**University of
Zurich**^{UZH}

**Zurich Open Repository and
Archive**

University of Zurich
University Library
Strickhofstrasse 39
CH-8057 Zurich
www.zora.uzh.ch

Year: 2018

Gestational age-dependent fetal fluid dynamics in the ovine developmental model: establishment of surrogate markers for the differentiation of stem cell origin

Kehl, Debora ; Görtz, Sabrina ; Wang, Craig ; Hoerstrup, Simon P ; Bleul, U ; Weber, Benedikt

Abstract: The ovine developmental model represents the standard in vivo model for studies involving maternofetal physiology, amniotic fluid (AF) research, and fetal cell therapy prior to human clinical use. Although being close to the human fetal anatomy, 2 separate extraembryonic fluid compartments remain during gestation, known as the amnion and the allantois. A clear distinction between AF versus allantoic fluid (AL) is therefore indispensable for correct scientific conclusions with regard to human translation. In the presented study, the biochemical composition of AF and AL was evaluated in ovine gravid uteri postmortem (n = 31) over the entire gestation. Four parameters, consisting of Na⁺, Cl⁻, Mg²⁺, and total protein, have been found to allow for specific discrimination of the 2 fetal fluids at all gestational phases and therefore as potential surrogate parameters for gestational age. In addition, volumetric changes of the developing fetus and the 2 fetal fluid cavities were analyzed by contrast-enhanced computed tomography (n = 12). AF showed a significant, linear volumetric increase over gestation, whereas AL volume maintained relatively static independent of gestational age. These results serve as a basis for future studies by providing surrogate markers enabling a reliable distinction of isolated fetal fluids and contained cells in the ovine developmental model over the entire gestation.

DOI: <https://doi.org/10.1159/000499504>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://www.zora.uzh.ch/178610>

Journal Article

Published Version

Originally published at:

Kehl, Debora; Görtz, Sabrina; Wang, Craig; Hoerstrup, Simon P; Bleul, U; Weber, Benedikt (2018). Gestational age-dependent fetal fluid dynamics in the ovine developmental model: establishment of surrogate markers for the differentiation of stem cell origin. *Cells, Tissues, Organs*, 206(4-5):208-217.

DOI: <https://doi.org/10.1159/000499504>

Gestational Age-Dependent Fetal Fluid Dynamics in the Ovine Developmental Model: Establishment of Surrogate Markers for the Differentiation of Stem Cell Origin

Debora Kehl^a Sabrina Görtz^a Craig Wang^b Simon P. Hoerstrup^{a, c-e}
Ulrich Bleul^f Benedikt Weber^{a, c, d, g}

^aInstitute for Regenerative Medicine (IREM), University of Zurich, Zurich, Switzerland; ^bDepartment of Mathematics, University of Zurich, Zurich, Switzerland; ^cCenter for Applied Biotechnology and Molecular Medicine (CABMM), University of Zurich, Zurich, Switzerland; ^dZurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland; ^eWyss Zurich, University of Zurich, and ETH Zurich, Zurich, Switzerland; ^fClinic of Reproductive Medicine, Department of Farm Animals, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland; ^gSkin and Endothelium Research Division (SERD), Department of Dermatology, Medical University of Vienna, Vienna, Austria

Keywords

Amniotic fluid · Allantoic fluid · Developmental model · Fetal fluid volumes · Amniotic fluid stem cells

Abstract

The ovine developmental model represents the standard in vivo model for studies involving maternofetal physiology, amniotic fluid (AF) research, and fetal cell therapy prior to human clinical use. Although being close to the human fetal anatomy, 2 separate extraembryonic fluid compartments remain during gestation, known as the amnion and the allantois. A clear distinction between AF versus allantoic fluid (AL) is therefore indispensable for correct scientific conclusions with regard to human translation. In the presented study, the biochemical composition of AF and AL was evaluated in ovine gravid uteri postmortem ($n = 31$) over the entire gestation. Four parameters, consisting of Na^+ , Cl^- , Mg^{2+} , and total protein, have been found to allow for specific discrimination of the 2 fetal fluids at all gestational phases and therefore as potential surrogate parameters for gestational age. In addition,

volumetric changes of the developing fetus and the 2 fetal fluid cavities were analyzed by contrast-enhanced computed tomography ($n = 12$). AF showed a significant, linear volumetric increase over gestation, whereas AL volume maintained relatively static independent of gestational age. These results serve as a basis for future studies by providing surrogate markers enabling a reliable distinction of isolated fetal fluids and contained cells in the ovine developmental model over the entire gestation.

