# Canine amniotic fluid at birth: from a discarded sample to a potential diagnostic of neonatal maturity

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## Abstract

The definition of new reliable markers for neonatal maturity evaluation is crucial in canine clinical practice. Concerns about the safety of amniotic sampling in pregnant dogs have prevented its collection for diagnostic purposes. Moreover, amniotic fluid had been considered waste material until the latest studies reported amniocentesis as a reliable and safe procedure, even in the canine species. In our study, amniotic fluid (n = 63) collected at birth from ten dogs undergoing elective Caesarean sections at term was analysed to discover new potential indices of canine neonatal maturity. Based on gestational age, mothers and puppies were divided into two groups: the early group (≤65 days from luteinizing hormone (LH) surge, n=5) and the late group (>65 days from LH surge, n=5). Amniotic parameters of the lightest and heaviest puppy in individual/each litter, with a birth weight difference of at least 20% among littermates, were also compared. In particular, the content of lecithin, sphingomyelin, surfactant protein A (SP-A), cortisol, and pentraxin 3 (PTX3) in amniotic fluid, which is considered predictive of foetal development in humans, were investigated. Maternal serum SP-A and cortisol were also measured simultaneously.

All amniotic parameters were detectable in canine amniotic fluid. Interestingly, the concentrations of different amniotic parameters correlated with each other. Lecithin was positively correlated with

sphingomyelin ($p<0.0001$), maternal SP-A ($p<0.0005$), and the ratio of amniotic and maternal cortisol ($p<0.004$). Amniotic SP-A was inversely correlated to maternal SP-A ($p<0.05$), lecithin ($p<0.005$), and lecithin-sphingomyelin ratio ($p<0.05$). A positive correlation was also recorded between amniotic and maternal cortisol ($p<0.008$). Considering that all puppies were born alive and mature, these data could provide a potential range of expected amniotic values in full-term new-born dogs. Furthermore, since gestational age was positively correlated with both maternal and amniotic cortisol ($p<0.0001$) and amniotic PTX3 ($p<0.05$), amniotic fluid seems to be an attractive, innovative, and minimally invasive matrix with potential diagnostic and prognostic utility for the investigation of canine maturity.

Key words: amniotic fluid, foetal maturity, dog

## 1. Introduction

The elective caesarean section is becoming increasingly popular in both humans and dogs, even when not strictly necessary (Salas Garcia et al. 2018; Loeb and Evans, 2020). Prenatal recognition of foetal maturity is pivotal in planning the parturition date and ensuring the birth of viable and healthy neonates. A combination of clinical, hormonal, radiographic, and ultrasonographic parameters is currently used to predict foetal readiness in dogs (Siena et al., 2021). However, some limits of inaccuracy may affect the estimation, especially when the day of ovulation is unknown (Siena et al., 2021). We speculated that amniotic fluid could provide crucial information on neonatal maturity in dogs as in humans where scientific evidence already exists (Leung-Pineda and Gronowski, 2010). The lecithin/sphingomyelin ratio is commonly used to preventively diagnose neonatal respiratory distress syndrome in babies with a ratio of 2:1 or greater representative of mature foetal lungs (Varner et al., 2013; Ogbejesi and Tadi, 2021). Amniotic surfactant protein A (SP-A) is positively correlated with the lecithin/sphingomyelin ratio in human amniotic fluid and with perinatal outcomes (Pryhuber et al., 1991). SP-A concentrations are reported to increase with advancing gestation, reaching a peak

at term, then decreasing during spontaneous labour (Pryhuber et al., 1991; Wali et al., 1992; Chaiworapongsa et al., 2008). The lecithin/sphingomyelin ratio has also been positively correlated with amniotic cortisol in humans (Diver et al., 1982).

Although exogenous glucocorticoids are known to stimulate foetal lung development in several species, including dog (Vannucchi et al., Bolt et al., 2001), their role in foetal maturity is still debated (Garbrecht et al., 2006). Moreover, PTX3 is a physiologic constituent of the human amniotic fluid and is assumed to be related to gestational age and the onset of labour (Rovere-Querini et al., 2006; Cruciani et al., 2010; Martin 2014).

In Veterinary Medicine, the literature on amniotic fluid is lacking and mainly refers to equine and bovine (Zaremba et al., 1997; Castagnetti et al., 2007) or ovine species as a model for humans (Mimmi et al., 2015), while in dogs the potential diagnostic role of amniotic fluid is far from known (Groppetti et al., 2015; Tal et al., 2019; Plavec et al., 2022). Basic research on the composition of amniotic fluid at birth is mandatory to deepen the knowledge of the factors involved in foetal maturity that allow the identification of immature puppies, thereby improving neonatal care and favouring their chances of survival. The first aim of this study was to evaluate the presence and concentration of specific components of the amniotic fluid collected at birth. In particular, lecithin, sphingomyelin, SP-A, cortisol, and PTX3 have been investigated. An analytic method for quantifying amniotic lecithin and sphingomyelin that has never been described in dogs was validated. SP-A and cortisol were also measured in maternal serum at birth. Furthermore, a possible correlation between these amniotic parameters and some clinical aspects such as gestational age, new-borns respiratory rate as an expression of lung function, litter size, and birth weight were explored.

## 2. Materials and methods

This study complied with Italian animal experimentation and ethics laws and was also approved by the Ethical Committee of the Università degli Studi di Milano (OPBA_77_2017). Amniotic sampling was performed at birth at Caesarean-section on residual fluid waste in the canine species.

