authors read and approved the final manuscript.
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11 API/2015/60
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13 **Competing interests** None declared.
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16 Patient consent obtained.
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19 **Ethics approval** The Human Research Ethics Committee (Comité de Protection des
20 Personnes EST II, process number **15/494**)
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ABSTRACT

Introduction

2-D ultrasound Shear Wave Elastography (SWE) could be considered as a new noninvasive tool for monitoring fetal lung development based on evaluation of mechanical properties during pregnancy. Interesting results are available concerning use of SWE on developing organs, especially on premature infants and animals models. The main objective in this study is to evaluate feasibility of 2-D SWE in human fetal lungs between 24 and 34 weeks of gestation (WG). Secondary objective is to modelize fetal lung-to-liver elastography ratio (LLE ratio) and to assess variations between normal lung and lung surfactant-enriched after a corticosteroids course indicated for a threatened preterm labor (TPL).

Methods and analysis

A prospective case-control study will be performed between 24 and 34 WG. Fetal lungs and liver will be explored by SWE into two groups: fetuses of women with an uncomplicated pregnancy (control group) and fetuses of women with a TPL requiring administration of corticosteroids (cases group). LLE Ratio will be defined as the value of the lung elasticity divided by the value of the liver elasticity.

Primary judgment criterion is the value of elasticity modulus expressed in kilopascal. Lungs and liver will be explored through three measurements to define the most reproducible regions with the lowest intra and inter-observer variability. Feasibility will be evaluated by assessing the number of exams performed and the number of exams with interpretable results. Intra and inter-observer reproducibility will be evaluated by means of the intra-class correlation coefficient.

Ethics and dissemination

Approval of the study protocol was obtained from the human ethical research committee (Comité de Protection des Personnes EST II, process number 15/494) and the French National Agency for Medicines and Health Products Safety (process number 2015-A01575-44). All participants will sign a statement of informed consent.

Trial Registration number: NCT02870608

Key words: Shear wave elastography, elasticity, fetal, lung.

Strengths and limitation of the study

- This study will be the first one to demonstrate applicability of Shear Wave Elastography in human fetal lungs.
- Pharmacovigilance data will be collected at birth and 3 months later in order to assess safety of the device.
- Different factors will be taken into account to understand variability of measures: acquisition depth, number of measurements and representative values such as median or mean values.
- We shall not attempt to define reference value of fetal lung elasticity or to correlate SWE value with respiratory function at birth.

INTRODUCTION:

Background

Prenatal evaluation of fetal lung maturity (FLM) is still a challenge and different approaches have been reported with invasive biological tools or fetal imaging including ultrasounds and magnetic resonance imaging (MRI). FLM is mainly correlated with surfactant level and numerous tests have been developed to quantify this production: lecithin/sphingomyelin ratio (L/S), laminar body count, testing for Phosphatidyl glycerol with thin-layer chromatography, and estimation of the surfactant/albumin ratio using polarized light. All of these tests have been shown to have good sensitivity and negative predictive values; however, they all have a low positive predictive value and need invasive procedure^{1,2}. Thus, amniocentesis for FLM became obsolete³.

Lung and liver signal or echogenicity were often compared as ratio to assess FLM in several studies⁴⁻¹⁰. Non-invasive assessment of FLM has been first attempted using ultrasonography quantified by gray level histogram^{4,11,12}. These studies demonstrated sonographic changes associated with lung maturation but weak correlations between echogenicity and respiratory distress syndrome (RDS). Quantitative analysis with specific software has been secondly proposed to improve robustness. Fetal lung texture analysis with this method demonstrated a strong correlation with gestational age, biological test on amniotic fluid and risk of neonatal respiratory morbidity^{13, 14}. Additionally, several studies found a significant association between fetal lung-to-liver signal intensity ratio and estimated gestational age with fetal Magnetic Resonance Imaging (MRI). Actually, this technique is mainly used to assess fetal lung development and volume, especially in case of congenital diaphragmatic hernia or severe oligohydramnios. Nevertheless, the accuracy of both MRI and ultrasounds for estimating long-term respiratory outcomes remains limited¹⁵⁻¹⁹. These previous technique propose prenatal evaluation of lung maturity based on imaging features, but prediction of the pulmonary function through prenatal biomechanical properties seems relevant. With this hypothesis, we advanced the concept of “prenatal functional imaging” and shear wave elastography (SWE) could be a promising tool.

SWE is a recent ultrasound technology using acoustic radiation force imaging (ARFI), which enables to assess the stiffness of tissues in real time through a color quantitative elastogram²⁰. SWE assesses tissue elasticity (E), which is the tendency of tissue to resist deformation with an applied force. In locally homogeneous and purely elastic medium, we

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3 can calculate Young's modulus (kPa) from the shear wave speed ($\text{m}\cdot\text{s}^{-1}$) with the formula: $E=$
4 $3\rho\cdot c_{\text{sw}}^2$ (ρ being the medium density and c_{sw} the shear wave speed). The stiffness at any
5 location of the ROI can be sampled using measurement tools to obtain a quantitative
6 evaluation either in terms of shear wave speed or Young's modulus. A low shear wave speed
7 corresponds to a soft tissue, while a shear wave high speed indicates a stiff tissue. Study of
8 deep organs is possible with SWE because this method does not require any compression-
9 relaxation sequence on the target organ. One of the advantages of SWE over other
10 elastography methods is that the generation of the mechanical impulse is operator-
11 independent. SWE is expanding its range nowadays by its promising role for examination of
12 various organs (liver, thyroid) and for aiding discrimination of lesion characteristic, especially
13 breast or prostate tumors²¹⁻²⁴.