© 2019 S. Karger AG, Basel

Introduction

The ovine developmental model represents a standard in vivo model for the assessment of maternofetal disease and fetal therapeutic applications prior to human clinical

Ulrich Bleul and Benedikt Weber contributed equally as senior authors to the study.

use. Even though it does not completely recapitulate human pregnancy, several scientific questions involving the entire duration of pregnancy cannot be addressed in the human situation given major ethical and medical restrictions. Consequently, the ovine model is well accepted and regularly used as part of preclinical fetal studies, such as fetal tracheal [Kunisaki et al., 2006; Gray et al., 2012] and diaphragmatic [Fuchs et al., 2004; Kunisaki et al., 2006; Turner et al. 2011] reconstruction, heart valve replacement [Weber et al., 2012], wound healing [Klein et al., 2011], maternofetal interactions (placental development/function) [Barry and Anthony, 2008], or cell transplantations [Emmert et al., 2013]. Also, studies on ovine fetal adnexa-derived (stem) cells [Cremonesi et al., 2011] or ovine amniotic fluid (AF)-derived (stem) cells [Weber et al., 2016] have been conducted revealing cellular phenotypes largely comparable to the human counterpart.

However, although being close to the human fetal anatomy, the ovine maternofetal model maintains 2 separate extraembryonic fluid compartments during the entire gestation, which are known as the amnion and the allantois [Wintour et al., 1986]. In humans, only a single cavity, known as the AF cavity, is present after the 3rd to 5th week of conception where the transient allantoic fluid (AL) compartment is lost [Pansky, 1982]. As a consequence, when dealing with studies mimicking the human physiology (such as fetal preclinical trials), this anatomical difference has to be appreciated. In particular in studies which involve the AF (or cells thereof), a clear distinction between AF and AL harvest seems indispensable to allow for correct interpretation of the data. This is even more exemplified by recent studies showing significant differences in pluripotency-related gene expression of cells isolated from the ovine AF versus AL compartment [Kehl et al., 2017] as well as proteomic analyses on both fluids [Riding et al., 2008]. Therefore, several groups have investigated the biochemical composition of AF and AL showing differences in electrolyte and protein components between both fetal fluids in mares [Zanella et al., 2014], sheep [Benson and Wintour, 1995; Tangelakis et al., 1995; Anitha and Thangavel, 2011; Weber et al., 2016], and feline models [Fresno et al., 2012]. These biochemical changes can be influenced by maternal and external factors [Benson et al., 1995; Tangelakis et al., 1995] as well as the significant volumetric changes of the ovine fetal fluid compartments over the course of gestation [Prestes et al., 2001; McCarty et al., 2009; Anitha and Thangavel, 2011]. In spite of these first insights into the biochemical profiles and reports on the anatomical changes of the ovine fetus in utero, less is understood about the biochemical composition changes of both AF and AL over the

entire duration of gestation. So far, most of the published studies in the ovine model only analyzed fetal fluid composition and volumes after the 10th week of gestation punctually [Lingwood and Wintour, 1984; Benson et al., 1995; Tangelakis et al., 1995; Prestes et al., 2001; Robertson et al., 2009]. However, these evaluations extending the entire gestation are mandatory for future preclinical evaluations involving AF harvest in the ovine model and its translation into the human counterpart.

The presented study therefore analyzes volumetric changes in the fetus as well as the surrounding fetal fluid cavities by computed tomography (CT) and correlates these findings with the biochemical profile obtained from both, AF and AL, over the entire duration of ovine gestation. The analyses should enable a more reliable harvest of fetal fluids and a better comparison of fetal fluid dynamics in the ovine versus the human situation for future preclinical evaluations of maternofetal disease and fetal therapeutic applications.

Materials and Methods

Harvest of Ovine Gravid Uteri

Ovine gravid uteri ($n = 52$) of single and multiple gestations were collected postmortem from a local abattoir (Hinwil, Switzerland). Crown-rump length (CRL) of each fetus was determined in order to allocate it to one of the three gestational age groups according to previously published standards [Sivachelvan et al., 1996]. Gestational age group 1 is defined by a CRL ≤ 10 , group 2 by a CRL >10 to ≤ 20 , and group 3 by a CRL >20 . For CT examination ($n = 12$), gravid uteri were directly stored at $4-8^{\circ}\text{C}$, and measurements were performed within 48 h. Within 2 h after slaughter, both fetal fluids (AF and AL) were harvested from the same animal ($n = 40$) as previously published [Weber et al., 2016]. In brief, the uterus and the fetal fluid cavities were opened surgically, and the fetal fluids were aspirated with a 50-mL syringe and a 10-gauge needle under visual guidance. After centrifugation for 10 min at 1,600 RPM, the supernatant was stored overnight at 4°C for the subsequent differential fluid analyses.

Imaging of Ovine Gravid Uteri

For CT imaging, the uteri were placed in a supine position. Imaging was performed using a multidetector CT scanner (Brilliance 16; Philips AG, Zurich, Switzerland). Transverse slices of the whole organs with a slice thickness of 2 mm were made using a voltage peak of 120 kVp and a current of 250 mA; 2–20 mL of contrast medium (Ioversol, Optiray 300; Guerbet, Switzerland) were injected into the amnion and allantois cavity under ultrasound guidance (MyLab One; Esaote, Maastricht; 8-MHz linear probe) depending on the particular size of the cavity. The fluid volumes were evaluated in a soft tissue and bone window with a window width of 520 Hounsfield units (HU), and a window level of 100 HU.