### *2.1. Animals and clinical records*

Ten purebred pregnant bitches undergoing elective Caesarean-section and their 63 puppies were enrolled in this study. Breed, age, body weight at proestrus, litter size, and gestational age calculated from the deduced LH surge and the single mating day have been herein summarized (Table 1). Reproductive cycle from proestrus to parturition was monitored as previously described (Groppetti et al., 2015) to deduce luteinizing hormone (LH) surge and accurately date gestational age. Scientific literature indicates 65±2 days from LH surge as the best time to perform elective C-section in dogs (Kim et al., 2007; Smith, 2007; Michel et al., 2011). However, the variability of pregnancy length due to maternal, foetal, and environmental factors make precise dating uncertain. Thus, accurate pre-partum monitoring is required to properly schedule surgery. In the present study, Caesarean-section was planned when parturition was deemed oncoming on the basis of maternal rectal temperature, signs of anorexia, nesting, lactation, and blood progesterone (Enzyme Linked Fluorescent Assay, MiniVidas, BioMérieux, France) (Brugger et al., 2011; Groppetti et al., 2015c), and puppies were estimated fully developed by means of X-ray and ultrasound evaluations made with a 4-8 Mz microconvex and an 8–12 MHz linear electronic multi-frequency probe (Esaote, MyLab™ Five VET, Italy) (Kim et al., 2007; Smith, 2007; Gil et al., 2015; Roos et al., 2018). Surgeries occurred 63 to 66 days after the estimated LH surge that is, 65.1 ± 1.1 days from LH surge and 60.7 ± 0.8 days from the single mating. Based on gestational age, mothers and puppies were arbitrarily divided into two groups: early group (≤65 days from LH surge, five dams and their 34 puppies) and late group (>65 days from LH surge, five dams and their 29 puppies) as shown in Table 1.

Immediately after birth, residual fluid from nasal and oral cavity of each puppy was removed with a bulb syringe. Pulmonary auscultation was performed within 5 minutes after delivery and respiratory rate (RR) recorded for all puppies. Each puppy was weighted at birth. In litters with a difference of at least 20% birth weight (bW) among littermates, amniotic parameters of the lightest and heaviest

puppy were compared. Since there is no data on the value of the variation within the litter of bW to be considered physiological, an arbitrary threshold of 20% was established as representative of the litter heterogeneity.

### *2.2. Sample collection*

Amniotic fluid (AF) was collected during Caesarean-section at the moment of the puppy extraction. The fluid was aspirated with a 20 mL sterile syringe that delicately pierced the wall of the amniotic sac. The puppy was held upright with the head up and the needle inserted in the most declivous portion to avoid injuring the puppy with the syringe needle.

Each sample was immediately centrifuged at 500 x g for 15 min and then the supernatant was stored at -80°C until analysis (lecithin and sphingomyelin, SP-A, cortisol), while the pellet was lysed with 1 mL of TRIreagent (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20°C until RNA extraction (PTX3).

Concurrently, at the time of anaesthesia induction, a maternal blood sample (1.5 mL) was collected from the cephalic vein into serum tubes, immediately centrifuged at 1500 x g for 10 min at RT, and serum stored at -20°C until SP-A and cortisol measurement.

### *2.3. Amniotic fluid composition analysis*

#### *2.3.1. Lecithin and sphingomyelin extraction and HPLC-MS analysis*

Lecithin and sphingomyelin extraction was performed from aliquots (0.2 mL) of amniotic fluid samples mixed with 0.4 mL chloroform followed by 0.2 mL of methanol. Samples were vortexed for 1 min and centrifuged at 5000 rpm on $4^0$C (Centrifuge SORVALL™ ST 8 SERIES, Thermo Fisher, San Jose, CA, USA). The bottom chloroform layer was collected and dried under a stream of $N_2$. In order to perform HPLC-Q-Extactive-Orbitrap® *High resolution mass spectrometry* analysis, samples were reconstituted in 1 mL of acetonitrile. An aliquot of each sample (0.2 mL) was transferred to an autosampler vial and injected. Chromatography was accomplished on an HPLC Surveyor MS

quaternary pump, a Surveyor AS autosampler with a column oven and a Rheodyne valve with a 20 µL loop system (Thermo Fisher Scientific). Analytical separation was carried out by hydrophilic interaction liquid chromatography (HILIC) using a column 75 × 2.6 mm, 100 Å, Kinetex, with a 4 × 2 mm i.d. HILIC guard column (Phenomenex, Torrance, CA, USA). The mobile phase was run as a gradient that consisted of acetonitrile (A) and water solution of ammonium formate buffer, 20 mM, pH 3.2 (B). The flow rate was 0.25 mL/min. The gradient was initiated with 95% eluent A with a linear decrease up to 50% in 10 min. This condition was maintained for 2 min. The mobile phase was returned to initial conditions at 12 min, followed by 8 min re-equilibration period (total run time: 20 min). The column and sample temperatures were 30°C and 5°C, respectively.

The mass spectrometer Thermo Q-Exactive Plus (Thermo Scientific) was equipped with a heated electrospray ionisation (HESI) source. Capillary temperature and vaporiser temperature were both set at 320 and 280°C, respectively, while the electrospray voltage operating in positive was adjusted at 3.30 kV. Sheath and auxiliary gas were 35 and 15 arbitrary units, with S lens RF level of 60. The mass spectrometer was controlled by Xcalibur 3.0 software (Thermo Fisher Scientific). The exact mass of the compounds was calculated using Qualbrowser in Xcalibur 3.0 software. The full scan (FS) and data-independent acquisition (DIA) in positive mode were used for both screening and quantification purposes. Resolving power of FS adjusted on 70,000 FWHM at *m/z* 200, with scan range of *m/z* 500-850, while fragmentation in DIA was performed with resolving power set at 17500. For FS, automatic gain control (AGC) was set at $3e^6$, with an injection time of 200 ms, while for DIA the AGC was fixed to $2e^5$, with the maximum injection time of 100 ms. Fragmentation of precursors was optimised with normalised collision energy (NCE) of 15eV.