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22 There is to date no publication regarding its use on human fetuses. The Food and Drug
23 Administration (FDA) has so far, not yet approved use of SWE for obstetric clinical
24 applications because of the paucity of data in the literature. Although there is no report about
25 apparent histological changes with shear wave, the absence of others bioeffects could not be
26 discarded and further studies are recommended²⁵. However, setting parameters respect
27 mechanical and thermal indexes for soft-tissues and bone, which are necessary to allow an
28 obstetric examination defined by the FDA²⁶. Thus, there is a debate concerning safety of SWE
29 in fetal medicine. Herman et al studied the models and regulatory considerations for transient
30 temperature rise during ARFI, and found that any transient increase in temperature caused by
31 pulse bursts might still be within the safe limits determined by the (FDA)²⁷. Others authors
32 showed that transducer heating was below 1°C for the current clinical applications of ARFI.
33 According to experimental studies that simulate the heating of soft tissue during ARFI, they
34 demonstrate that ARFI on soft tissue is safe, provided that thermal index must be monitored
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22 Interesting and reassuring results are available concerning use of SWE on developing
23 organs, especially on premature infants and animals models. Principals organs explored are
24 the liver, brain and lungs. Quarello et al demonstrated the possibility to use SWE in non-
25 human primate fetuses and its feasibility to explore fetal organs. They found that elasticity
26 values were related to organs and gestational age³⁰. Concerning fetal lungs elasticity,
27 measurements of the proximal lungs seem to increase throughout the pregnancy. Concerning
28 use of SWE on premature infants, results are more numerous and include exploration of the
29 liver or the brain. Alison et al. have demonstrated feasibility and reproducibility of liver

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3 stiffness in preterm neonates with intra uterine growth restriction (IUGR) and gestational age
4 at birth between 26-31 weeks of gestation (WG)³¹. Others studies reported contribution of
5 SWE to the diagnosis of biliary atresia in neonates^{32,33}. Kim et al described variation of brain
6 elasticity in different regions in healthy neonates born between 28 and 40 WG³⁴. Su et al
7 quantitatively evaluated the effect of acoustic radiation force impulse imaging (ARFI) in
8 neonatal brain development. The authors found that full-term neonates had significantly
9 higher elasticity values than preterm neonates and there were no reported immediate adverse
10 events³⁵. Others teams work on application of this technology to neonates as a supplementary
11 tool to detect early ischemic brain injury³⁶.

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18 If we describe fetal thorax centered on a 4-chamber view of the fetal heart with SWE,
19 the following observations can be made (figure 1):
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- 21 - SWE is able to make distinction between fetal soft and stiff tissues. Fetal ribs
22 appear red colored mapped whereas proximal lung appears blue colored.
- 23 - A homogeneous colored area can be obtained on the proximal lung with a
24 color scale ranging from 0 to 48 kPa. A homogeneous ROI is important for
25 the reliability of the results.
- 26 - SWE can differ according to the acquisition depth. Proximal lung appears
27 with a blue homogenous distribution whereas distal lung appears with green
28 homogenous distribution.
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37 Objectives

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40 The main objective in this study is to evaluate feasibility of 2-D SWE in human fetal
41 lungs between 24 and 34 weeks of gestation (WG). Secondary objective is to modelize fetal
42 lung-to-liver elastography ratio (LLE ratio) and to assess variations between normal lung and
43 lung surfactant-enriched after a corticosteroids course indicated for a threatened preterm labor
44 (TPL).
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METHODS/DESIGN

Study design and setting

A prospective case-control study will be performed at the University Hospital of Besançon, (Besançon, France), Department of Obstetrics and Gynecology. Fetal lungs and liver will be explored by SWE between 24 and 34 WG in two groups: fetuses of women with an uncomplicated pregnancy will be considered as control group, and fetuses of women with a threatened preterm labor requiring administration of corticosteroids will be enrolled as cases. The first eligible patient was enrolled in May 2016 and we plan to enroll for 18 months. End of follow up of children will be completed approximately six months after the last birth.

In this pilot study, corticosteroids generate an effect model interesting for assessment of LLE ratio variation in the cases. We take advantage of the action of corticosteroids to explore another medium with different supposed biomechanical properties. Indeed, corticosteroids accelerated development of type 2 pneumocytes leading to surfactant production. Similarly, there is a significant increase in saturated phosphatidylcholine content in fetal lung, modifying propagation of shear waves potentially³⁷.

Control group will be recruited during routine prenatal visits or ultrasounds according to the French ultrasounds schedule (ultrasound screening during the second trimester between 20-25 WG and the third trimester between 30-35 WG³⁸). Cases will be enrolled during their hospitalization for threatened preterm delivery (figure 2). The design, conduction and reporting of this study will follow the norms of the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement³⁹.