1

(For legend see next page.)

Volumetric Assessment of Ovine Fetal Fluid Cavities

Volumetric measurements were performed with the Philips Intellispace Portal. Using the 3D segmentation mode on the volume explorer, regions of interest (ROI) were selected according to the injected contrast medium. ROI were verified on all planes. Volumes were calculated by the software in cubic centimeters (cm³), and the amount of contrast medium (mL) injected was subtracted from the obtained results. Separate measurements were conducted for AF and AL compartments for each animal. Fetal volume was determined by selecting the fetal body as ROI and calculating the corresponding fetal volume. Extremities were not selected given their poor recognition at early gestational ages.

Differential Ovine Fetal Fluid Analysis

To monitor gestational age differences in the composition of AF and AL, values of total protein (TP), albumin, sodium (Na⁺), chloride (Cl⁻), and magnesium (Mg²⁺) were defined in harvested fetal fluids using the TP Gen.2 (Ref. 05171385190), the ISE indirect Na-K-CL for Gen.2 (Ref. 108), and the Mg Gen.2 (Ref. 05171911190) of Roche/Hitachi Cobas analyzer (Roche Diagnostics, Switzerland).

Statistical Analysis

Quantitative data are presented as means $\pm$ SD (GraphPad Prism, GraphPad Software Inc., USA). Receiver-operating characteristic curves (ROC) were generated for visualization of sensitivity (at a certain specificity) of parameters in the differential fetal fluid analysis (SPSS 21.0; IBM, USA). Differences in AF versus AL and gestational age groups were analyzed using the Wilcoxon signed-rank test, and p values <0.05 or z values $>\pm 1.96$ were considered statistically significant. Correlations were analyzed with either the Pearson (ρ) or Spearman's rank correlation coefficient (R) according to defined significance levels: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, or additive bayesian networks for dependency structure discovery using graphical modeling (R version 3.3.3, Austria) [Kratzer et al., 2016]. Overall, for statistical analyses, values below the detection level were set to zero (in TP and partially Cl⁻).

Results

Volumetric Assessment of Ovine Fetal Fluid Cavities – Correlation to CRL

Volumetric measurements of the fetus by CT (Fig. 1) revealed a significant correlation between the CRL and the fetal volume over gestation ($R = 0.98$, $p < 0.001$) (Fig. 2a). Given that CRL is defined as an indicator of the

gestational age in this study, the volumetric assessment of the fetus further exemplifies the fetal development over time (Fig. 2d). When analyzing the change of AF and AL over either the fetal volume or the CRL, identical results were found. AL volume maintained relatively static with a mean of 157 ± 77 ml, and no correlation to gestational age or fetal volume ($R = 0.45$, $p = 0.14$) and CRL ($R = 0.46$, $p = 0.13$) was found (Fig. 2b). AF represented the major fluid within the fetal cavity and significantly increased in a linear fashion over gestation, with positive correlations found for fetal volume ($\rho = 0.90$, $p < 0.001$) and CRL ($\rho = 0.97$, $p < 0.001$) (Fig. 2b). Therefore, the total fetal fluid is mainly influenced by the increase in AF and also significantly correlates with the fetal volume ($\rho = 0.89$, $p < 0.001$) and the CRL ($\rho = 0.95$, $p < 0.001$). No significant differences were found in the amount of AF and AL at early gestation ($p = 0.81$), whereas there is some evidence for differences at mid and late gestation ($p = 0.06$ and $p = 0.06$) (Fig. 2c). The total fluid per fetal volume significantly decreases over gestation ($R = -0.96$, $p < 0.001$) for AF ($R = -0.90$, $p < 0.001$) and AL ($R = -0.90$, $p < 0.001$) (Fig. 2e). In particular at early gestation (group 1), a much higher fluid volume to fetal size/volume was found, with equal amounts of AF and AL (group 1: $p = 0.81$) (Fig. 2f). At mid and late gestation, the fluid volume to fetal size/volume was found to be relatively static, but there was with weak evidence showing a higher AF than AL amount (group 2: $p = 0.06$, group 3: $p = 0.06$) (Fig. 2f). Correlation analyses (Fig. 2g, h) revealed strong dependence between fetal size, amnion volume, and fetal volume. However, correlation becomes independent when the effect of amnion is removed, further stressing that the major cavity increasing over time is based on AF. The independence of the allantois demonstrates its overall stable volume, which is not in any relation with the course of gestation.

