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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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Patient consent obtained.

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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 obsolete[3].

Lung and liver signal or echogenicity were often compared as ratio to assess FLM in several studies[4-10]. 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 limited [15-19]. 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, which enables to assess the stiffness of tissues in real time [20]. In locally homogeneous and purely elastic medium, we can calculate Young's modulus (kPa) from the shear wave speed ($m.s^{-1}$) by using SWE with the formula: $E= 3\rho.c_{sw}^2$ (ρ being the medium density and c_{sw} the shear wave speed). The stiffness at any location of

the ROI can be sampled using measurement tools to obtain a quantitative evaluation either in terms of shear wave speed or Young's modulus. A low shear wave speed corresponds to a soft tissue, while a shear wave high speed indicates a stiff tissue. Study of deep organs is possible with SWE because this method does not require any compression- relaxation sequence on the target organ. One of the advantages of SWE over other elastography methods is that the generation of the mechanical impulse is operator-independent. SWE is expanding its range nowadays by its promising role for examination of various organs (liver, thyroid) and for aiding discrimination of lesion characteristic, especially breast or prostate tumors [21-24].

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 recommended [25-27]. 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].

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 age [30]. 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 WG [31]. 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 WG [34]. 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 events [35]. Others teams work on application of this technology to neonates as a supplementary tool to detect early ischemic brain injury [36].

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 potentially [37].

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 WG [38]. 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) statement [39].

Ethical considerations

The study respects the ethical standards established in the declaration of Helsinki [40].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 26,2006 [41]. 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

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

As explained in the background, 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. In this study, fetuses will be included from 24 to 34 WG, over the period of organogenesis. In the patient information sheet, it is specified that previous studies explored liver or brain of premature infants with SWE. Alison et al. studied liver stiffness in preterm neonates with intra uterine growth restriction (IUGR) and gestational age at birth between 26-31 WG [31]. Franchi-Abella et al evaluate feasibility and diagnostic accuracy of SWE for the assessment of liver stiffness in 96 patients and 7 were preterm newborns between 30-35 WG [42]. Both studies did not report adverse outcomes and were approved by institutional review board. Moreover, consent form mentions conclusions of an experimental study of the biological effects associated with SWE in brains of neonatal mice. Results indicated that using SWE doe not cause detectable histologic changes. Potential effects on intracellular signaling pathways were detected when the SWE scanning lasted more than 30 min [25]. Thus, patients will be informed that our study is focused on fetuses with a gestational age similar to preterm newborn included in the above-mentioned studies. Moreover, exploration with SWE will concern only lung and liver with duration of less than 10 min.

Participants

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

For the control group, women will be matched by gestational age and will be recruited during their pregnancy monitoring. Information concerning the study will be given as early as the second trimester by obstetrician or midwives during medical visits or ultrasounds. The

complementary inclusion criteria are following: major pregnant women, uncomplicated pregnancy between 24 and 34 WG, singleton pregnancy with eutrophic fetus, signature of consent and affiliation to health insurance scheme (figure 2). If they are willing to participate, a first SWE exam will be proposed at "day 0", followed by a second one in two days "day 2". Patients who come back for an SWE exam performed outside current medical visit will receive financial compensation for travel expenses. As well as cases, control patients will be stratified by gestational age between 24 and 34 WG.

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

Variables and measuring technique

- **Prenatal variables**

Primary judgment criterion is the value of elasticity modulus (E) (Young's modulus) expressed in kilopascal (kPa). A Logic E9 system (General Electric Medical System, Milwaukee, WI, USA) with CE certification CE LNE/G-MED [CE0459] will be used for this study, equipped with abdominal convex probe 1-6 MHz (C1-6-D probe). To provide robust shear wave and optimize its detection, Logiq E9 uses innovative techniques. Comb-push Ultrasound SWE generates multiple shear wave sources into the region of interest (ROI) and uses directional filtering to remove the interference. The TAST technique is able to robustly track shear waves and correct for the sequential tracking delay [43]. This technique has the merit of rapid and solid reconstruction of a large elasticity map with the only single acquisition by generating multiple shear waves from multiple unfocused push beams.

The shear wave acquisition measurement protocol is saved in a specific preset that will be used for every patient: Mode 2D: Harmonic imaging central depth = 6 cm, frequency = 6 MHz, Acoustic Power 100%; Mode elastography: Shear wave signal frequency ranges from 50 to 400 kHz, Acoustic power of the pushing beam: 100%, Tracking power: 100%, color scale ranging from 0 to 48 kPa. Each measurement has to be performed on a homogeneous area to be valid. Operators will perform 2 cycles of 3 elasticity measurements while systematically repositioning the probe on each target organ. A cycle includes: 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) (figure 3). Implementation of Shear

Wave elastography displays 2D images of Young's Modulus in the target organ. In real time, the elasticity appears color coded, where blue identifies deformable tissue and red indicates rigid tissues. The stiffness at any location will be sampled using a round ROI of 5 mm. Distance between the probe and the target organ will be collected. The average stiffness in the ROI will be automatically recorded by the system in a worksheet. The investigators will adjust the position of the ROI using the B-mode image for guidance and take care to obtain the most homogeneous color coded ROI before stiffness estimation (figure 4). Technical failure was defined as failure to obtain a homogeneous color map in more than 50% in the sampling area. To test the inter-observer variability, a second observer will successively perform measurements on 30 fetuses. All measurements will be carried out directly by ultrasound on the target organ and the second operator will be blinded to the feasibility and results obtained by the first one.

Evaluation of fetal weight will be performed during each exam according to Hadlock formula based on Cephalic circumference (CC), Abdominal circumference (AC) and femoral length (FL): log10 EPF = 1,326 + 0,0107 PC + 0,0438 PA + 0,158 LF + 0,00326 (PA x LF) [44].

- **Postnatal variables**

The following pharmacovigilance data will be collected at birth and 3 months later in order to assess safety of the device:

- At birth in both groups: term of birth, weight, neonatal transfer, Apgar Score and evaluation of respiratory distress by Silverman Score [45].

- Three months after birth: medical history, health problems, respiratory diseases or symptoms, liver disease or symptoms and number of hospitalization since birth.

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. Different limiting factors that can affect the results will be evaluated, especially reduced amniotic fluid, fetal position and maternal body mass index. Intra and inter-observer reproducibility will be evaluated by means of the intra-class correlation coefficient (ICC). Further, each sets of measurements for evaluation of inter- and intra-observer variability will be compared by calculating the following parameters as described by Bland and Altman and graphically presented as Bland-Altman scatter plots (46). Only repeatable and reproducible values of each ROI will be considered to modelize LLE ratio.

- **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 will be used to determine if data set is well modeled by a normal distribution. The relationship of quantitative variables to each other will be tested using Pearson's or Spearman's correlation as appropriate.

Qualitative variable will be expressed as frequencies and quantitative variables will be displayed as the mean ± SD if normally distributed or as the median ± IQR (Inter Quartile Range) if asymmetrically distributed. Qualitative variables will be analyzed using a χ^2 test or Fisher test.

For statistical analyses, the level of statistical significance will be set at 5% ($p<0.05$). Statistical analysis will be performed with statistical software SAS for Windows, version 9.4 and MedCalc software, version 15.

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 cytidylyltransferase [37]. 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–

7 days after the initial dose [49,50]. Two approaches can be proposed after a complete course of corticosteroids. On the one hand, SWE of fetal lung could increase because corticosteroids accelerate FLM, and we could expect an advanced ripening by one WG. On the other hand, increase in phospholipids content in fetal lung after corticosteroids could increase viscosity and lead to dispersion of shear wave speed and attenuation.