Ethical considerations

The study respects the ethical standards established in the declaration of Helsinki⁴⁰. Investigators undertake to respect law no. 2004-806 of August 9, 2004 relative to public health policy on clinical trials, made effective by implementing Decree 2006-477 of April 26, 2006⁴¹. Approval of the study protocol was obtained from the human ethical research committee (Comité de Protection des Personnes EST II, process number **15/494**) and the French National Agency for Medicines and Health Products Safety (process number **2015-A01575-44**). The study is registered on clinicaltrials.gov with the following number: **NCT02870608**. All participants will sign a statement of informed consent and will be allowed to abandon the study at any time, with no negative consequences. All procedures of the study

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3 will be confidential. The study will involve collection and storage of ultrasound images and
4 study participants will therefore be thoroughly informed. All variables (patient parameters,
5 data items, data elements) will be aggregated into electronic Case report Forms.
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8 As explained in the background, The Food and Drug Administration (FDA) has so far,
9 not yet approved use of SWE for obstetric clinical applications because of the paucity of data
10 in the literature. In this study, fetuses will be included from 24 to 34 WG, over the period of
11 organogenesis. In the patient information sheet, it is specified that previous studies explored
12 liver or brain of premature infants with SWE. Alison et al. studied liver stiffness in preterm
13 neonates with intra uterine growth restriction (IUGR) and gestational age at birth between 26-
14 31 WG³¹. Franchi-Abella et al evaluate feasibility and diagnostic accuracy of SWE for the
15 assessment of liver stiffness in 96 patients and 7 were preterm newborns between 30-35 WG
16⁴². Both studies did not report adverse outcomes and were approved by institutional review
17 board. Moreover, consent form mentions conclusions of an experimental study of the
18 biological effects associated with SWE in brains of neonatal mice. Results indicated that
19 using SWE do not cause detectable histologic changes. Potential effects on intracellular
20 signaling pathways were detected when the SWE scanning lasted more than 30 min²⁵. Thus,
21 patients will be informed that our study is focused on fetuses with a gestational age similar to
22 preterm newborn included in the above-mentioned studies. Moreover, exploration with SWE
23 will concern only lung and liver with duration of less than 10 min in respect of biophysical
24 safety indexes: Thermal index $Ti \leq 0.7$ and Mechanical index $Mi < 1.9$.
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37 **Participants**

38 Cases will be recruited during hospitalization for a threatened preterm labor requiring
39 administration of corticosteroids. The complementary inclusion criteria are: pregnant women
40 aged 18 years or more, singleton pregnancy with eutrophic fetus, signature of consent and
41 affiliation to health insurance scheme (figure 2). Patients will be stratified by gestational age
42 between 24 and 34 WG, with one class every WG (10 classes in total) and 5 patients per class.
43 After obtaining consent, a SWE exam will be performed before the first intramuscular
44 administration of corticosteroids “day 0” (betamethasone 12mg). A second SWE exam will be
45 performed within 24 h after the second intramuscular administration of corticosteroids “day
46 2”. A third SWE will be performed between the fifth and the seventh day following the first
47 administration of corticosteroids “day 5-7”, if the women are not early discharge at home.
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3 For the control group, women will be matched by gestational age and will be recruited
4 during their pregnancy monitoring. Information concerning the study will be given as early as
5 the second trimester by obstetrician or midwives during medical visits or ultrasounds. The
6 complementary inclusion criteria are following: major pregnant women, uncomplicated
7 pregnancy between 24 and 34 WG, singleton pregnancy with eutrophic fetus, signature of
8 consent and affiliation to health insurance scheme. If they are willing to participate, a first
9 SWE exam will be proposed at “day 0”, followed by a second one in two days “day 2”.
10 Patients who come back for an SWE exam performed outside current medical visit will
11 receive financial compensation for travel expenses. As well as cases, control patients will be
12 stratified by gestational age between 24 and 34 WG.

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20 Exclusion criteria common to both groups are: Fetal lung or liver pathologies,
21 inclusion in another medical study, patients under legal incapacity.

22 23 24 25 **Variables and measuring technique**

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27 • **Prenatal variables**

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30 Primary judgment criterion is the value of Young’s modulus (E) (elasticity modulus)
31 expressed in kilopascal (kPa). A Logic E9 system (General Electric Medical System,
32 Milwaukee, WI, USA) with CE certification CE LNE/G-MED [CE0459] will be used for this
33 study, equipped with abdominal convex probe 1-6 MHz (C1-6-D probe). This technique
34 includes a real-time visualization of a color quantitative elastogram coupled to a B-mode
35 image. To provide robust shear wave and optimize its detection, Logiq E9 uses innovative
36 techniques. Comb-push Ultrasound SWE generates multiple shear wave sources into the
37 region of interest (ROI) and uses directional filtering to remove the interference. The TAST
38 technique is able to robustly track shear waves and correct for the sequential tracking delay⁴³.
39 This technique has the merit of rapid and solid reconstruction of a large elasticity map
40 (elastogram) with the only single acquisition by generating multiple shear waves from
41 multiple unfocused push beams.