Differential Fetal Fluid Analysis

AF and AL Purity Assessment – Cutoff Values of Electrolytes and Total Protein

Harvested ovine fetal fluids ($n = 40$) were compared to reference values from an existing database [Weber et al., 2016], including TP, albumin, Na⁺, Cl⁻, and Mg²⁺, in order to assess the purity of the fetal fluids and exclude possible collateral contamination. Using this reference pool [Weber et al., 2016], ROC and cutoff values (cutoffs for AF: Na⁺ ≥ 117.5 mmol/L, Cl⁻ ≥ 95 mmol/L, Mg²⁺ ≤ 0.88 mmol/L, TP ≤ 1 g/L) were established (Fig. 3a). At 100% sensitivity levels, the specificity values to detect inaccurate fluid harvest and contamination was 100% for TP, 90% for Na⁺ and Cl⁻, and 86.7% for Mg²⁺. TP ($z = -4.646$)

Fig. 1. Computed tomography (CT) of ovine fetal fluid cavities over gestation. CT pictures of early, mid, and late gestation were obtained to measure fetal fluid volumes of amniotic (AF) and allantoic (AL) fluid. **a** Contrast agent (Ioversol, Opitray 300) was used to visualize both cavities. Three-dimensional reconstructions of the ovine amnion (**b**), allantois (**c**), and merged (**d**) show the in vivo architecture of the fetal fluid cavities during pregnancy. Scale bar, 4 cm.

Fig. 2. Volumetric assessment of ovine amniotic (AF) and allantoic fluid (AL) cavities over gestation. **a** Crown-rump length (CRL) significantly correlates with fetal volume, which is an indicator for gestational age (**d**). **b, c** The total fetal adnexal fluid is mainly influenced by AF over gestation and increases in a linear fashion, whereas AL maintains relatively static independent of gestational age. **e, f** Overall higher fetal adnexal fluid per fetal volume is found

at early gestation. During gestation, higher AF than AL amounts are detected also in relation to fetal volume. **g** Spearman's rank correlation matrix displays all correlation analyses conducted with each coefficient and the according significance level (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$), demonstrating graphically that there is a strong dependence between AF volume and gestation, but not with AL volume and gestation (**h**).

Fig. 3. Differential analysis of ovine amniotic (AF) and allantoic fluid (AL) during gestation. **a** Receiver-operating characteristic curve (ROC) shows sensitivity levels to detect inaccurate fetal fluid harvest and contaminations, consisting of 100% for total protein (TP), 90% for Na and Cl, and 86.7% for Mg. **b** Scatterplots of fetal fluid samples included in the study. The red lines demonstrate cutoff values that have been established by the use of reference val-

ues from an existing database. **c** Photometric or colometric detection of Na⁺, Cl⁻, Mg²⁺, and TP of harvested fetal fluids showed increasing/decreasing values throughout gestation. Spearman's rank correlation matrices for AL (**d**) and AF (**e**) display all correlations detected; graphical modeling results are displayed for AL (**f**) and AF (**g**). CRL, crown-rump length.

and Mg²⁺ ($z = -4.860$) levels were significantly higher in AL than AF, which confirms that they detect AL contamination with high sensitivity. On the other hand, Na⁺ ($z = -4.860$) and Cl⁻ levels ($z = -4.860$) were significantly higher in AF than AL, suggesting that they detect AF contamination with high sensitivity. Consequently, AF samples with Mg²⁺ and TP values over the defined cutoff values and AL samples with Na⁺ and Cl⁻ values over the defined cutoff values were excluded based on contamination according to our reference pool. Hemorrhagic contami-

nation of the fetal fluids potentially causing the differences in the TP amount could be excluded by the lack of albumin detection. If the amount of fetal fluid harvested was insufficient for the differential fluid analyses, the respective fetuses were excluded from further analyses ($n = 2$). Taken together, 9/40 fetuses had to be excluded as their pure AF or AL origin could not be confirmed, whereas fetal fluid samples included into the study are displayed on the corresponding scatter plot ($n = 31$) (Fig. 3b).

Correlation – Electrolytes and TP versus CRL

Measurements of AF and AL biochemical components from gravid uteri postmortem ($n = 31$) showed several significant correlations with the CRL of the fetus (Fig. 3c), representing a surrogate marker for the gestational age. Spearman's rank correlation matrices display correlations for each fluid: AL (Fig. 3d) and AF (Fig. 3e). While Na^+ levels of AF decreased significantly throughout pregnancy ($R = -0.88$, $p < 0.001$), no significant correlation could be detected in AL ($R = -0.09$, $p = 0.64$). Cl^- levels of AF ($R = -0.83$, $p < 0.001$) and AL ($R = -0.69$, $p < 0.001$) decreased significantly throughout pregnancy. On the other hand, Mg^{2+} showed a significant increase in AF ($R = 0.72$, $p < 0.001$) and AL ($R = 0.69$, $p < 0.001$) fluid. TP levels only showed an increase in AL throughout gestation ($R = 0.62$, $p < 0.001$), while the values for AF were mostly below the detection level (< 2 g/L).