Different factors will be taken into account in this study to understand variability of measures: acquisition depth, number of measurements and representative values such as median or mean values. Shear wave velocity can be different according to the acquisition depth and this parameter is gradually underestimated with increasing depth. Effect is greatest in stiff tissues but little effect is seen in soft target [51-53]. This phenomenon might be explained by a damping of the acoustic push pulse that generates the shear waves by both increased attenuation and increased stiffness of the target [54]. Thus, we expected low variation between proximal and distal lung because they may be considered as soft tissues. There are also debates about the acquisition number during SWE and the designers of the device did not provided advices. The number of measurements (NMs) reported in the recent literature on various organs is inconsistent (commonly seen are 3, 4 and 5 NMs) [55-56]. Quarello et al reported 3 NMs on fetal lungs in pregnant baboons and ICC for and inter observer variability were better for an average value of three measurements [57]. For Alison et al, 9 NMs were performed on liver in preterm neonates with intra uterine growth restriction (3 measurements from three different liver segments). Measurements showed high reproducibility on average values (ICC = 0.94–0.98 for intra-operator, 0.86 for inter-operator) [31]. One objective of our protocol is to determine the NM's necessary to evaluate fetal lung SWE with a good inter and intra-observer reproducibility. Thus, each value will be analyzed to determine if mean lung and liver SWE can be achieved through only 1 valid measurement or more, and if its performance is equivalent to 2 or 3 valid measurements.

To sum up, we hope that the results of this study will contribute to clarify applicability of SWE on human fetal lung between 24 and 34WG. SWE could be a new tool for reliable prediction of lung development through biomechanical properties. This study will be the first one to propose a protocol of measurement underlying limiting factors.

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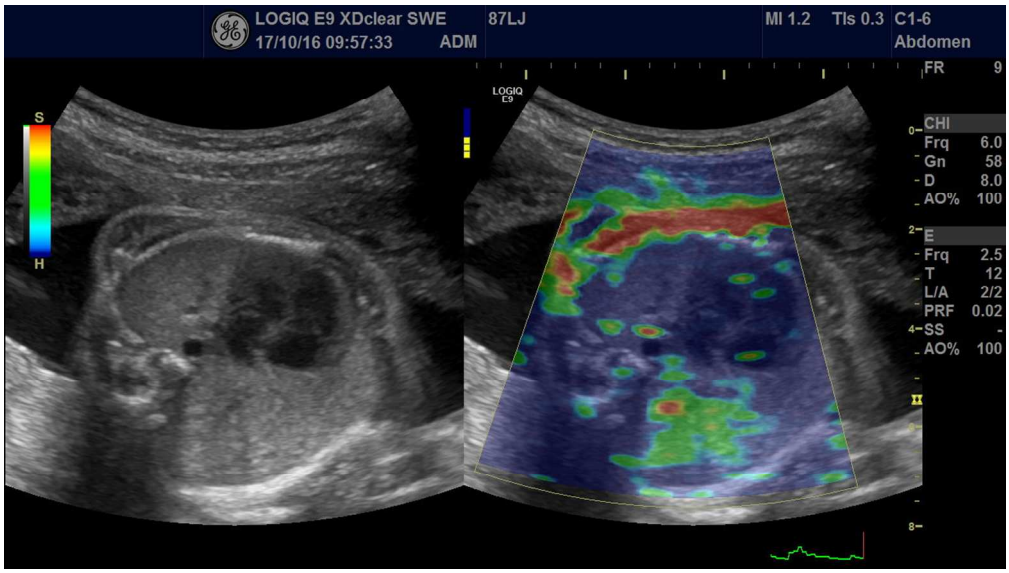
Figure 1: SWE on fetal thorax centered on a 4-chamber view of the fetal heart.

Figure 2: Design of the study.

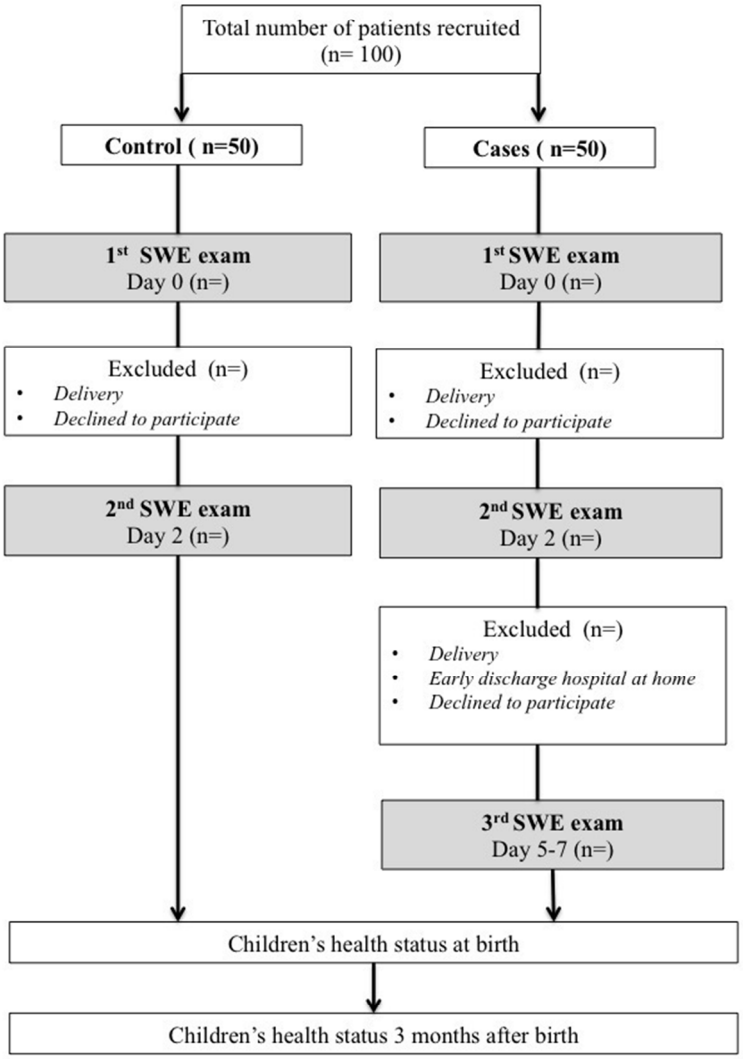
Figure 3: Measurement sites with SWE: 3 measurements on the proximal lung (anterior "P1", medium "P2" and posterior portion "P3"), 3 measurements on the distal lung (anterior "D1", medium "D2" and posterior portion "D3") and 3 measurements on 3 liver segments (IV, V, VI) according to Couinaud classification.

Figure 4: 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.