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50 The shear wave acquisition measurement protocol is saved in a specific preset that will be
51 used for every patient: 2D-Mode: Harmonic imaging central depth = 6 cm, frequency = 6
52 MHz, Acoustic Power 100%; Elastography-Mode: Shear wave signal frequency ranges from
53 50 to 400 kHz, Acoustic power of the pushing beam: 100%, Tracking power: 100%, color
54 scale ranging from 0 to 48 kPa. Biophysical safety indices: Thermal index $Ti \leq 0.7$ and
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3 Mechanical index $M_i < 1,9$ (table 1).
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5 Fetal lungs will be voluntarily approached laterally to systematically obtain proximal
6 and distal lungs regarding the distance to the probe. Each measurement has to be performed
7 on a homogeneous elastogram to be valid. Operators will perform 2 cycles of 9 Young's
8 modulus measurements while systematically repositioning the probe on each target organ.
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10 A cycle includes: 3 measurements on the proximal lung (anterior "P1", medium "P2" and
11 posterior portion "P3"), 3 measurements on the distal lung (anterior "D1", medium "D2" and
12 posterior portion "D3") and 3 measurements on 3 liver segments (IV, V, VI) (figure 3).
13 Implementation of Shear Wave elastography displays 2D images of Young's Modulus in the
14 target organ. In real time, the elasticity appears color coded, where blue identifies deformable
15 tissue and red indicates rigid tissues. The Young's modulus value at any location will be
16 sampled using a round ROI of 5 mm. Distance between the probe and the target organ will be
17 collected. The average elasticity in the ROI will be automatically recorded by the system in a
18 worksheet. The investigators will adjust the position of the ROI using the B-mode image for
19 guidance and take care to obtain the most homogeneous color coded ROI before Young's
20 modulus estimation (figure 4). Technical failure was defined as failure to obtain a
21 homogeneous elastogram in more than 50% in the sampling area. To test the inter-observer
22 variability, a second observer will successively perform measurements on 30 fetuses. All
23 measurements will be carried out directly by ultrasound on the target organ. The second
24 observer will perform measurements just after the first one and will be blinded to the
25 feasibility and results obtained by the first one.
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38 Estimation of Fetal Weight Estimation (EFW) will be performed during each exam
39 according to Hadlock formula based on Cephalic circumference (CC), Abdominal
40 circumference (AC) and femoral length (FL): $\log_{10} EFW = 1,326 + 0,0107 PC + 0,0438 PA$
41 $+ 0,158 LF + 0,00326 (PA \times LF)$ 44.
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45 • **Postnatal variables**
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48 The following pharmacovigilance data will be collected at birth and 3 months later in
49 order to assess safety of the device:
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51 - At birth in both groups: term of birth, weight, neonatal transfer, Apgar Score and
52 evaluation of respiratory distress by Silverman Score 45.
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54 - Three months after birth: medical history, health problems, respiratory diseases or
55 symptoms, liver disease or symptoms and number of hospitalization since birth.
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Limitation of Bias

Use of sampling method with 5 patients per class of gestational age will limit repartition bias between control and cases. Inclusion of any pregnant women attending Besançon University maternity for the control group, and inclusion of all women with a threatened preterm labor will limit risk of selection bias. Risk of loss will be limited in the cases group between “day 0” and “day 2” because patients will remain hospitalized for corticosteroids administration. Nevertheless, risk of loss is most important between “day 2” and “day 5-7” because of possible early discharge at home after the second administration of corticosteroids. For ethical and medical reasons, cases early discharge at home cannot be convened in “day 5-7”. Risk of loss will be limited in the control group because of a financial compensation for travel expenses. To limit inter-observer variation, expert sonographers will perform elastography exams.

Study size

Because of the paucity of data regarding SWE on human fetal lung, the sample size calculation was based on measurements of fetal lungs in pregnant baboons and extrapolations made by clinicians, with the following assumptions: average expected value of elasticity coefficient for fetal lung will be 2 KPa at 24 WG and 4 KPa at 34 WG. We hypothesized a linear increase of elasticity coefficient during pregnancy in the control group and a decrease in cases exposed to corticosteroids. At “day 2”, we therefore expect in the cases a variation of 0.2 KPa (corresponding to one week of lung maturation during gestation). Forty-two patients per group will be needed to have 90% power to statistically demonstrate such a difference (0.2 versus 0.057) assuming an α risk of 0.05 and standard deviation of 0.2.

Proposed statistical analysis

- **Technical validation:**

Feasibility will be evaluated by assessing the number of exams performed and the number of exams with interpretable results. All the variables and data that will be stored for each SWE exam are summarized in table 1. Intra and inter-observer reproducibility will be evaluated by the intra-class correlation coefficient (ICC) with 95% IC. Intra-observer

reproducibility will be calculated using the two cycles of measure, inter observer reproducibility between the operators will be calculated for means of the two cycles of measures if intra-observer is high⁴⁶. Only repeatable and reproducible values of each ROI will be considered to modelize LLE ratio.