Correlation analyses using graphical modeling revealed strong dependence between Na^+ and Cl^- levels in the amnion compartment but not with Mg^{2+} levels when corrected for fetal size (Fig. 3g). In the allantois compartment, all measured electrolytes except for Na^+ depend on size (Fig. 3f). However, Mg^{2+} is independent of Cl^- given TP and independent of size given Cl^- and TP, whereas TP is independent of size given Cl^- .

Discussion

In a recent investigation, our group showed significant differences in the cellular phenotype of cells isolated from the ovine AF versus AL compartment, including proliferation capacities and the expression of multi- and pluripotency-related genes [Kehl et al., 2017]. Also, proteomic analyses of both fluids revealed a significant difference in the composition of both fetal fluids [Riding et al., 2008]. Therefore, when dealing with animal models recapitulating the human physiology for preclinical trials, anatomical differences have to be appreciated. This implies that a clear distinction between ovine AF and AL is indispensable for correct interpretation of data obtained using the ovine developmental model. This aspect is of particular importance considering the recent controversial discussions on the interpretation of findings in the field of stem cell research, which might serve as a basis for clinical treatments in the near future [Estes et al., 2019].

AF is a highly dynamic compartment that changes in volume and composition during pregnancy. It not only provides mechanical cushioning, but also contains nutrients and growth factors for fetal growth and develop-

ment. AF volume results from a balance between fetal fluid production (urination, expiration) and reabsorption (swallowing, intramembranous absorption) in a defined pattern through pregnancy [Underwood et al., 2005]. Increasing electrolyte permeability during gestation allows for the exponential increase in fetal weight. In humans, AF volume increases linear to fetal size until mid gestation (~ 25 weeks), where AF volume is no longer increasing in a linear fashion given complete keratinization of the fetal skin. A plateau is reached near term gestation (~ 28 weeks), and thereafter its volume declines (~ 42 weeks) [Brace and Wolf, 1989; Underwood et al., 2005]. This AF plateau and decline has not been found in our ovine model studied, consistent with previous reports [Bazer et al., 2012]. Volume changes within the ovine AF during the last half of gestation have been described as quite variable with stable, increased, and/or decreased quantities [Lingwood and Wintour, 1984]. Here, AF showed a significant increase over the entire gestation in a linear fashion, whereas AL maintained relatively static with smaller volumes compared to AF, as previously reported [Bazer et al., 2012]. Interestingly, fluid dynamics described by Bazer et al. [2012] did show the same AL behavior with a small decrease around gestational weeks 7–10, followed by an increase afterwards. These consistent findings emphasize fluid control mechanisms not only in AF, as determined by continuous measurements of swallowing, and lung liquid and urine production [Faber et al., 2005; Yang et al., 2005; Robertson et al., 2009] but also in the allantois. Its function consists of collecting liquid waste from the embryo and gas exchange. There are also reports suggesting the allantois as a reservoir for nutrients, given its fusion with the chorion to form the chorioallantoic placenta during placentation [Bazer et al., 1989]. This independence of the AL compartment has also been shown by the graphical modeling displayed in the presented data, where a clear anatomical independence of this compartment was shown.

In the presented study, 3 electrolytes and TP were selected based on their clear discriminative potential between AF and AL. They represent hallmark biomarkers for the accurate isolation of fetal fluids in the ovine developmental model [Benson et al., 1995; Tangalakakis et al., 1995; Jobe et al., 2000; Weber et al., 2016]. Hence, collateral contaminations can be easily excluded. This includes the evaluation of fetal fluid function and composition during fetal interventions on the one hand, and well-defined isolation of fetal cells for cell-based tissue regeneration on the other. In this regard, the presented work shows that not only the mean values of each fluid show a

sensitive and specific discrimination, but also that changes over the entire gestation still enable accurate discrimination of AF and AL.