Lecithin and sphingomyelin detection was based on calculated exact mass of the protonated molecular ions along with their isotopic patterns and phosphocholine fragment (*m/z* = 184.0735), and on retention time (RT) of corresponding chemical standards. Extracted ion chromatograms (EICs) were obtained following the *m/z* signals for the molecular ions of two phospholipids enrolled herein: $[M+H]^+$ of lecithin 760.58508 with RT at 9.89min and $[M+H]^+$ 731.60615 for sphingomyelin

(RT=10.07min). Lecithin and sphingomyelin chromatograms are shown in Figure 1. Quantification of both compounds was performed using external calibration curve (range 0.5-50 μg/mL) with standards of lecithin and sphingomyelin provided by Avanti Polar Lipids, Inc (Alabama, USA). Limits of detection (LODs) and quantification (LOQs) were determined at lower concentrations 0.1, 0.5 and 1 μg/mL, were LOD was defined as the minimum concentration at which the molecular ion had been identified (mass error<2 ppm) in FS mode, while LOQ was set as the minimum concentration where both the molecular ion and phosphocholine fragment from DIA spectrum had been quantified. Therefore, LOD and LOQ for both compounds was set at 0.1 μg/mL and 1 μg/mL, respectively.

*2.3.2. Surfactant protein A*

Surfactant protein A (SP-A) in maternal serum and amniotic fluid were titrated using a commercial sandwich ELISA assay kit (LifeSpan BioSciences, Seattle, WA, USA) according to the manufacturer's instructions. Briefly, 100 μL of each sample was added in duplicates, and incubated for 1 hour at 37°C. Then, the supernatant was aspirated and 100 μL of detection reagent A (biotin-conjugated detection antibody) was added to each well and incubated for 1 hour at 37°C. After reagents removal, the plate was washed 3 times, and 100 μL of detection reagent B (Avidin-Horseradish Peroxidase conjugate) was added and the plate incubated for 30 minutes at 37°C. A final wash, repeated 5 times, was done and 90 μL of TMB substrate solution was added to each well, and incubated for 15 minutes at 37°C. Finally, 50 μL of stop solution was added and the plate absorbance was read at 450 nm. The minimal detectable concentration of SP-A was 6.25 pg/ml, and we obtained an interassay of 6.52% and an intra-assay variation of 6.58%.

*2.3.3. Cortisol*

Cortisol concentration in maternal serum and AF was determined using a quantitative test based on ELFA technique (Enzyme Linked Fluorescent Assay, MiniVidas, bioMérieux) as previously reported

(Groppetti et al., 2015b). According to the manufacturer the minimal detectable concentration of this kit is 5.51 nmol/L, the inter-assay CVs were 3.1%, and the intra-assay CVs were 3.7%. The ratio of amniotic and maternal cortisol (AF/M CORT) was calculated as mean of the amniotic cortisol value of the litter divided by maternal serum cortisol concentration.

*2.4. RNA extraction, retrotranscription and Real Time PCR for PTX3 detection*

Total RNA was extracted using the TRI Reagent (Thermo Fisher Scientific, Waltham, MA USA) following the manufacturer's instructions. The concentration and quality of RNA was determined using a spectrophotometer (BioPhotometer, Eppen-dorf, Hamburg, Germany) at 260/280 nm wavelength. Total RNA (2 μg) was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA, USA), according to the manufacturer's instructions. Dog PTX3 primers were designed based on dog PTX3 sequence XM_003433174 using Primer Express Software (Applied Biosystems) and selected to produce amplicons spanning 2 exons. All the samples were also tested with β-Actin primers as housekeeping gene (Spichiger et al., 2005). Primers were purchased from Sigma-Aldrich S.r.l, Milano and their sequences are listed in Table 2. Specificity of primers was evaluated by sequencing the amplicon generated by the primers using a cDNA from placental tissue sample (Eurofins Genomics, Ebersberg, Germany). The cDNA obtained from each sample was used as a template for Real-Time PCR in an optimized 25 μl reaction volume using Sybr Green chemicals, as previously described (Riva et al., 2010). Real-Time quantitative PCR was carried out in the 7000 Sequence Detection System (Applied Biosystems), at the following thermal cycle conditions, 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C, as previously described (Riva et al., 2010). Each sample was amplified by Real-Time PCR in duplicate. The expression of canine target genes was normalized using the calculated beta actin cDNA expression (mean) of the same sample and run. A duplicate no-template control (NTC) was also included in each plate. The relative quantification of each gene was calculated with the "delta Ct"

method (Schmittgen & Livak, 2008). The values obtained were multiplied by "10,000" to transform them into Arbitrary Unit (AU).

### *2.5. Statistical analysis*

Statistical analyses were performed using GraphPad Prism 6 (La Jolla, CA, USA) considering statistically significant values at $p<0.05$. Descriptive statistics were expressed as mean ± standard error. Shapiro-Wilk test was used to verify the distribution of data. Mann-Whitney test was used to compare PTX3 expression in amniotic fluid, amniotic cortisol and respiratory rate in early and late puppies. Two-tails rho tests of Spearman $\rho$ were used to reveal correlations (bivariate linear correlations) among gestational age and RR versus each of the investigated amniotic parameters, as well as correlations between maternal serum SP-A and cortisol versus each of the amniotic puppy parameters.