Table 1: Variables and data stored for each Shear Wave Elastography Exam.
ROI (Region of interest)

Maternal	Fetal	Technical
<ul style="list-style-type: none"> ▪ Age (years) 	<ul style="list-style-type: none"> ▪ Presentation: <ul style="list-style-type: none"> - Cephalic - Breech - Transverse 	<ul style="list-style-type: none"> ▪ Young's modulus (kPa) in 9 ROI: <ul style="list-style-type: none"> - Proximal lung: P1, P2, P3 - Distal lung: D1, D2, D3 - Liver: IV, V, VI
<ul style="list-style-type: none"> ▪ Weeks of gestation 	<ul style="list-style-type: none"> ▪ Estimated fetal weight (grams) 	<ul style="list-style-type: none"> ▪ Distance between probe and target ROI for each measurement (cm)
<ul style="list-style-type: none"> ▪ Body mass Index (kg/m²) 	<ul style="list-style-type: none"> ▪ Placenta: <ul style="list-style-type: none"> - Position: anterior, posterior, lateral, fundal - Thickness (cm) 	<ul style="list-style-type: none"> ▪ Biophysical safety indices: <ul style="list-style-type: none"> - Mechanical index (Mi) - Thermal index (Ti)
<ul style="list-style-type: none"> ▪ Subcutaneous adipose tissue thickness (cm) 	<ul style="list-style-type: none"> ▪ Amniotic fluid index (cm) 	

- **Clinical evaluation**

Evolution of fetal lung SWE, fetal liver SWE and lung-to-liver-SWE ratio will be assessed by group and overall. Lung-to-liver-SWE Ratio will be initially defined as the value of the lung elasticity divided by the value of the liver elasticity.

SWE values before corticosteroids administration will be compared between cases and control groups. If there is a statistically significant difference, a confusional bias will have to be sought between fetal lung elasticity values and threatened preterm labor. All results will be presented as “delta” variation between two measurements in order to limit “non-comparability bias” between both groups. If corticosteroids affect fetal lung elasticity, “delta before/after” will be more important in cases than controls.

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3 Analysis of mean differences between groups will be carried out using a Student's test or
4 Wilcoxon test according to distribution of data for quantitative variables. Shapiro-Wilk test
5 will be used to determine if data set is well modeled by a normal distribution. The relationship
6 of quantitative variables to each other will be tested using Pearson's or Spearman's
7 correlation as appropriate.
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11 Qualitative variable will be expressed as frequencies and quantitative variables will be
12 displayed as the mean \pm SD if normally distributed or as the median \pm IQR (Inter Quartile
13 Range) if asymmetrically distributed. Qualitative variables will be analyzed using a χ^2 test or
14 Fisher test.
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18 For statistical analyses, the level of statistical significance will be set at 5% ($p < 0.05$).
19 Statistical analysis will be performed with statistical software SAS for Windows, version 9.4
20 and MedCalc software, version 15.
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DISCUSSION

2D-SWE could be considered as a new noninvasive tool for monitoring fetal lung development based on evaluation of mechanical properties during pregnancy. However, standardization for measuring SWE in human fetal lung is necessary to demonstrate applicability of the technique, and to understand limiting factors. This pilot study will focus on technical performances before considering clinical applications. In this protocol, SWE will be used after fetal period of organogenesis and pharmacovigilance data will be collected at birth and 3 months later in order to assess safety of the device.

With the study design, we shall not attempt to define reference value of fetal lung elasticity during pregnancy or to correlate SWE value with respiratory function at birth. The main objective is to evaluate feasibility and reproducibility of 2D-SWE in human fetal lung between 24 and 34 WG and to modelize a new LLE ratio. Indeed, each target organ will be explored through three measurements to define the most relevant and reproducible regions with the lowest intra and inter-observer variability. Use of LLE ratio is interesting because it allows comparison between two tissues in which the liver is taken as a reference organ. Several studies indicate that liver signal or echogenicity remain constant through gestation suggestive of a regular evolution of the tissue characteristics^{47,48}. Thus, we will compare evolution of SWE values between two organs subjected to the same technical variations, especially for depth. Both lung and liver tissue density increase during pregnancy and we expect a linear increase of SWE values of these organs. In case of variations of lung SWE values after a course of corticosteroids, LLE ratio should be modified. If we suppose that liver elasticity will be constant between two separate exams of 48h, LLE ratio will decrease if corticosteroids lead to attenuation of shear wave speed. Conversely, if lung elasticity increases after a course of corticosteroids, LLE ratio will also increase. **Finally, LLE ratio will be used to increase the reliability of the results and to standardize the study.**

One advantage of this protocol is to evaluate SWE in three tissues with different supposed biomechanical properties: normal lung, lung with increase of surfactant synthesis after corticosteroids course, and liver. Corticosteroids generate an effect model interesting for assessment of LLE's ratio variation in the cases because of a significant increase of phosphatidylcholine content in fetal lungs. This effect is mediated by an activation of choline-phosphate cytidyltransferase³⁷. Given the placental transfer and time to induce cellular mechanism of transcription, an artificial delay of 24h seems to be necessary to produce