Average Cl^- and Na^+ levels were significantly higher in AF and showed a significant decrease over pregnancy in AF for both electrolytes but in AL only for Cl^- . This is consistent with independent studies in mares [Zanella et al., 2014], sheep [Ross et al., 1988; Prestes et al., 2001], and feline models [Fresno et al., 2012]. The decrease in Na^+ and Cl^- in AL is suggested to be based on the increasing reabsorption ability of the fetal kidneys by means of the Na^+ - K^+ -ATPase activity [Fresno et al., 2012] and the increased renal responsiveness to plasma aldosterone [Lingwood et al., 1978]. In humans, lower Na^+ concentrations are found by the end of pregnancy given an almost completely developed fetal kidney with Na^+ reabsorption of about 85–95% of the filtration load [Oliveira et al., 2002]. It has been shown that the origin of AF in humans varies through pregnancy. During early gestation, AF is similar to fetal plasma, and the production is due to diffusion of water and solutes between the fetus and the not-yet-keratinized fetal skin and the surfaces of the amnion, placenta, and umbilical cord [Parolini et al., 2009; Estes, 2019]. At late gestation, the majority of the fluid is produced by the fetus itself by secretion from the respiratory tract or by production of urine [Oliveira et al., 2002; Estes, 2019]. Thus, at late gestation, only 28% of ovine AL osmolality is based on electrolyte contribution, given major developmental changes in metabolic activity [Wales and Murdoch, 1973]. Interestingly, overall Na^+ , K^+ , and Cl^- concentrations have shown to account for a significantly greater proportion of AF osmolality in humans (97%) compared to ovine AF alone (86%) or hypothetically combined with ovine AL [Albuquerque et al., 1999]. These results demonstrated increased concentrations of nonelectrolyte solutes in the ovine model such as sugars and proteins compared to the human situation [Albuquerque et al., 1999]. High TP values were found in AL, with increasing values over time, whereas TP levels below the detection limit were measured for AF. In pregnant sheep, TP values only <1 g/L have been detected in the AF [Benson and Wintour, 1995; Tangalakakis et al., 1995; Prestes et al., 2001; Zanella et al., 2014], which would be below the detection threshold of the method used in the presented study. In humans, AF proteins (0.2–7 g/L) are mainly of maternal origin, and their principal clearance mechanism is based on fetal swallowing [Tisi et al., 2004]. Low levels of TP in AF is necessary to maintain the osmotic pressure between AF and maternal blood [Tong et al., 2009]. Among these proteins, proteomic analysis revealed peptides involved in cellular movement, organ develop-

ment, cell-to-cell signaling, cellular growth, and proliferation [Cho et al., 2007]. In humans, these proteins get into the AF by different routes, such as the maternal uterine tissue, umbilical cord, AF cells, fetal urine, meconium, and transudation through fetal skin [Tisi et al., 2004]. Accordingly, AF is not the result of simple filtration from the maternal blood, but independently providing a pathway for the transport of regulatory proteins for fetal development [Tong et al., 2009]. However, one has to emphasize that all the measured values themselves might differ from physiological values due to postmortem harvest. As an example, the meconium, which is usually retained in the fetus until after birth, can be expelled in certain cases into the fetal cavities. However, Na^+ , K^+ , and TP values detected in living sheep [Albuquerque et al., 1999; Bazer et al., 2012] covered the same range as described in the presented study, suggesting minimal compositional changes in the fetal fluids postmortem. Furthermore, Mg^{2+} levels in human and ovine AF were consistent, which has been hypothesized as an indicator for pregnancy complications such as preeclampsia and diabetes [Bocos Terraz et al., 2011]. Overall, changes in the composition along gestation represent changes in metabolic and transport activity as well as different contributions of fetal and extraembryonic tissues.

In conclusion, these results provide information on ovine fetal fluid dynamics and benchmark values for pre-clinical studies examining volumetric and compositional changes in AF as well as the isolated (stem) cells thereof. These results serve as a basis for future studies by providing surrogate markers enabling a reliable distinction between isolated fetal fluids and contained cells in the ovine developmental model. However, this study also highlights the necessity of a better characterization of the physiology of currently used animal models for more accurate extrapolation of gained knowledge to the human situation, which also seems indispensable with regard to ethical principles in animal research.

Acknowledgements

The authors would like to thank Ursula Steckholzer, Christine Lohmann, and Matthias Dennler for their technical assistance. Differential fluid analyses were performed in collaboration with the Institute of Clinical Chemistry, University Hospital Zurich, Switzerland. This work was supported by the Swiss National Science Foundation (project 310030_143992), the Alfred and Anneliese Sutter-Stöttner-Foundation (doctoral dissertation Debora Kehl, Münschwilen, Switzerland), Forschungskredit Postdoc of the University of Zurich, Switzerland, Fonds Medical Research, and the Foundation for Research in Science and the Humanities at the University of Zurich, Switzerland.

Statements of Ethics

As tissues were harvested postmortem (from the local slaughterhouse), no ethical permission was required for this study.

Disclosure Statement

The authors declare no competing or conflicting financial interests.

References

Albuquerque CA, Nijland MJ, Ross MG. Human and ovine amniotic fluid composition differences: implications for fluid dynamics. *J Matern Fetal Med*. 1999 May-Jun;8(3):123–9.

Anitha A, Thangavel A. Biochemical profile of ovine amniotic and allantoic fluids. *Talminadu J Vet Anim Sci*. 2011;7:262–7.

Barry JS, Anthony RV. The pregnant sheep as a model for human pregnancy. *Theriogenology*. 2008 Jan;69(1):55–67.

Bazer FW (Brace A, editor). *Allantoic fluid: Regulation of volume and composition*. Fetal and Neonatal Body Fluids R. Cornell (NY): Perinatology Press; 1989. pp. 135–57.