Furthermore, when a birth weight difference of at least 20% was recorded among the littermates, the amniotic parameters of the lightest and heaviest puppy in the litter were compared using a Mann-Whitney test.

## 3. Results

### *3.1. Animals*

All 63 puppies, 34 males and 29 females, were born alive and mature in the appearance that is, normal in conformation, hair and nails development and attitude. Litter size ranged from 2 to 11 puppies (6.3 ± 3.2). Respiratory rate ranged 23.2 ± 9.9 bpm. Birth weight of puppies was 236 to 770 gr (438.5 ± 140.5). Seven out of ten litters showed a variation in bW 20% or more allowing a comparison between the lightest and heaviest littermates. Five out of 63 amniotic samples were missed for technical problems, therefore amniotic analysis was performed on a total of 58 AFs.

### *3.2. Dog amniotic liquid composition*

All the molecules investigated (lecithin, sphingomyelin, SP-A, cortisol and PTX3) were detected in all the amniotic samples. In particular, the concentration of lecithin ranged from 1.5 to 49.2 μg/mL (19.4 ± 12.6; median 17.4 μg/mL), those of sphingomyelin from 0.7 to 32.1 μg/mL (8.7 ± 6.4; median 7.2 μg/mL), SP-A from 14.3 to 31.5 pg/mL (21.5 ± 2.8; median 21.3 pg/mL), cortisol from 2.0 to 9.8 ng/mL (4.8 ± 1.7; median 4.3 ng/mL), and PTX3 from 32.6 to 1654211.6 AU (14797.8 ± 28426.9; median 1988.84 AU). Lecithin and sphingomyelin ratio (L/S) ranged from 0.5 to 13.4 (3.0 ± 2.4; median 2.5).

### *3.3. Maternal serum SP-A and cortisol*

Maternal serum concentration of SP-A ranged from 11.5 to 8.9 pg/mL (11.0 ± 5.0; median 11.2 pg/mL). Maternal serum concentration of cortisol was 77.9 to 20.8 ng/mL (39.4 ± 14.6; median 39.1 ng/mL). The ratio of amniotic and maternal cortisol (AF/M CORT) ranged from 0.2 to 0.04 (0.12 ± 0.12; median 0.07). Similarly, to what is described in humans (Varma et al. 1979), the individual results of AF/M CORT ratio have been divided into two subgroups by an arbitrary line at 0.16 and related to L/S ratio. Interestingly all puppies with AF/M CORT higher than 0.16 showed an L/S ratio higher than 2.8, except for one (13 out of 14 puppies), while the majority (31 out of 44) of puppies with AF/M CORT lower than 0.16 showed an L/S ratio lower than 2.8 (Figure 2, Appendix 1 and 2).

### *3.4. Correlations among amniotic and maternal parameters*

Lecithin and sphingomyelin were positively correlated to each other ($p<0.0001$; $r=0.54$), to maternal SP-A ($p<0.0005$; $r=0.44$ and $r=0.46$, respectively) and to the ratio of amniotic and maternal cortisol ($p<0.004$; $r=0.53$ and $r=0.37$, respectively). Lecithin was negatively correlated to amniotic SP-A ($p<0.005$; $r=-0.38$) and L/S was negatively correlated to both amniotic SP-A and maternal cortisol ($p<0.05$; $r=-0.26$ and $r=-0.28$, respectively). Amniotic SP-A was negatively correlated to maternal SP-A ($p<0.05$; $r=-0.32$), while amniotic cortisol was positively correlated to maternal cortisol

(p<0.008; r=0.34). Significant correlations recorded in this study are shown in Table 3. Maternal SP-A decreased with the increase of bitches' age (p<0.0001; r=-0.59). Litter size did not impact AF composition. No difference was found in the composition of the amniotic fluid between the heaviest and the lightest puppies of the same litter.

### *3.5. Differences between early and late puppies*

Based on statistical comparison, the two groups of dogs, i.e. early and late, were similar in term of maternal age (p-value=0.69), body weight (p-value=0.14) and litter size (p-value=0.58).

Gestational age was negatively correlated with both maternal and amniotic cortisol (p<0.0001; r=-0.56 and r=-0.5, respectively), and positively with PTX3 (p<0.05; r=0.28) as shown in Table 3. In particular, early puppies had higher concentrations of amniotic cortisol than late puppies (p=0.0003, Figure 3A). On the contrary, amniotic PTX3 showed a higher tendency (p<0.1) in puppies with longer gestational age calculated from mating (Figure 3B). Respiratory rate was significantly lower in early (19.6 ± 8.3 breaths/minute) than late puppies (25 ± 9.8 breaths/minute) (p<0.005, Figure 3C).

## 4. Discussion

Technical skills required to perform amniocentesis with an acceptable risk level have limited its application in dogs, leading to poor knowledge of canine amniotic fluid composition through pregnancy. In the present study, we investigated for the first time the presence and concentration of some components of canine amniotic fluid based on human evidence. Interestingly, all the selected parameters were measurable in the dogs' amniotic and blood samples. Some of the investigated molecules were already reported in dogs but in substrates other than amniotic fluid.

Literature attributes foetal pulmonary development to the rise of surfactant in the amniotic fluid, mainly due to its lecithin content (Whitfield, 1973) which impedes the collapse of the pulmonary alveoli and ensures the neonate to breathe autonomously after birth (Rossi et al., 2007). Indeed, lecithin and sphingomyelin ratio (L/S) in amniotic fluid is routinely used as a prognostic marker of

foetal maturity in humans (St. Clair et al., 2008). L/S has also been determined in animals, with only one study in dogs reporting amniotic L/S values of 3.62 ± 0.44 in puppies born from elective Caesarean-section (Silva et al., 2015). In the present study, we obtained similar values (2.9 ± 2.4) even though the surgery was performed before the onset of the clinical signs of labour, while Silva and colleagues awaited until the onset of the first stage of parturition.