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3 surfactant in pneumocytes type 2. The benefit of corticosteroid administration is greatest at 2–
4 7 days after the initial dose^{49,50}. Two approaches can be proposed after a complete course of
5 corticosteroids. On the one hand, SWE of fetal lung could increase because corticosteroids
6 accelerate FLM, and we could expect an advanced ripening by one WG. On the other hand,
7 increase in phospholipids content in fetal lung after corticosteroids could increase viscosity
8 and lead to dispersion of shear wave speed and attenuation.
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14 Different factors will be taken into account in this study to understand variability of
15 measures: acquisition depth, number of measurements and representative values such as
16 median or mean values. Shear wave velocity can be different according to the acquisition
17 depth and this parameter is gradually underestimated with increasing depth. Effect is greatest
18 in stiff tissues but little effect is seen in soft target⁵¹⁻⁵³. This phenomenon might be explained
19 by a damping of the acoustic push pulse that generates the shear waves by both increased
20 attenuation and increased stiffness of the target⁵⁴. Thus, we expected low variation between
21 proximal and distal lung because they may be considered as soft tissues. There are also
22 debates about the acquisition number during SWE and the designers of the device did not
23 provided advices. The number of measurements (NMs) reported in the recent literature on
24 various organs is inconsistent (commonly seen are 3, 4 and 5 NMs)⁵⁵⁻⁵⁶. Quarello et al
25 reported 3 NMs on fetal lungs in pregnant baboons and ICC for and inter observer variability
26 were better for an average value of three measurements⁵⁷. For Alison et al, 9 NMs were
27 performed on liver in preterm neonates with intra uterine growth restriction (3 measurements
28 from three different liver segments). Measurements showed high reproducibility on average
29 values (ICC = 0.94–0.98 for intra-operator, 0.86 for inter-operator)³¹. One objective of our
30 protocol is to determine the NM's necessary to evaluate fetal lung SWE with a good inter and
31 intra-observer reproducibility. Thus, each value will be analyzed to determine if mean lung
32 and liver SWE can be achieved through only 1 valid measurement or more, and if its
33 performance is equivalent to 2 or 3 valid measurements.
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48 To sum up, we hope that the results of this study will contribute to clarify applicability
49 of SWE on human fetal lung between 24 and 34WG. SWE could be a new tool for reliable
50 prediction of lung development through biomechanical properties. This study will be the first
51 one to propose a protocol of measurement underlying limiting factors.
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3 **Figure 1:** (a) B-mode image of the fetal thorax centered on a 4-chamber view of the fetal
4 heart, using an abdominal convex probe 1-6 MHz. (b) Elastogram of the fetal thorax showing
5 a color-coded elasticity map: blue identifies deformable tissue and red indicates rigid tissues
6 (kPa).
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10 **Figure 2:** Design of the study.
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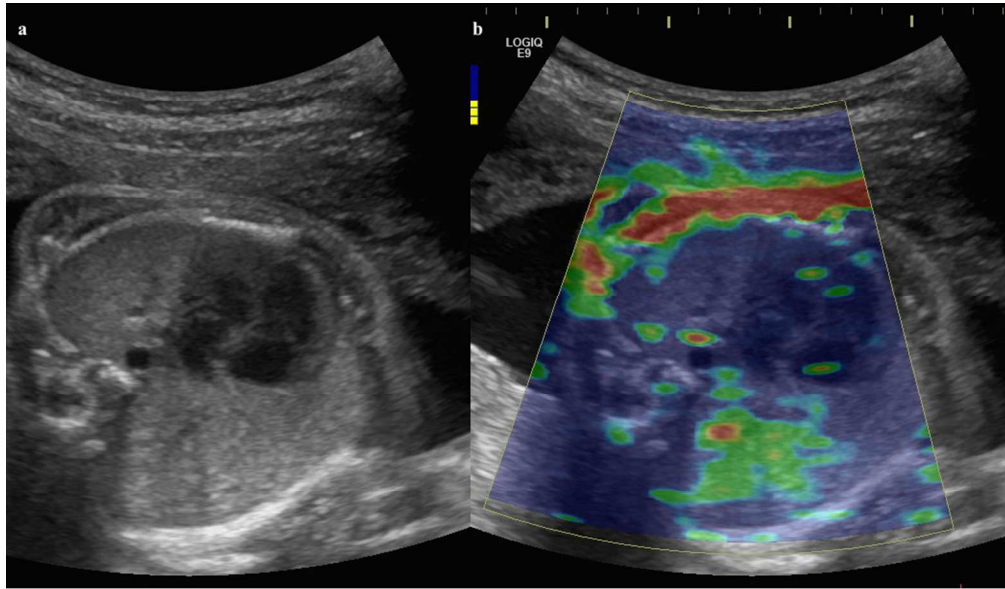
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13 **Figure 3:** Measurement sites with 2D comb-push SWE using an abdominal convex probe 1-6
14 MHz (C1-6-D probe). Color scale ranging from 0 to 48 kPa. Sufficient color maps covering
15 more than 50% of the sampling area are obtained considered as a technical success. Regions
16 of interest (ROI) are placed on homogeneous elastograms:
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- 19 - (a,b) 3 measurements on the proximal lung (anterior “P1”, medium “P2” and posterior
20 portion “P3”)
- 21 - (a,c) 3 measurements on the distal lung (anterior “D1”, medium “D2” and posterior
22 portion “D3”)
- 23 - (d-f) 3 measurements on 3 liver segments (IV, V, VI)