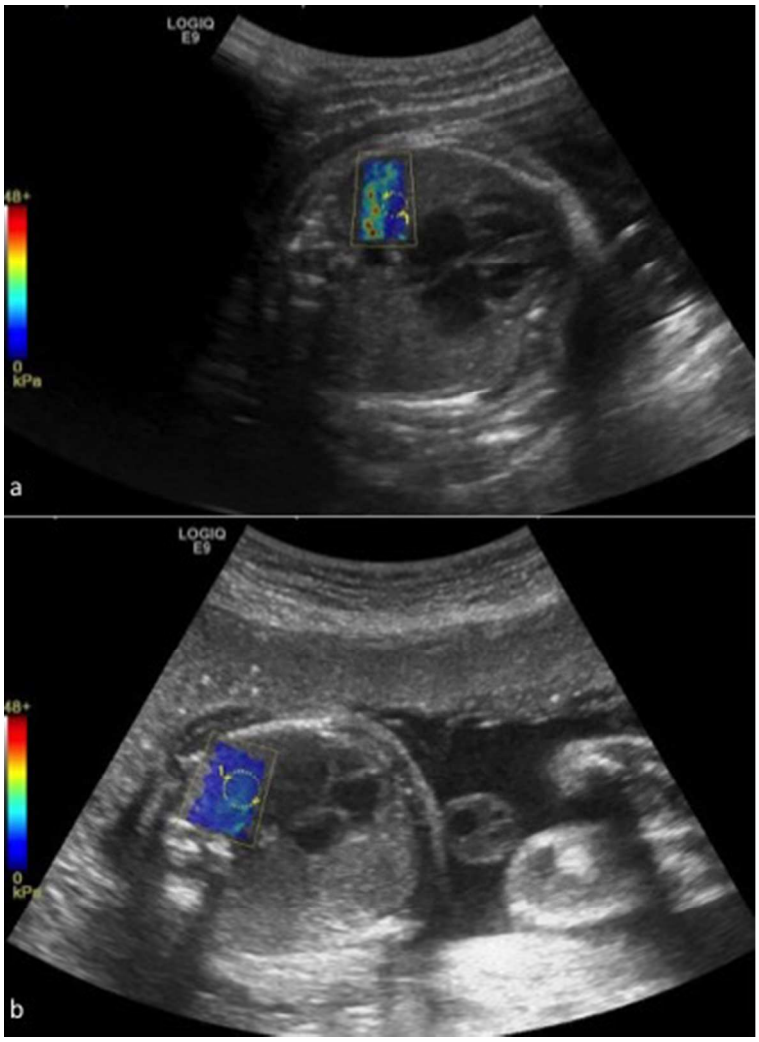
2-D ultrasound SWE on fetal thorax centered on a 4-chamber view of the fetal heart.

108x60mm (300 x 300 DPI)



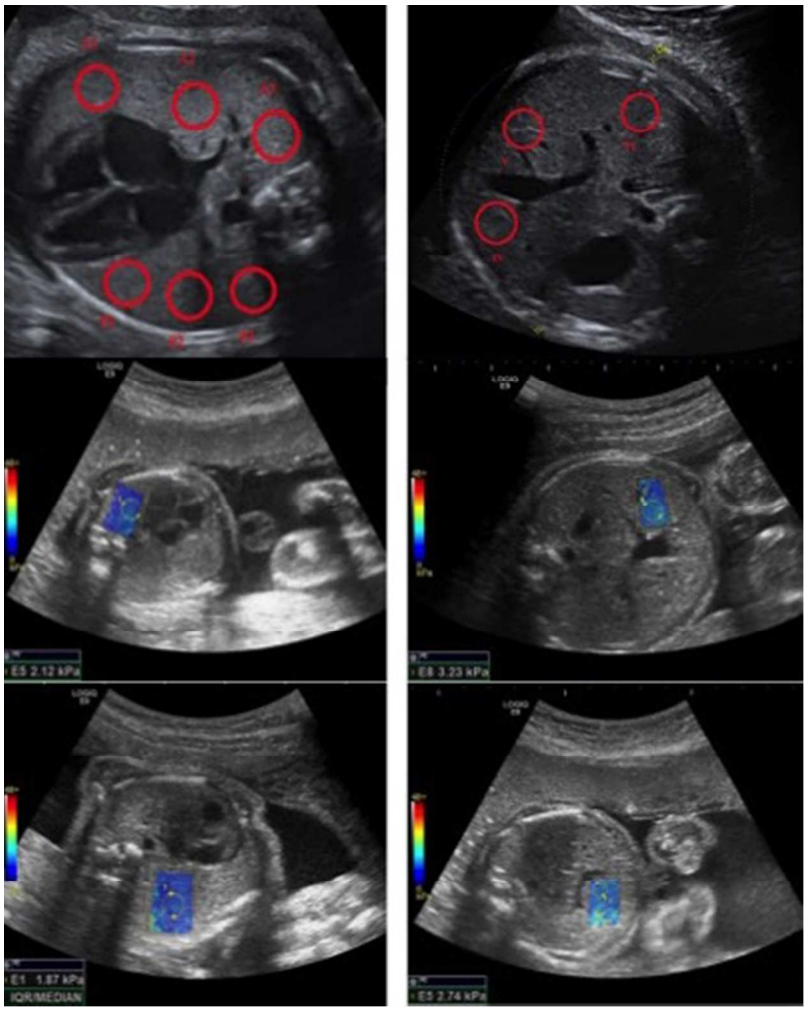
Design of the study.

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)



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

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

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Competing interests None declared.

Patient consent obtained.

Ethics approval The Human Research Ethics Committee (Comité de Protection des Personnes EST II, process number **15/494**)

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 obsolete[3].

Lung and liver signal or echogenicity were often compared as ratio to assess FLM in several studies[4-10]. 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 limited [15-19]. 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 elastogram [20]. 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

can calculate Young's modulus (kPa) from the shear wave speed ($m.s^{-1}$) with the formula: $E= 3\rho.c_{sw}^2$ (ρ being the medium density and c_{sw} the shear wave speed). The stiffness at any location of the ROI can be sampled using measurement tools to obtain a quantitative evaluation either in terms of shear wave speed or Young's modulus. A low shear wave speed corresponds to a soft tissue, while a shear wave high speed indicates a stiff tissue. Study of deep organs is possible with SWE because this method does not require any compression-relaxation sequence on the target organ. One of the advantages of SWE over other elastography methods is that the generation of the mechanical impulse is operator-independent. SWE is expanding its range nowadays by its promising role for examination of various organs (liver, thyroid) and for aiding discrimination of lesion characteristic, especially breast or prostate tumors [21-24].

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 recommended [25]. 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[26]. Thus, there is a debate concerning safety of SWE in fetal medicine. Herman et al studied the models and regulatory considerations for transient temperature rise during ARFI, and found that any transient increase in temperature caused by pulse bursts might still be within the safe limits determined by the (FDA) [27]. Others authors showed that transducer heating was below 1°C for the current clinical applications of ARFI. According to experimental studies that simulate the heating of soft tissue during ARFI, they demonstrate that ARFI on soft tissue is safe, provided that thermal index must be monitored [28,29].

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 age [30]. 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 (WG) [31]. 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 WG [34]. 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 events [35]. Others teams work on application of this technology to neonates as a supplementary tool to detect early ischemic brain injury [36].

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 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/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 potentially [37].

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 WG [38]. 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) statement [39].

Ethical considerations

The study respects the ethical standards established in the declaration of Helsinki [40].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 26,2006 [41]. 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

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

As explained in the background, 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. In this study, fetuses will be included from 24 to 34 WG, over the period of organogenesis. In the patient information sheet, it is specified that previous studies explored liver or brain of premature infants with SWE. Alison et al. studied liver stiffness in preterm neonates with intra uterine growth restriction (IUGR) and gestational age at birth between 26-31 WG [31]. Franchi-Abella et al evaluate feasibility and diagnostic accuracy of SWE for the assessment of liver stiffness in 96 patients and 7 were preterm newborns between 30-35 WG [42]. Both studies did not report adverse outcomes and were approved by institutional review board. Moreover, consent form mentions conclusions of an experimental study of the biological effects associated with SWE in brains of neonatal mice. Results indicated that using SWE doe not cause detectable histologic changes. Potential effects on intracellular signaling pathways were detected when the SWE scanning lasted more than 30 min [25]. Thus, patients will be informed that our study is focused on fetuses with a gestational age similar to preterm newborn included in the above-mentioned studies. Moreover, exploration with SWE will concern only lung and liver with duration of less than 10 min in respect of biophysical safety indexes: Thermal index Ti $\leq$ 0.7 and Mechanical index Mi < 1,9.