Currently, four types of amniotic surfactant proteins have been characterized in humans: SP-A, SP-B, SP-C, and SP-D (Chaiworapongsa et al., 2008-2). The SP-A is involved in the synthesis, regulation, and homeostasis of surfactants (Chaiworapongsa et al., 2008-2; Gupta and Gupta, 2012). In humans, the increase of SP-A during the third trimester of pregnancy is considered predictive of foetal lung maturity (Shimizu et al., 1989) and initiation of labour (Sotiriadis et al., 2015). So far, none of these proteins have been analysed in the canine amniotic fluid. Serum SP-A concentration is reported to be <2.0 ng/mL in healthy dogs, and 3 to 7 ng/ml in dogs with pulmonary problems (i.e., tumours, injuries) (Sore et al, 2013). In literature, few studies indicate serum and amniotic SP-A reference values in pregnant women that vary from about 6 to 74 ng/mL (Cho K et al, 1999; Greene et al, 2002; Kale et al., 2020) and 0.4 to 25 μg/mL (Shimizu 1989; Miyamura et al., 1994; Cho K et al, 1999; Chaiworapongsa et al., 2008-2), respectively. SP-A concentrations detected in serum and amniotic fluid collected at Caesarean-section in our caseload were 10.75 ± 8.5 ng/mL and 21.5 ± 2.8 pg/mL, respectively. Serum and amniotic SP-A values were inversely proportional, suggesting a different secretory mechanism between the maternal and foetal compartments. Furthermore, contrary to humans, the SP-A concentrations were much lower in amniotic fluid than maternal serum, probably due to the different placental type: haemochorial in the woman *versus* endotheliochorial in the dog (Furukawa et al., 2014), allowing a diverse crossing of these proteins.

Glucocorticoids seem to play a central role in foetal lung maturation either by inducing specific enzymes and lecithin in the surfactant synthesis pathway or by enhancing the release of surfactant (Kitterman et al., 1981; Post et al., 1986; Liggins, 1994; Pepe et al., 2003; Rossi et al., 2007).

Amniotic cortisol values agreed with previously published data in canine AF collected at birth (Groppetti et al., 2015) and were directly correlated with maternal serum concentrations. Although the maternal peripartum increase in serum cortisol is a commonly trusted signal of the starting parturition in many species, including the dog (Hoffmann et al., 2004; Baan et al 2008; Kota et al., 2013; Wang et al., 2018), irregular and high individual variability in cortisol concentrations have also been reported in the bitch and is therefore not mandatory for normal parturition (Kowalewski et al., 2020).

Pentraxins 3 (PTX3) belongs to an evolutionarily conserved and multi-functional superfamily of proteins involved in regulating of innate immune response against microbial invasion of the amniotic cavity. Preterm parturition, with intact or ruptured membranes, is associated with a maternal systemic inflammatory response with PTX3 as a marker of preeclampsia (Cruciani et al., 2010; Porte et al., 2019). Only a few human studies investigated PTX3 during physiological pregnancy, suggesting an increase in the maternal serum PTX3 concentrations with advancing gestational age with the concentration peaking during labour (Rovere-Querini et al., 2006; Larsson et al., 2011; Martin 2014). In veterinary medicine, the role of PTX3 is still poorly understood in pig, chickens and ruminants as anti-microbial molecule (Crisci et al., 2014; Filipe et al., 2018; Burkhardt et al., 2019). No data about PTX3 in canine amniotic samples are available. In the present study, PTX3 mRNA was detected in all AF samples, showing higher values in late puppies than in early ones. The latter supports a link between amniotic PTX3, foetal development and gestational age in dogs and humans. We hypothesise that a greater expression of PTX3 mRNA in the amniotic fluid could be due to a more marked maturity of innate immunity in the puppy.

Furthermore, respiratory rate of puppies was recorded at birth as a reflection of lung function. Healthy puppies are expected to have a RR equal or greater than 15 bpm (Plavec et al., 2022; Groppetti et al., 2010). We recorded a significant higher RR in late than early puppies without other correlations with amniotic and clinical parameters. As the birth weight is deemed an indicator of neonatal maturity both in humans (Kramer, 1987) and dogs (Wootton et al., 1983; Mugnier et al., 2019), we compared

amniotic concentrations of the lightest and the heaviest puppy in litter with a birth weight difference of at least 20% among littermates. No differences resulted, possibly due to the small number of litters with relevant (≥ 20%) birth weight differences among littermates (7 out 10 litters). It would be interesting to assess this aspect on a large-scale population.