24 Operator will perform 2 cycles of 9 Young’s modulus measurements while systematically
25 repositioning the probe and each target ROI.
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28 **Figure 4:** 2D comb-push SWE on proximal fetal lung. Color scale ranging from 0 to 48 kPa.
29 On image (a), elastogram is not homogeneous and is degraded by artifact on the left side due
30 to rib shadowing. By moving the probe to another location, a more homogeneous elastogram
31 (b) is obtained allowing measurement.
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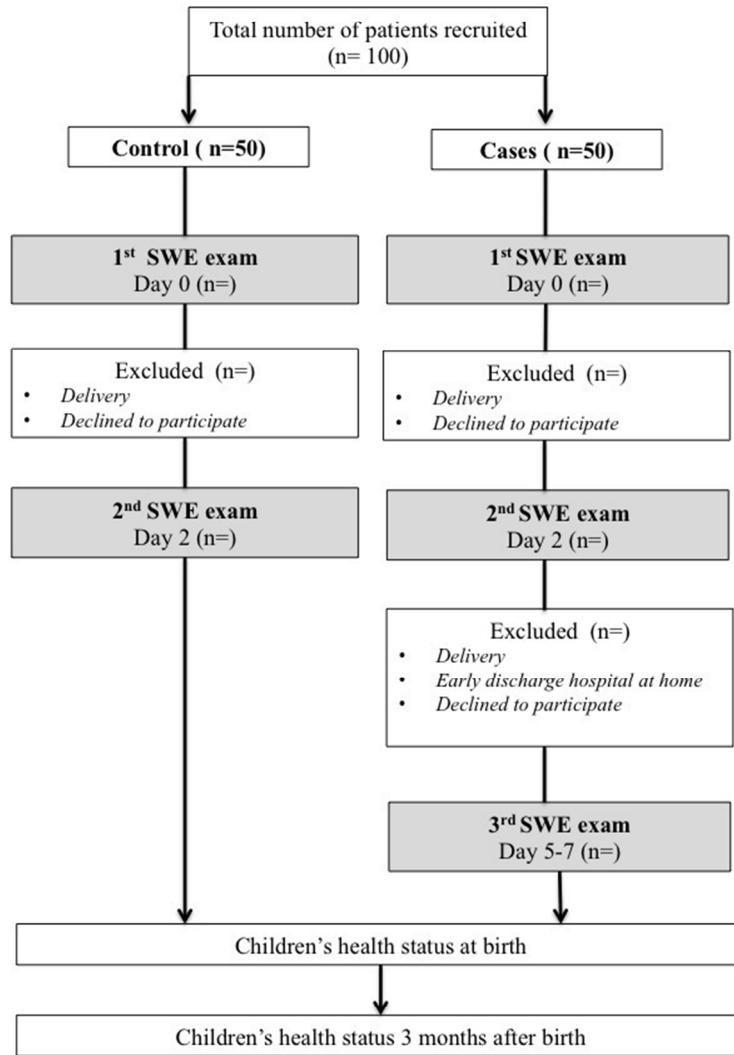
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(a) B-mode image of the fetal thorax centered on a 4-chamber view of the fetal heart, using an abdominal convex probe 1-6 MHz. (b) Elastogram of the fetal thorax showing a color-coded elasticity map: blue identifies deformable tissue and red indicates rigid tissues (kPa).

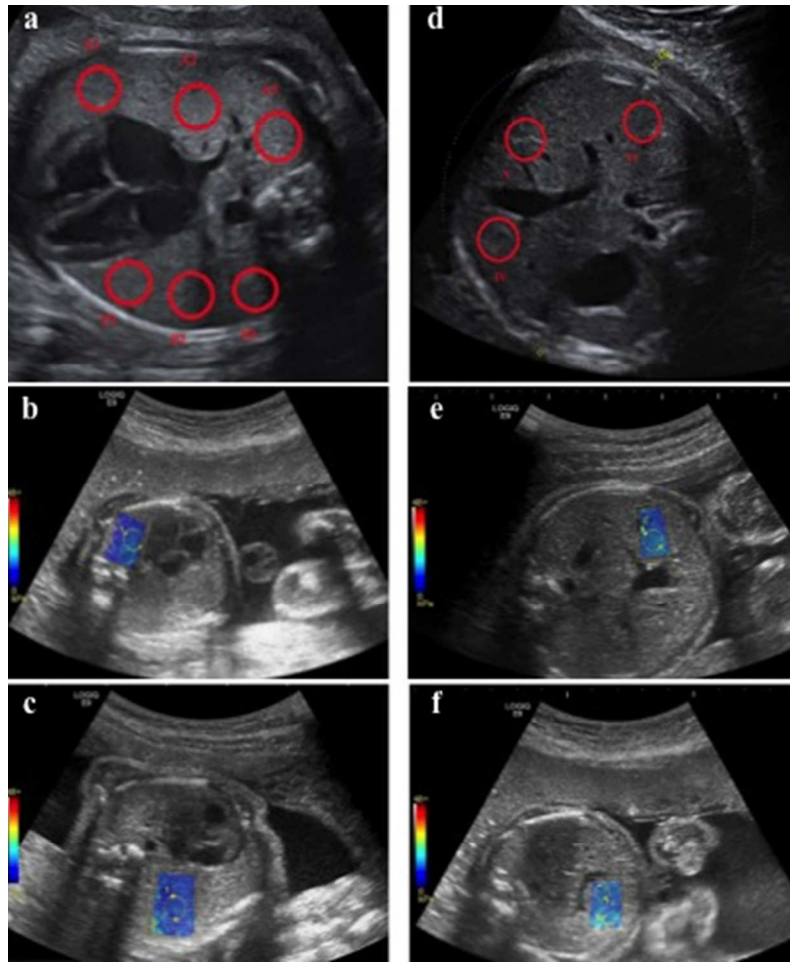
88x51mm (300 x 300 DPI)

Review only



Design of the study.