Participants

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

For the control group, women will be matched by gestational age and will be recruited during their pregnancy monitoring. Information concerning the study will be given as early as

the second trimester by obstetrician or midwives during medical visits or ultrasounds. The complementary inclusion criteria are following: major pregnant women, uncomplicated pregnancy between 24 and 34 WG, singleton pregnancy with eutrophic fetus, signature of consent and affiliation to health insurance scheme. If they are willing to participate, a first SWE exam will be proposed at "day 0", followed by a second one in two days "day 2". Patients who come back for an SWE exam performed outside current medical visit will receive financial compensation for travel expenses. As well as cases, control patients will be stratified by gestational age between 24 and 34 WG.

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

Variables and measuring technique

- **Prenatal variables**

Primary judgment criterion is the value of Young's modulus (E) (elasticity modulus) expressed in kilopascal (kPa). A Logic E9 system (General Electric Medical System, Milwaukee, WI, USA) with CE certification CE LNE/G-MED [CE0459] will be used for this study, equipped with abdominal convex probe 1-6 MHz (C1-6-D probe). This technique includes a real-time visualization of a color quantitative elastogram coupled to a B-mode image. To provide robust shear wave and optimize its detection, Logiq E9 uses innovative techniques. Comb-push Ultrasound SWE generates multiple shear wave sources into the region of interest (ROI) and uses directional filtering to remove the interference. The TAST technique is able to robustly track shear waves and correct for the sequential tracking delay [43]. This technique has the merit of rapid and solid reconstruction of a large elasticity map (elastogram) with the only single acquisition by generating multiple shear waves from multiple unfocused push beams.

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

Fetal lungs will be voluntarily approached laterally to systematically obtain proximal

and distal lungs regarding the distance to the probe. Each measurement has to be performed on a homogeneous elastogram to be valid. Operators will perform 2 cycles of 9 Young's modulus measurements while systematically repositioning the probe on each target organ.

A cycle includes: 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) (figure 3). Implementation of Shear Wave elastography displays 2D images of Young's Modulus in the target organ. In real time, the elasticity appears color coded, where blue identifies deformable tissue and red indicates rigid tissues. The Young's modulus value at any location will be sampled using a round ROI of 5 mm. Distance between the probe and the target organ will be collected. The average elasticity in the ROI will be automatically recorded by the system in a worksheet. The investigators will adjust the position of the ROI using the B-mode image for guidance and take care to obtain the most homogeneous color coded ROI before Young's modulus estimation (figure 4). Technical failure was defined as failure to obtain a homogeneous elastogram in more than 50% in the sampling area. To test the inter-observer variability, a second observer will successively perform measurements on 30 fetuses. All measurements will be carried out directly by ultrasound on the target organ. The second observer will perform measurements just after the first one and will be blinded to the feasibility and results obtained by the first one.

Estimation of Fetal Weight Estimation (EFW) will be performed during each exam according to Hadlock formula based on Cephalic circumference (CC), Abdominal circumference (AC) and femoral length (FL): log10 EFW = 1,326 + 0,0107 PC + 0,0438 PA + 0,158 LF + 0,00326 (PA x LF) [44].

- **Postnatal variables**

The following pharmacovigilance data will be collected at birth and 3 months later in order to assess safety of the device:

- At birth in both groups: term of birth, weight, neonatal transfer, Apgar Score and evaluation of respiratory distress by Silverman Score [45].

- Three months after birth: medical history, health problems, respiratory diseases or symptoms, liver disease or symptoms and number of hospitalization since birth.

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 high [46]. 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
▪ **Age (years)**	▪ **Presentation:** - Cephalic - Breech - Transverse	▪ **Young's modulus (kPa) in 9 ROI:** - Proximal lung: P1, P2, P3 - Distal lung: D1, D2, D3 - Liver: IV, V, VI
▪ **Weeks of gestation**	▪ **Weight (grams)**	▪ **Distance between probe and target ROI for each measurement (cm)**
▪ **Body mass Index (kg/m2)**	▪ **Placenta:** - Position: anterior, posterior, lateral, fundal - Thickness (cm)	▪ **Biophysical safety indices**: - Mechanical index (Mi) - Thermal index (Ti)
▪ **Subcutaneous adipose tissue thickness (cm)**	▪ **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.

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

will be used to determine if data set is well modeled by a normal distribution. The relationship of quantitative variables to each other will be tested using Pearson's or Spearman's correlation as appropriate.

Qualitative variable will be expressed as frequencies and quantitative variables will be displayed as the mean ± SD if normally distributed or as the median ± IQR (Inter Quartile Range) if asymmetrically distributed. Qualitative variables will be analyzed using a χ^2 test or Fisher test.

For statistical analyses, the level of statistical significance will be set at 5% ($p<0.05$). Statistical analysis will be performed with statistical software SAS for Windows, version 9.4 and MedCalc software, version 15.

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 cytidylyltransferase [37]. 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–

7 days after the initial dose [49,50]. Two approaches can be proposed after a complete course of corticosteroids. On the one hand, SWE of fetal lung could increase because corticosteroids accelerate FLM, and we could expect an advanced ripening by one WG. On the other hand, increase in phospholipids content in fetal lung after corticosteroids could increase viscosity and lead to dispersion of shear wave speed and attenuation.

Different factors will be taken into account in this study to understand variability of measures: acquisition depth, number of measurements and representative values such as median or mean values. Shear wave velocity can be different according to the acquisition depth and this parameter is gradually underestimated with increasing depth. Effect is greatest in stiff tissues but little effect is seen in soft target [51-53]. This phenomenon might be explained by a damping of the acoustic push pulse that generates the shear waves by both increased attenuation and increased stiffness of the target [54]. Thus, we expected low variation between proximal and distal lung because they may be considered as soft tissues. There are also debates about the acquisition number during SWE and the designers of the device did not provided advices. The number of measurements (NMs) reported in the recent literature on various organs is inconsistent (commonly seen are 3, 4 and 5 NMs) [55-56]. Quarello et al reported 3 NMs on fetal lungs in pregnant baboons and ICC for and inter observer variability were better for an average value of three measurements [57]. For Alison et al, 9 NMs were performed on liver in preterm neonates with intra uterine growth restriction (3 measurements from three different liver segments). Measurements showed high reproducibility on average values (ICC = 0.94–0.98 for intra-operator, 0.86 for inter-operator) [31]. One objective of our protocol is to determine the NM's necessary to evaluate fetal lung SWE with a good inter and intra-observer reproducibility. Thus, each value will be analyzed to determine if mean lung and liver SWE can be achieved through only 1 valid measurement or more, and if its performance is equivalent to 2 or 3 valid measurements.

To sum up, we hope that the results of this study will contribute to clarify applicability of SWE on human fetal lung between 24 and 34WG. SWE could be a new tool for reliable prediction of lung development through biomechanical properties. This study will be the first one to propose a protocol of measurement underlying limiting factors.