The subsequent estimation of these amniotic parameters as potential markers of neonatal immaturity faces some restraint since for ethical reasons it was not possible to engage premature puppies in this study. In the clinical setting, the elective Caesarean-section is scheduled when the puppies are deemed mature, so our comparison involved early and late neonates based on gestational age, but the puppies were always estimated as at the end of their development. It is reasonable to speculate a more relevant impact on amniotic parameters in case of pathological conditions such as premature, late parturition or abortion. However, some interesting results emerged from this preliminary study such as an association between maternal serum cortisol and gestational age with higher values in early puppies compared to the late ones. Similarly, a rise in amniotic cortisol concentrations is reported in case of premature rupture of the membrane in humans (Cohen et al., 1976). Due to a less individual variation in the ratio of amniotic and maternal cortisol (AF/M CORT) compared to amniotic cortisol alone, in humans, AF/M CORT was proposed to distinguish immature from mature new-born babies and a cut off 0.1 was identified (Varma et al., 1979). By analogy with humans, we identify a threshold value of 0.16 to distinguish early and late puppies. In our caseload, except for one, an AF/M CORT ratio higher than 0.16 was observed only in puppies with L/S higher than 2.8. However, the reverse was not evident. Indeed, AF/M CORT values below 0.16 were not associated with a low L/S ratio. This point deserves further investigation with large-scale studies, but it could be due to the difference in the development of puppies of the same pregnancy in polytocic animals when compared to humans. Furthermore, a possible relation between maternal size/weight and the composition of amniotic fluid can be supposed. To date, no literature exists supporting this hypothesis. Only a few studies on diet and metabolic status suggested such an association (Koski and Fergusson, 1992; Cheung et al., 2018).

## 5. Conclusions

Although our study represents a population of 10 individuals of different breeds, our data demonstrate a strong indication of the predictive value of the canine amniotic fluid. Nonetheless, further studies are necessary to make this data a robust, minimally invasive marker of neonatal maturity. Most advances in canine amniocentesis techniques are needed to understand the diagnostic and prognostic potential of amniotic fluid in canine species to allow more conscious planning of the Caesarean-section.

This study showed the feasibility of lecithin, sphingomyelin, SP-A, cortisol, and PTX3 measurement in canine amniotic fluid. Although amniotic fluid collected at birth is considered a negligible biological discard in dogs, it can potentially represent an innovative and non-invasive biological sample for recognizing neonatal development. Further investigations are required to establish the continuation and development of appropriate procedures to know when and in what cases it could be possible to implement amniotic fluid testing.


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## CRediT authorship contribution statement

**F. Riva**: Metodology, Data curation, Writing – original draft; **J. Filipe**: Validation, Formal analysis data Writing - review & editing; **R. Pavlovic**: Methodology, Formal analysis, Writing - review & editing; **A.M. Luciano**: Writing - review & editing, Supervision; **P. Dall'Ara**: Formal analysis, Writing - review & editing; **F. Arioli**: Methodology, Writing - review & editing; **A. Pecile**:

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612 Table 1. Animals enrolled in this study

613

614

| Breed | Age (ys) | BW (kg) | *n* of pups | Gestational age (days) | | Group |
|---|---|---|---|---|---|---|
| | | | | from LH rise | from mating | |
| German Shepherd dog | 7 | 34.5 | 8 | 63 | 60 | Early |
| French bully | 3 | 12.5 | 6 | 64 | 60 | Early |
| French bully | 3.5 | 11.4 | 2 | 64 | 60 | Early |
| American bully | 2.5 | 38.5 | 11 | 65 | 60 | Early |
| American bully | 2 | 23.8 | 2 | 65 | 60 | Early |
| Bernese Mountain dog | 3 | 56.2 | 8 | 66 | 61 | Late |
| American bully | 3 | 23.3 | 4 | 66 | 61 | Late |
| American bully | 2.5 | 28.5 | 5 | 66 | 61 | Late |
| German Shepherd dog | 5 | 28.3 | 6 | 66 | 62 | Late |
| Rhodesian ridgeback | 7 | 40.5 | 11 | 66 | 62 | Late |

615

616

617 BW means maternal body weight at proestrus; *n* means number of pups; Early group means ≤65
618 days from LH surge and 60 days from the single mating; Late group means >65 days from LH
619 surge and ≥61 days from the single mating

620

621

622

623

| Gene | Protein | Sequence (5' → 3') | Amplicon | GI |
|---|---|---|---|---|
| PTX3 | Pentraxin 3 | F: GCCGGCAGGTTGTGAAAC<br>R: CCAGATGCAGGCACTGAAAGA | 112 bp | 1952714952 |
| β-actin | β -actin | F: TCCCTGGAGAAGAGCTACGA<br>R: CTTCTGCATCCTGTCAGCAA | 243 bp | 5597004 |

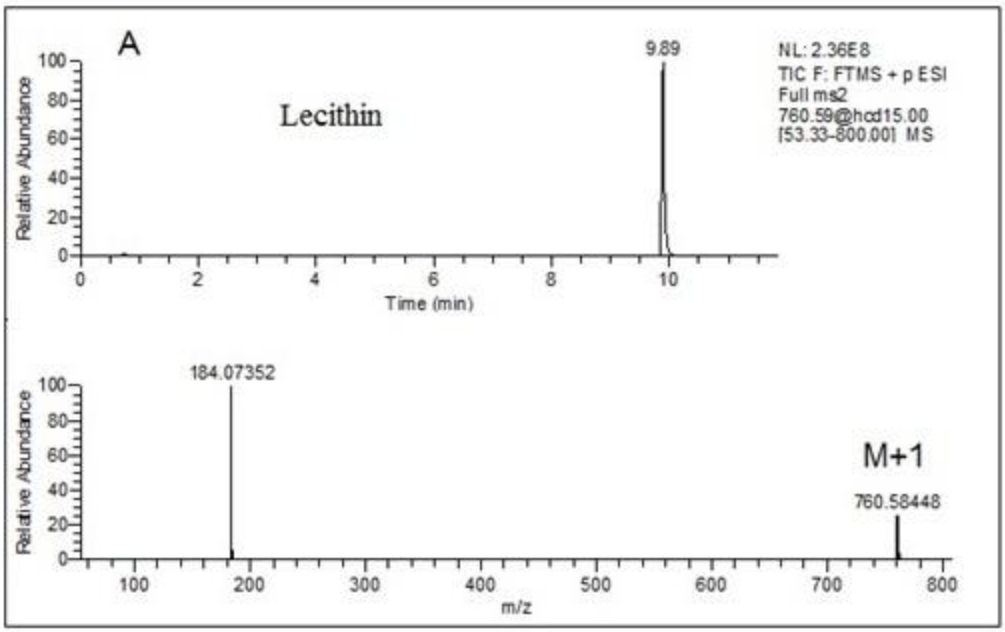
Table 2: Selected primers sequences and respective amplicons size