Bazer FW, Spencer TE, Thatcher WW. Growth and development of the ovine conceptus. *J Anim Sci*. 2012 Jan;90(1):159–70.

Benson CA, Wintour EM. The effect of bilateral fetal adrenalectomy on fluid balance in the ovine fetus. *J Physiol*. 1995 Nov;489(Pt 1):235–41.

Bocos Terraz JP, Izquierdo Álvarez S, Bancalero Flores JL, González López A, Escanero Marcén JF. Magnesium concentration in amniotic fluid in the early weeks of the second trimester of pregnancy. *BMC Res Notes*. 2011 Jun;4(1):185.

Brace RA, Wolf EJ. Normal amniotic fluid volume changes throughout pregnancy. *Am J Obstet Gynecol*. 1989 Aug;161(2):382–8.

Cho CK, Shan SJ, Winsor EJ, Diamandis EP. Proteomics analysis of human amniotic fluid. *Mol Cell Proteomics*. 2007 Aug;6(8):1406–15.

Cremonesi F, Corradetti B, Lange Consiglio A. Fetal adnexa derived stem cells from domestic animal: progress and perspectives. *Theriogenology*. 2011 May;75(8):1400–15.

Emmert MY, Weber B, Wolint P, Frauenfelder T, Zeisberger SM, Behr L, et al. Intramyocardial transplantation and tracking of human mesenchymal stem cells in a novel intra-uterine pre-immune fetal sheep myocardial infarction model: a proof of concept study. *PLoS One*. 2013;8(3):e57759.

Estes M. Expression of Concern. *Circulation*. 2019 Jan;139(3):e5–6.

Faber J, Anderson D, Hohimer R, Yang Q, Giraud G, Davis L. Function curve of the membranes that regulate amniotic fluid volume in sheep. *Am J Physiol Heart Circ Physiol*. 2005 Jul;289(1):H146–50.

Author Contributions

D.K., B.W., and U.B. conceived the project and designed the experiments. U.B. harvested ovine gravid uteri postmortem and performed CT imaging. D.K. analyzed obtained images and performed volumetric assessment analyses. D.K. and S.G. performed and analyzed differential fluid analysis. C.W. and D.K. conducted statistical analysis of all data. Funding for the project was obtained by D.K., B.W., U.B., S.G., and S.P.H. D.K. wrote the manuscript with the contribution of B.W., U.B., and S.G.

Fresno L, Rodriguez-Gil JE, Rigau T, Pastor J, Rivera del Alamo MM. Modulation of the biochemical composition of amniotic and allantoic fluids as a control mechanism of feline foetal development. *Placenta*. 2012 Jun;33(6):522–7.

Fuchs JR, Kaviani A, Oh JT, LaVan D, Udagawa T, Jennings RW, et al. Diaphragmatic reconstruction with autologous tendon engineered from mesenchymal amniocytes. *J Pediatr Surg*. 2004;39:834–8; discussion 834–8.

Gray FL, Turner CG, Ahmed A, Calvert CE, Zurakowski D, Fauza DO. Prenatal tracheal reconstruction with a hybrid amniotic mesenchymal stem cells-engineered construct derived from decellularized airway. *J Pediatr Surg*. 2012 Jun;47(6):1072–9.

Jobe AH, Newnham JP, Willet KE, Moss TJ, Gore Ervin M, Padbury JF, et al. Endotoxin-induced lung maturation in preterm lambs is not mediated by cortisol. *Am J Respir Crit Care Med*. 2000 Nov;162(5):1656–61.

Kehl D, Generali M, Görtz S, Geering D, Slamecka J, Hoerstrup SP, et al. Amniotic Fluid Cells Show Higher Pluripotency-Related Gene Expression Than Allantoic Fluid Cells. *Stem Cells Dev*. 2017 Oct;26(19):1424–37.

Klein JD, Turner CG, Steigman SA, Ahmed A, Zurakowski D, Eriksson E, et al. Amniotic mesenchymal stem cells enhance normal fetal wound healing. *Stem Cells Dev*. 2011 Jun;20(6):969–76.

Kratzer G, Pittavino M, Lewis F, Furrer R. abn: an R package for modelling multivariate data using additive Bayesian networks. Technical Report. 2016.

Kunisaki SM, Freedman DA, Fauza DO. Fetal tracheal reconstruction with cartilaginous grafts engineered from mesenchymal amniocytes. *J Pediatr Surg*. 2006a Apr;41(4):675–82; discussion 675–82.

Kunisaki SM, Fuchs JR, Kaviani A, Oh JT, LaVan DA, Vacanti JP, et al. Diaphragmatic repair through fetal tissue engineering: a comparison between mesenchymal amniocyte- and myoblast-based constructs. *J Pediatr Surg*. 2006b Jan;41(1):34–9; discussion 34–9.