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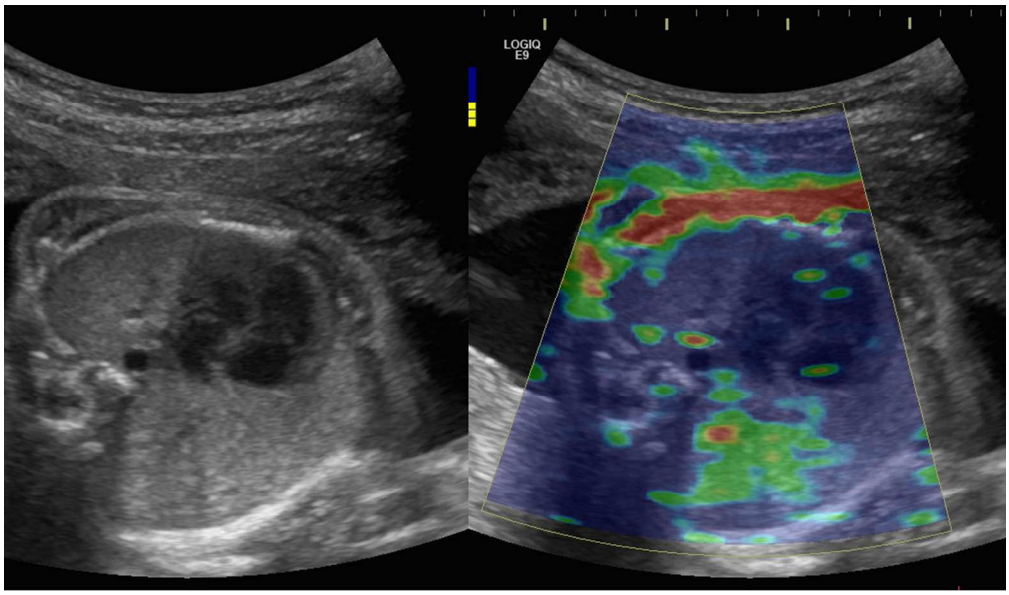
Figure 1: Elastogram on fetal thorax centered on a 4-chamber view of the fetal heart, using an abdominal convex probe 1-6 MHz (C1-6-D probe). Elasticity appears color-coded: blue identifies deformable tissue and red indicates rigid tissues (kPa).

Figure 2: Design of the study.

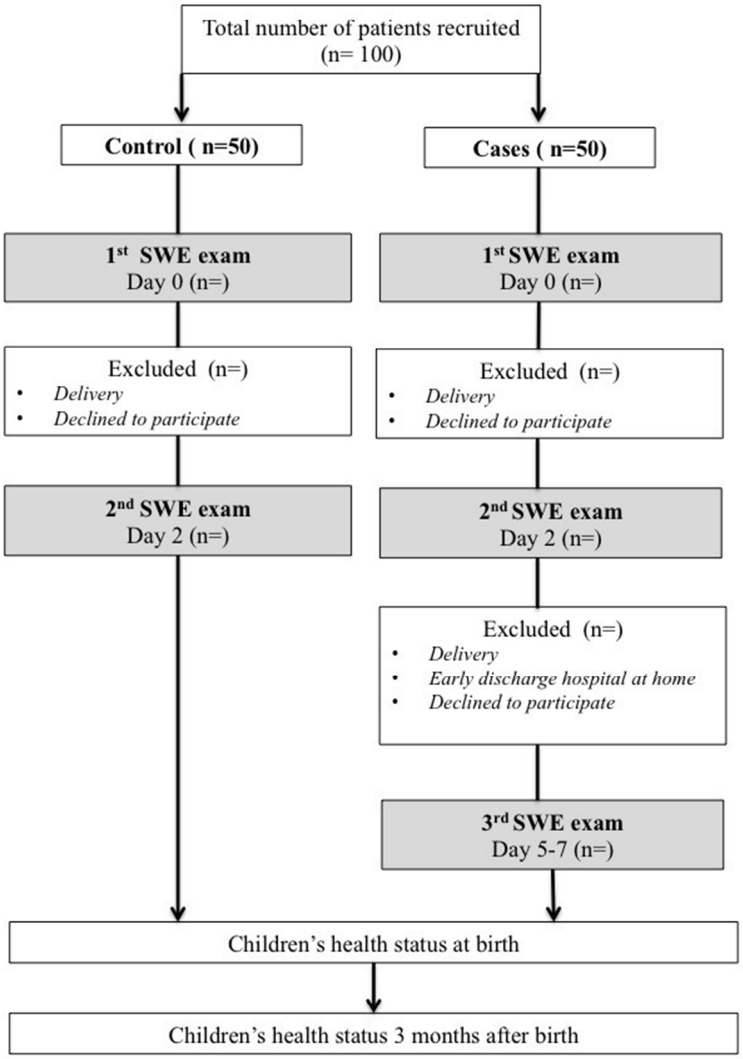
Figure 3: Measurement sites with 2D comb-push SWE using an abdominal convex probe 1-6 MHz (C1-6-D probe). Color scale ranging from 0 to 48 kPa. Sufficient color maps covering more than 50% of the sampling area are obtained considered as a technical success. Regions of interest (ROI) are placed on homogeneous 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.

Figure 4: 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.



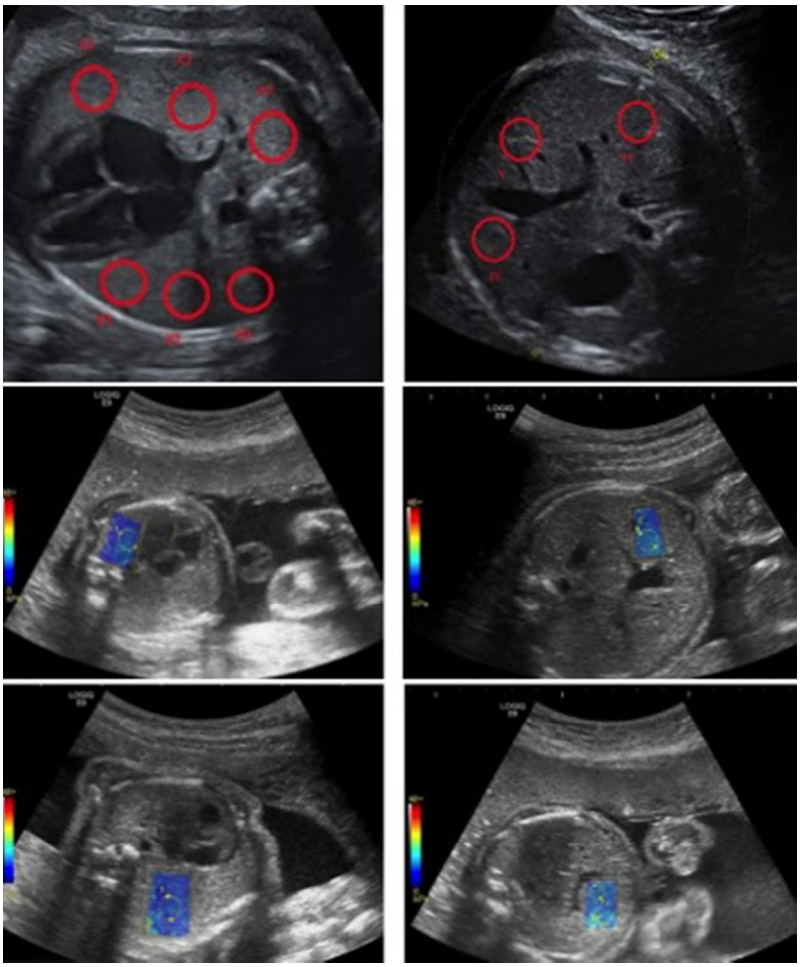
Elastogram on fetal thorax centered on a 4-chamber view of the fetal heart, using an abdominal convex probe 1-6 MHz (C1-6-D probe). Elasticity appears color-coded: blue identifies deformable tissue and red indicates rigid tissues (kPa).