Table 3. Significant correlations recorded among amniotic and maternal parameters and with gestational age.

| | | r | P |
|---|---|---|---|
| Amniotic lecithin | Amniotic sphingomyelin | 0.5444 | < 0.0001 |
| | L/S | 0.5168 | < 0.0001 |
| | Amniotic SP-A | - 0.3846 | 0.0029 |
| Amniotic sphingomyelin | L/S | - 0.3835 | 0.0027 |
| | PTX3 | 0.3133 | 0.0166 |
| Amniotic SP-A | Amniotic lecithin | - 0.3846 | 0.0029 |
| | L/S | - 0.2601 | 0.0486 |
| | Maternal SP-A | - 0.3203 | 0.0126 |
| Maternal SP-A | Amniotic lecithin | 0.4379 | 0.0005 |
| | Amniotic sphingomyelin | 0.4565 | 0.0003 |
| | Maternal age | - 0.5923 | < 0.0001 |
| Amniotic cortisol | Maternal cortisol | 0.3383 | 0.0077 |
| | Gestational age (from LH) | - 0.5004 | < 0.0001 |
| | Gestational age (from mating) | - 0.5651 | < 0.0001 |
| Maternal cortisol | L/S | -0.283 | 0.0297 |
| | Gestational age (from LH) | - 0.5647 | < 0.0001 |
| | Gestational age (from mating) | - 0.5004 | < 0.0001 |
| AF/M CORT | Amniotic lecithin | 0.5273 | < 0.0001 |
| | Amniotic sphingomyelin | 0.3714 | 0.0038 |
| Amniotic PTX3 | Amniotic sphingomyelin | 0.3133 | 0.0166 |
| | L/S | - 0.2985 | 0.0228 |
| | Gestational age (from mating) | 0.2816 | 0.0137 |

L/S indicates lecithin-sphingomyelin ratio in amniotic fluid; AF/M CORT indicates the ratio of amniotic and maternal cortisol