Lingwood B, Hardy KJ, Coghlan JP, Wintour EM. Effect of aldosterone on urine composition in the chronically cannulated ovine foetus. *J Endocrinol*. 1978 Mar;76(3):553–4.

Lingwood BE, Wintour EM. Amniotic fluid volume and in vivo permeability of ovine fetal membranes. *Obstet Gynecol*. 1984 Sep;64(3):368–72.

McCarty RC, Gronthos S, Zannettino AC, Foster BK, Xian CJ. Characterisation and developmental potential of ovine bone marrow derived mesenchymal stem cells. *J Cell Physiol*. 2009 May;219(2):324–33.

Oliveira FR, Barros EG, Magalhães JA. Biochemical profile of amniotic fluid for the assessment of fetal and renal development. *Braz J Med Biol Res*. 2002 Feb;35(2):215–22.

Pansky B. *Review of medical embryology*. New York: Macmillan; 1982.

Parolini O, Soncini M, Evangelista M, Schmidt D. Amniotic membrane and amniotic fluid-derived cells: potential tools for regenerative medicine? *Regen Med*. 2009 Mar;4(2):275–91.

Prestes NC, Chalhoub MC, Lopes MD, Takahira RK. Amniocentesis and biochemical evaluation of amniotic fluid in ewes at 70, 100 and 145 days of pregnancy. *Small Rumin Res*. 2001 Mar;39(3):277–81.

Riding GA, Jones A, Holland MK, Hill JR, Lehnert SA. Proteomic analysis of bovine conceptus fluids during early pregnancy. *Proteomics*. 2008 Jan;8(1):160–77.

Robertson P, Faber JJ, Brace RA, Louey S, Hohimer AR, Davis LE, et al. Responses of amniotic fluid volume and its four major flows to lung liquid diversion and amniotic infusion in the ovine fetus. *Reprod Sci*. 2009 Jan;16(1):88–93.

Ross MG, Ervin MG, Rappaport VJ, Youssef A, Leake RD, Fisher DA. Ovine fetal urine contribution to amniotic and allantoic compartments. *Biol Neonate*. 1988;53(2):98–104.

Sivachelvan M, Ali MG, Chibuzo G. Foetal age estimation in sheep and goats. *Small Rumin Res*. 1996;19(1):69–76.

Tangalakakis K, Moritz K, Shandley L, Wintour EM. Effect of maternal glucocorticoid treatment on ovine fetal fluids at 0.6 gestation. *Reprod Fertil Dev*. 1995;7(6):1595–8.

Tisi DK, Emard JJ, Koski KG. Total protein concentration in human amniotic fluid is negatively associated with infant birth weight. *J Nutr*. 2004 Jul;134(7):1754–8.

- Tong XL, Wang L, Gao TB, Qin YG, Qi YQ, Xu YP. Potential function of amniotic fluid in fetal development—novel insights by comparing the composition of human amniotic fluid with umbilical cord and maternal serum at mid and late gestation. *J Chin Med Assoc*. 2009 Jul;72(7):368–73.
- Turner CG, Klein JD, Steigman SA, Armant M, Nicksa GA, Zurakowski D, et al. Preclinical regulatory validation of an engineered diaphragmatic tendon made with amniotic mesenchymal stem cells. *J Pediatr Surg*. 2011 Jan; 46(1):57–61.
- Underwood MA, Gilbert WM, Sherman MP. Amniotic fluid: not just fetal urine anymore. *J Perinatol*. 2005 May;25(5):341–8.
- Wales RG, Murdoch RN. Changes in the composition of sheep fetal fluids during early pregnancy. *J Reprod Fertil*. 1973 May;33(2):197–205.
- Weber B, Emmert MY, Behr L, Schoenauer R, Brokopp C, Drögemüller C, et al. Prenatally engineered autologous amniotic fluid stem cell-based heart valves in the fetal circulation. *Biomaterials*. 2012 Jun;33(16):4031–43.
- Weber B, Kehl D, Bleul U, Behr L, Sammut S, Frese L, et al. In vitro fabrication of autologous living tissue-engineered vascular grafts based on prenatally harvested ovine amniotic fluid-derived stem cells. *J Tissue Eng Regen Med*. 2016 Jan;10(1):52–70.
- Wintour EM, Laurence BM, Lingwood BE. Anatomy, physiology and pathology of the amniotic and allantoic compartments in the sheep and cow. *Aust Vet J*. 1986 Jul;63(7):216–21.
- Yang Q, Davis L, Hohimer A, Faber J, Anderson D. Regulatory response to washout of amniotic fluid in sheep. *Am J Physiol Heart Circ Physiol*. 2005 Mar;288(3):H1339–43.
- Zanella LF, Takahira RK, Oña CM, Magalhães LC, Prestes NC. Biochemical profile of amniotic and allantoic fluid during different gestational phases in mares. *J Equine Vet Sci*. 2014; 34(3):403–6.