88x51mm (300 x 300 DPI)



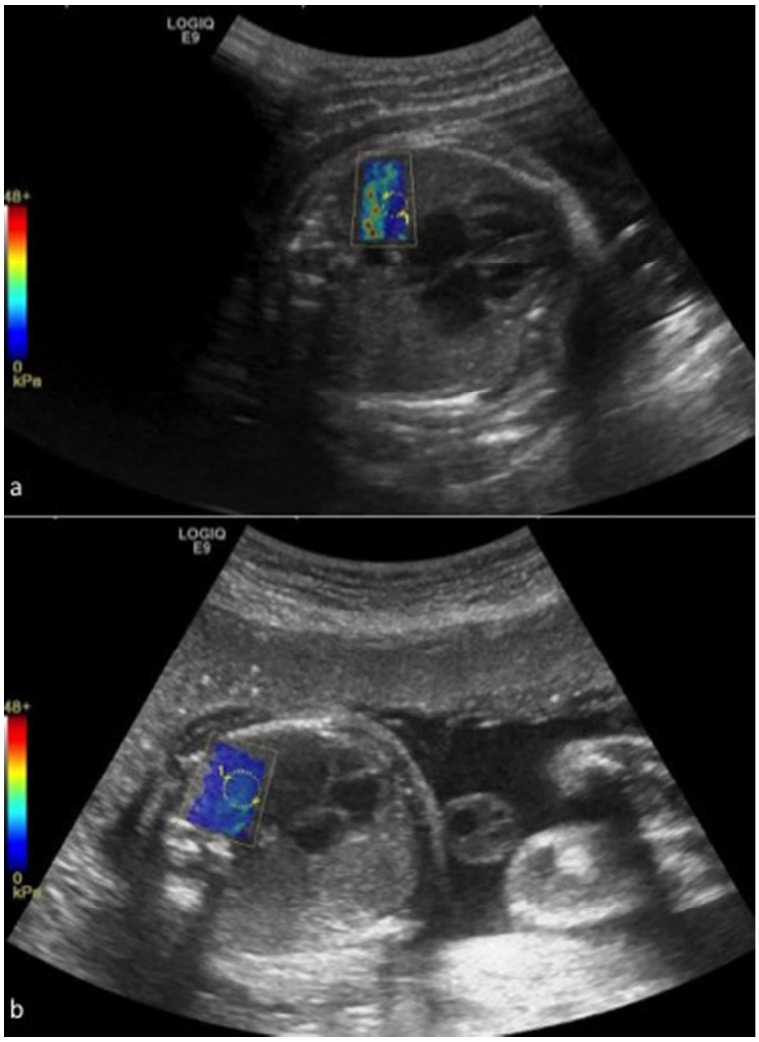
Design of the study.

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.

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

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Keywords:	shear wave elastography, lung, fetal, elasticity

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

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Competing interests None declared.

Patient consent obtained.

Ethics approval The Human Research Ethics Committee (Comité de Protection des Personnes EST II, process number **15/494**)

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 obsolete[3].

Lung and liver signal or echogenicity were often compared as ratio to assess FLM in several studies[4-10]. 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 limited [15-19]. 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 elastogram [20]. 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

can calculate Young's modulus (kPa) from the shear wave speed ($m.s^{-1}$) with the formula: $E= 3\rho.c_{sw}^2$ (ρ being the medium density and c_{sw} the shear wave speed). The stiffness at any location of the ROI can be sampled using measurement tools to obtain a quantitative evaluation either in terms of shear wave speed or Young's modulus. A low shear wave speed corresponds to a soft tissue, while a shear wave high speed indicates a stiff tissue. Study of deep organs is possible with SWE because this method does not require any compression-relaxation sequence on the target organ. One of the advantages of SWE over other elastography methods is that the generation of the mechanical impulse is operator-independent. SWE is expanding its range nowadays by its promising role for examination of various organs (liver, thyroid) and for aiding discrimination of lesion characteristic, especially breast or prostate tumors [21-24].

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 recommended [25]. 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[26]. Thus, there is a debate concerning safety of SWE in fetal medicine. Herman et al studied the models and regulatory considerations for transient temperature rise during ARFI, and found that any transient increase in temperature caused by pulse bursts might still be within the safe limits determined by the (FDA) [27]. Others authors showed that transducer heating was below 1°C for the current clinical applications of ARFI. According to experimental studies that simulate the heating of soft tissue during ARFI, they demonstrate that ARFI on soft tissue is safe, provided that thermal index must be monitored [28,29].

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 age [30]. 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 (WG) [31]. 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 WG [34]. 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 events [35]. Others teams work on application of this technology to neonates as a supplementary tool to detect early ischemic brain injury [36].

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 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/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 potentially [37].

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 WG [38]. 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) statement [39].

Ethical considerations

The study respects the ethical standards established in the declaration of Helsinki [40].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 26,2006 [41]. 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 clinicaltrial.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 study participants will therefore be thoroughly informed. All variables (patient parameters, data items, data elements) will be aggregated into electronic Case report Forms.

As explained in the background, 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. In this study, fetuses will be included from 24 to 34 WG, over the period of organogenesis. In the patient information sheet, it is specified that previous studies explored liver or brain of premature infants with SWE. Alison et al. studied liver stiffness in preterm neonates with intra uterine growth restriction (IUGR) and gestational age at birth between 26-31 WG [31]. Franchi-Abella et al evaluate feasibility and diagnostic accuracy of SWE for the assessment of liver stiffness in 96 patients and 7 were preterm newborns between 30-35 WG [42]. Both studies did not report adverse outcomes and were approved by institutional review board. Moreover, consent form mentions conclusions of an experimental study of the biological effects associated with SWE in brains of neonatal mice. Results indicated that using SWE doe not cause detectable histologic changes. Potential effects on intracellular signaling pathways were detected when the SWE scanning lasted more than 30 min [25]. Thus, patients will be informed that our study is focused on fetuses with a gestational age similar to preterm newborn included in the above-mentioned studies. Moreover, exploration with SWE will concern only lung and liver with duration of less than 10 min in respect of biophysical safety indexes: Thermal index Ti $\leq$ 0.7 and Mechanical index Mi < 1,9.

Participants

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

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

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

Variables and measuring technique

- **Prenatal variables**

Primary judgment criterion is the value of Young's modulus (E) (elasticity modulus) expressed in kilopascal (kPa). A Logic E9 system (General Electric Medical System, Milwaukee, WI, USA) with CE certification CE LNE/G-MED [CE0459] will be used for this study, equipped with abdominal convex probe 1-6 MHz (C1-6-D probe). This technique includes a real-time visualization of a color quantitative elastogram coupled to a B-mode image. To provide robust shear wave and optimize its detection, Logiq E9 uses innovative techniques. Comb-push Ultrasound SWE generates multiple shear wave sources into the region of interest (ROI) and uses directional filtering to remove the interference. The TAST technique is able to robustly track shear waves and correct for the sequential tracking delay [43]. This technique has the merit of rapid and solid reconstruction of a large elasticity map (elastogram) with the only single acquisition by generating multiple shear waves from multiple unfocused push beams.

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

Mechanical index Mi < 1,9 (table 1).

Fetal lungs will be voluntarily approached laterally to systematically obtain proximal and distal lungs regarding the distance to the probe. Each measurement has to be performed on a homogeneous elastogram to be valid. Operators will perform 2 cycles of 9 Young's modulus measurements while systematically repositioning the probe on each target organ.