60x81mm (300 x 300 DPI)

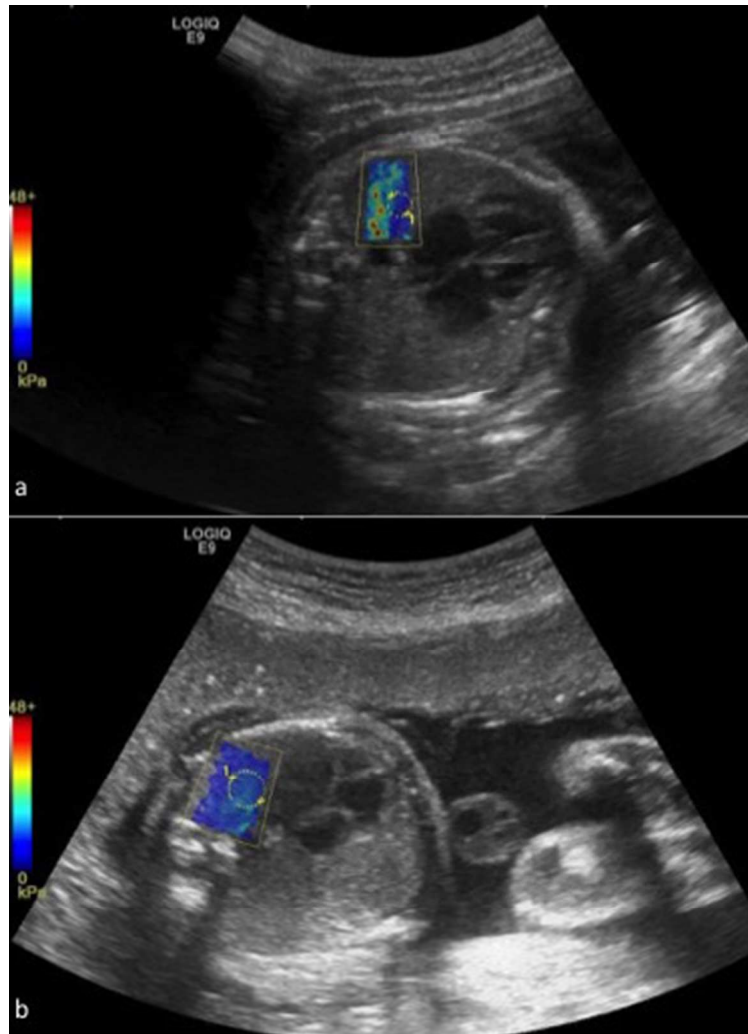


Measurement sites with 2D comb-push SWE using an abdominal convex probe 1-6 MHz (C1-6-D probe). Color scale ranging from 0 to 48 kPa. Sufficient color maps covering more than 50% of the sampling area are obtained considered as a technical success. Regions of interest (ROI) are placed on homogeneous elastograms:

- (a,b) 3 measurements on the proximal lung (anterior "P1", medium "P2" and posterior portion "P3")
- (a,c) 3 measurements on the distal lung (anterior "D1", medium "D2" and posterior portion "D3")
- (d-f) 3 measurements on 3 liver segments (IV, V, VI)

Operator will perform 2 cycles of 9 Young's modulus measurements while systematically repositioning the probe and each target ROI.

33x40mm (300 x 300 DPI)



2D comb-push SWE on proximal fetal lung. Color scale ranging from 0 to 48 kPa. On image (a), elastogram is not homogeneous and is degraded by artifact on the left side due to rib shadowing. By moving the probe to another location, a more homogeneous elastogram (b) is obtained allowing measurement.

31x43mm (300 x 300 DPI)

STROBE 2007 (v4) Statement—Checklist of items that should be included in reports of case-control studies

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(a) Indicate the study’s design with a commonly used term in the title or the abstract	3
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	3
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	5,6
Methods			
Study design	4	Present key elements of study design early in the paper	8
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	8
Participants	6	(a) Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls	8,9
		(b) For matched studies, give matching criteria and the number of controls per case	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	9,10
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	9,10,11
Bias	9	Describe any efforts to address potential sources of bias	11
Study size	10	Explain how the study size was arrived at	11
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	11,12
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	12
		(b) Describe any methods used to examine subgroups and interactions	12
		(c) Explain how missing data were addressed	12
		(d) If applicable, explain how matching of cases and controls was addressed	12
		(e) Describe any sensitivity analyses	12
Results			12

Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	8,9
		(b) Give reasons for non-participation at each stage	10
		(c) Consider use of a flow diagram	10
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	8,9
		(b) Indicate number of participants with missing data for each variable of interest	
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	
		(b) Report category boundaries when continuous variables were categorized	
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	
Discussion			
Key results	18	Summarise key results with reference to study objectives	13
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	15
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14
Generalisability	21	Discuss the generalisability (external validity) of the study results	14
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.