638 Fig 1



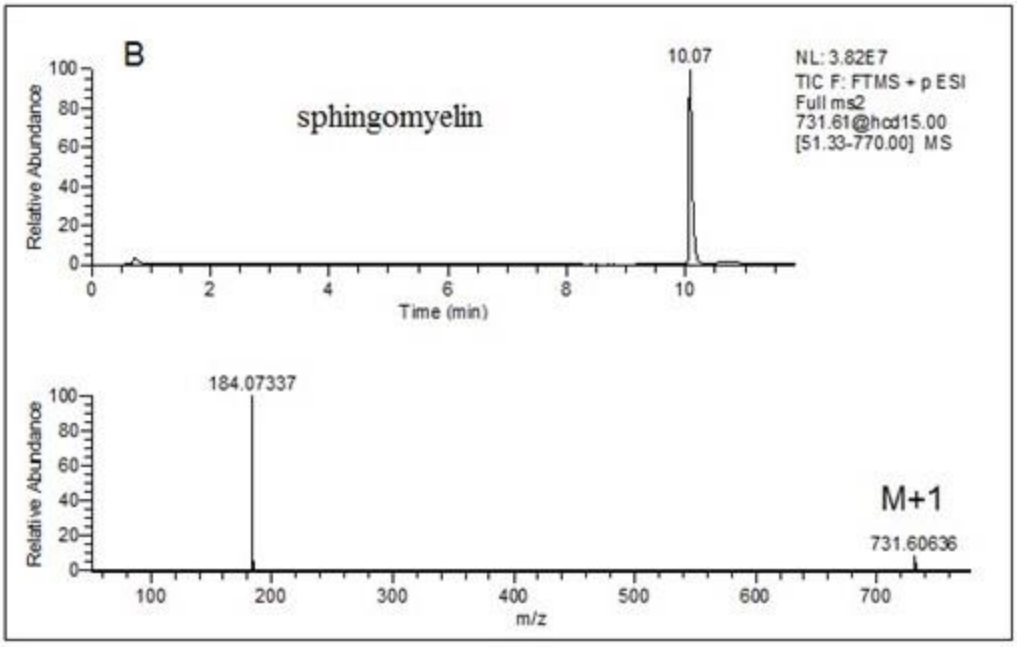




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Fig. 2

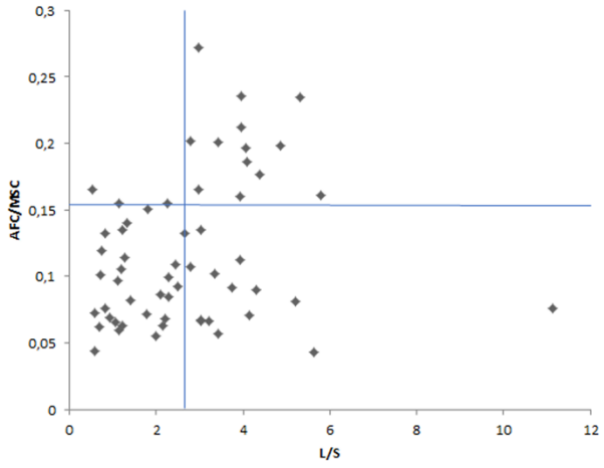
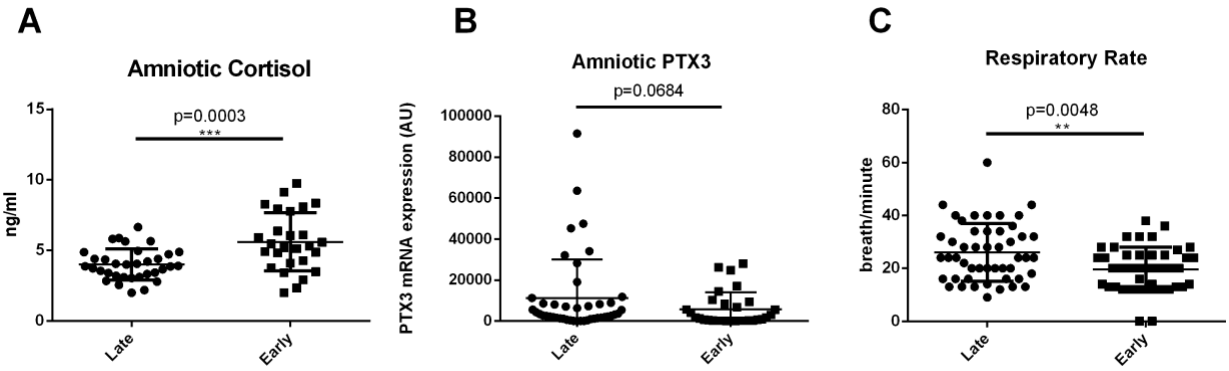

AFC/MSC
L/S
0,3
0,25
0,2
0,15
0,1
0,05
0
0
2
4
6
8
10
12

Fig. 3


A
Amniotic Cortisol
p=0.0003
***
ng/ml
15
10
5
0
Late
Early
B
Amniotic PTX3
p=0.0684
PTX3 mRNA expression (AU)
100000
80000
60000
40000
20000
0
Late
Early
C
Respiratory Rate
p=0.0048
**
breath/minute
80
60
40
20
0
Late
Early

Appendix 1

Comparison of individual AF/M CORT ratio higher than 0.16 and the respectively L/S ratio

| ID | AF/M CORT | L/S |
|---|---|---|
| 3.1 | 0.20 | 4.86 |
| 4.2 | 0.20 | 3.43 |
| 5.3 | 0.20 | 4.07 |
| 9.1 | 0.16 | 5.80 |
| 9.2 | 0.20 | 2.80 |
| 10.1 | 0.19 | 4.10 |
| 10.2 | 0.24 | 3.96 |
| 10.3 | 0.16 | 3.93 |
| 10.4 | 0.21 | 3.96 |
| 10.5 | 0.17 | 2.96 |
| 10.6 | 0.23 | 5.31 |
| 10.7 | 0.27 | 2.97 |
| 10.8 | 0.18 | 4.39 |
| | | |
| 8.6 | 0.17 | 0.54 |

ID numbers identify mother (first digit) and pup (second digit); AF/M CORT is the ratio of amniotic and maternal cortisol; L/S is the lecithin and sphingomyelin ratio

Appendix 2

Comparison of individual AF/M CORT ratio lower than 0.16 and the respectively L/S ratio

| ID | AF/M CORT | L/S |
|---|---|---|
| 1.3 | 0,07 | 1,78 |
| 1.4 | 0,07 | 2,21 |
| 1.5 | 0,06 | 1,23 |
| 1.6 | 0,07 | 1,06 |
| 1.7 | 0,05 | 2,00 |
| 1.8 | 0,06 | 2,14 |
| 2.4 | 0,09 | 2,49 |
| 4.1 | 0,15 | 1,81 |
| 4.3 | 0,11 | 2,78 |
| 5.1 | 0,10 | 2,28 |
| 5.2 | 0,04 | 0,59 |
| 5.4 | 0,08 | 2,27 |
| 5.5 | 0,13 | 2,64 |
| 5.7 | 0,15 | 2,25 |

| | | |
|---|---|---|
| 6.1 | 0,10 | 1,13 |
| 6.2 | 0,12 | 0,73 |
| 6.3 | 0,07 | 0,92 |
| 6.4 | 0,08 | 1,39 |
| 6.5 | 0,11 | 2,43 |
| 6.6 | 0,10 | 0,72 |
| 6.7 | 0,11 | 1,28 |
| 6.8 | 0,09 | 2,11 |
| 6.9 | 0,06 | 1,15 |
| 6.10 | 0,11 | 1,18 |
| 7.1 | 0,06 | 0,69 |
| 7.2 | 0,08 | 0,83 |
| 7.3 | 0,07 | 0,58 |
| 8.1 | 0,15 | 1,14 |
| 8.2 | 0,13 | 1,21 |
| 8.3 | 0,14 | 1,33 |
| 8.4 | 0,13 | 0,83 |
| | | |
| 1.1 | 0,07 | 3,21 |
| 1.2 | 0,08 | 11,14 |
| 2.1 | 0,07 | 3,03 |
| 2.2 | 0,07 | 3,03 |
| 2.3 | 0,13 | 3,03 |
| 2.5 | 0,08 | 5,20 |
| 2.6 | 0,04 | 5,63 |
| 3.2 | 0,11 | 3,94 |
| 5.6 | 0,07 | 4,14 |
| 5.9 | 0,09 | 3,75 |
| 5.10 | 0,06 | 3,43 |
| 7.4 | 0,09 | 4,30 |
| 7.5 | 0,10 | 3,35 |

673
674 ID numbers identify mother (first digit) and pup (second digit)
675
676

677