A cycle includes: 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) (figure 3). Implementation of Shear Wave elastography displays 2D images of Young's Modulus in the target organ. In real time, the elasticity appears color coded, where blue identifies deformable tissue and red indicates rigid tissues. The Young's modulus value at any location will be sampled using a round ROI of 5 mm. Distance between the probe and the target organ will be collected. The average elasticity in the ROI will be automatically recorded by the system in a worksheet. The investigators will adjust the position of the ROI using the B-mode image for guidance and take care to obtain the most homogeneous color coded ROI before Young's modulus estimation (figure 4). Technical failure was defined as failure to obtain a homogeneous elastogram in more than 50% in the sampling area. To test the inter-observer variability, a second observer will successively perform measurements on 30 fetuses. All measurements will be carried out directly by ultrasound on the target organ. The second observer will perform measurements just after the first one and will be blinded to the feasibility and results obtained by the first one.

Estimation of Fetal Weight Estimation (EFW) will be performed during each exam according to Hadlock formula based on Cephalic circumference (CC), Abdominal circumference (AC) and femoral length (FL): log10 EFW = 1,326 + 0,0107 PC + 0,0438 PA + 0,158 LF + 0,00326 (PA x LF) [44].

- **Postnatal variables**

The following pharmacovigilance data will be collected at birth and 3 months later in order to assess safety of the device:

- At birth in both groups: term of birth, weight, neonatal transfer, Apgar Score and evaluation of respiratory distress by Silverman Score [45].

- Three months after birth: medical history, health problems, respiratory diseases or symptoms, liver disease or symptoms and number of hospitalization since birth.

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 high [46]. 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
▪ **Age (years)**	▪ **Presentation:** - Cephalic - Breech - Transverse	▪ **Young's modulus (kPa) in 9 ROI:** - Proximal lung: P1, P2, P3 - Distal lung: D1, D2, D3 - Liver: IV, V, VI
▪ **Weeks of gestation**	▪ **Estimated fetal weight (grams)**	▪ **Distance between probe and target ROI for each measurement (cm)**
▪ **Body mass Index (kg/m2)**	▪ **Placenta:** - Position: anterior, posterior, lateral, fundal - Thickness (cm)	▪ **Biophysical safety indices**: - Mechanical index (Mi) - Thermal index (Ti)
▪ **Subcutaneous adipose tissue thickness (cm)**	▪ **Amniotic fluid index (cm)**	

- **<u>Clinical evaluation</u>**

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 will be used to determine if data set is well modeled by a normal distribution. The relationship of quantitative variables to each other will be tested using Pearson's or Spearman's correlation as appropriate.

Qualitative variable will be expressed as frequencies and quantitative variables will be displayed as the mean ± SD if normally distributed or as the median ± IQR (Inter Quartile Range) if asymmetrically distributed. Qualitative variables will be analyzed using a χ^2 test or Fisher test.

For statistical analyses, the level of statistical significance will be set at 5% ($p<0.05$). Statistical analysis will be performed with statistical software SAS for Windows, version 9.4 and MedCalc software, version 15.

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 cytidylyltransferase [37]. 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 2–7 days after the initial dose [49,50]. Two approaches can be proposed after a complete course of corticosteroids. On the one hand, SWE of fetal lung could increase because corticosteroids accelerate FLM, and we could expect an advanced ripening by one WG. On the other hand, increase in phospholipids content in fetal lung after corticosteroids could increase viscosity and lead to dispersion of shear wave speed and attenuation.

Different factors will be taken into account in this study to understand variability of measures: acquisition depth, number of measurements and representative values such as median or mean values. Shear wave velocity can be different according to the acquisition depth and this parameter is gradually underestimated with increasing depth. Effect is greatest in stiff tissues but little effect is seen in soft target [51-53]. This phenomenon might be explained by a damping of the acoustic push pulse that generates the shear waves by both increased attenuation and increased stiffness of the target [54]. Thus, we expected low variation between proximal and distal lung because they may be considered as soft tissues. There are also debates about the acquisition number during SWE and the designers of the device did not provided advices. The number of measurements (NMs) reported in the recent literature on various organs is inconsistent (commonly seen are 3, 4 and 5 NMs) [55-56]. Quarello et al reported 3 NMs on fetal lungs in pregnant baboons and ICC for and inter observer variability were better for an average value of three measurements [57]. For Alison et al, 9 NMs were performed on liver in preterm neonates with intra uterine growth restriction (3 measurements from three different liver segments). Measurements showed high reproducibility on average values (ICC = 0.94–0.98 for intra-operator, 0.86 for inter-operator) [31]. One objective of our protocol is to determine the NM's necessary to evaluate fetal lung SWE with a good inter and intra-observer reproducibility. Thus, each value will be analyzed to determine if mean lung and liver SWE can be achieved through only 1 valid measurement or more, and if its performance is equivalent to 2 or 3 valid measurements.

To sum up, we hope that the results of this study will contribute to clarify applicability of SWE on human fetal lung between 24 and 34WG. SWE could be a new tool for reliable prediction of lung development through biomechanical properties. This study will be the first one to propose a protocol of measurement underlying limiting factors.

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Figure 1: (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).

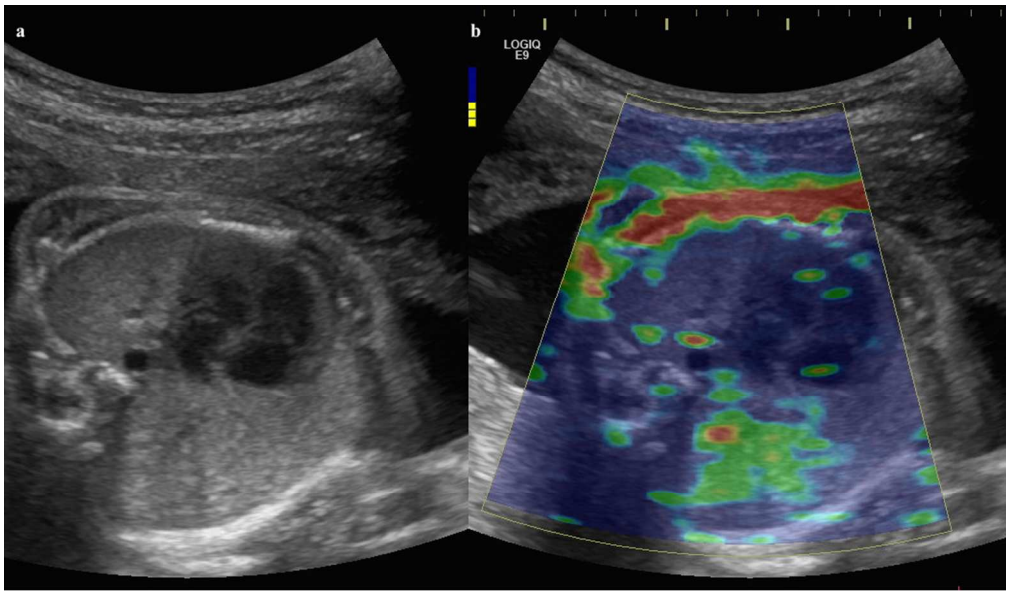
Figure 2: Design of the study.

Figure 3: 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)

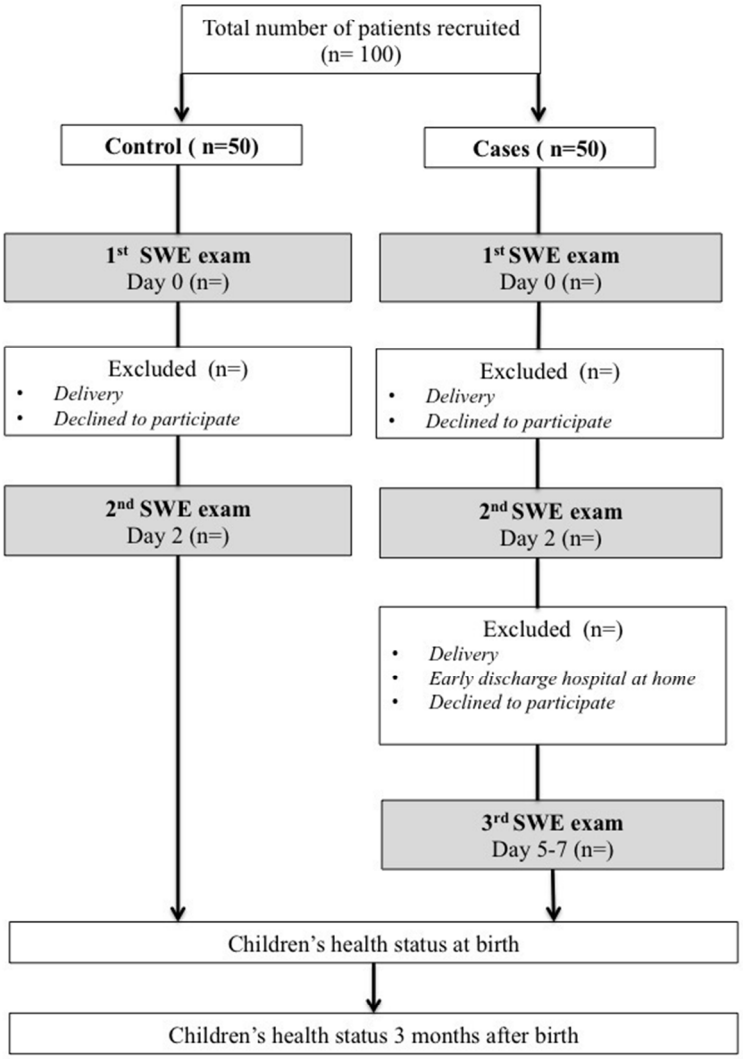
Operator will perform 2 cycles of 9 Young's modulus measurements while systematically repositioning the probe and each target ROI.

Figure 4: 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.



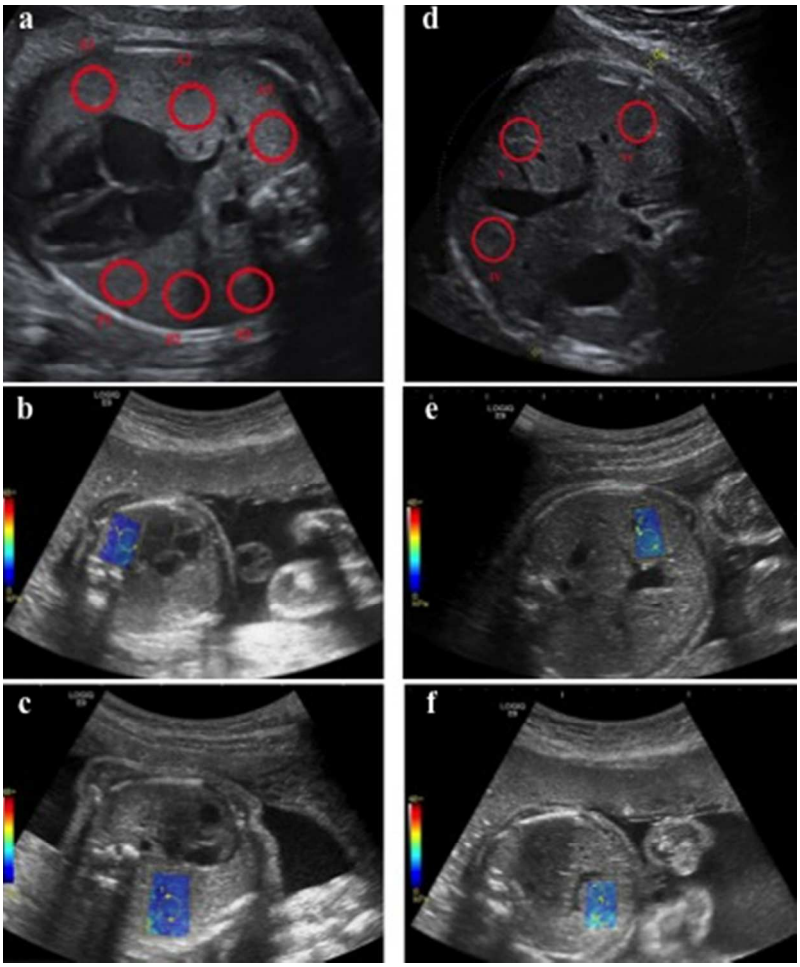
(a) B-mode image of the fetal thorax centered on a 4-chamber view of the fetal heart, using an abdominal convex probe 1-6 MHz. (b) Elastogram of the fetal thorax showing a color-coded elasticity map: blue identifies deformable tissue and red indicates rigid tissues (kPa).

88x51mm (300 x 300 DPI)



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")
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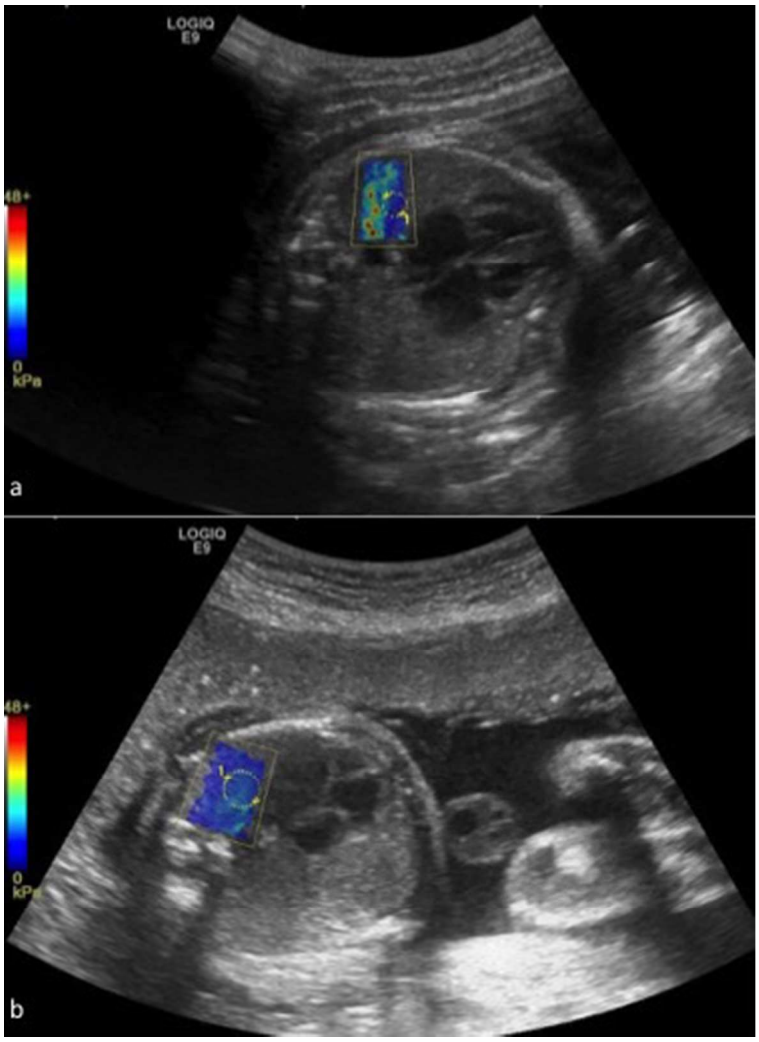
- (d-f) 3 measurements on 3 liver segments (IV, V, VI)
Operator will perform 2 cycles of 9 Young's modulus measurements while systematically repositioning the probe and each target ROI.

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.