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Amniotic Fluid at Canine Birth Reflects Neonatal IgG Titres Against CPV-2, CAdV-1, and CDV: A Non-Invasive Immunity Proxy

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ABSTRACT

Interest in the biochemical composition of amniotic fluid (AF) has expanded across human and veterinary research. Beyond its nutritive and protective roles for the fetus, AF is now recognized for its diagnostic, prognostic, and therapeutic potential. Neonatal dogs possess an immature immune system, rendering them vulnerable to severe pathogens such as canine parvovirus (CPV-2), canine adenovirus type 1 (CAdV-1), and canine distemper virus (CDV), which contribute significantly to early-life mortality. Among immunoglobulins, only IgG can traverse the placenta in limited amounts and has been identified within canine AF. The present investigation aimed to assess whether AF collected at birth could act as a non-invasive indicator of passive immunity in dogs. For this purpose, total and pathogen-specific IgG levels against CPV-2, CAdV-1, and CDV were analyzed in both maternal plasma and AF obtained during cesarean delivery. The vaccination background of each dam was also recorded. Considering that immune competence is influenced by gestational maturity, with premature neonates displaying underdeveloped innate and adaptive systems, IgG levels were examined in relation to amniotic concentrations of lecithin, sphingomyelin, cortisol, surfactant protein A, and pentraxin 3—biomolecules previously quantified in a study on fetal maturity using the same sample set. Finally, potential links between these parameters and neonatal outcomes were explored. The findings indicate that AF evaluation at birth can provide meaningful insights into early immune status in puppies, supporting its use as a minimally invasive approach for health monitoring and management in neonatal dogs.

Keywords: Dog, Amniotic fluid, Immunoglobulins, Parvovirus, Hepatitis, Distemper

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Introduction

The innate immune system develops during intrauterine life, whereas adaptive immunity matures postnatally and remains functionally limited in puppies until approximately 2–6 months of age. Passive protection from the dam—mainly transferred during gestation and particularly through colostrum—plays a crucial role in survival during the early weeks [1, 2].

Adaptive immunity involves pathogen-specific responses mediated by lymphocytes capable of generating immunologic memory [1]. At the same time, the innate system is relatively more advanced at birth; both innate and adaptive arms require several weeks to achieve full competence [3].

During gestation, the placenta shields the embryo and fetus from pathogen exposure, while AF contributes to maternal immune modulation that prevents fetal rejection [4]. The neonatal stage in dogs is considered highly vulnerable, with infections constituting a major cause of mortality [5, 6].

Vaccination remains the cornerstone of infectious disease prevention in both humans and companion animals. International guidelines distinguish vaccines as core—those essential for all dogs due to their protection against severe, globally prevalent diseases—and non-core, which are recommended based on exposure risk, environment, or lifestyle. Owing to their high pathogenicity and fatal potential, CPV-2, CAdV-1, and CDV are classified as core vaccines for all canines worldwide [7–9].

Passive immune transfer in puppies occurs primarily via colostrum intake [10, 11]. The endotheliochorial structure of the carnivore placenta restricts antibody transfer to only about 5–12%, consisting exclusively of IgG [10]. Despite their high molecular size, IgGs are the only antibody type that can traverse the canine placenta into fetal circulation, albeit in low quantities [12]. The transfer takes place mainly during the final 20 days of gestation [13], and these antibodies have also been detected in AF collected at birth [10]. Gestational age can significantly influence immune capability; preterm or early-born pups exhibit greater susceptibility to infection due to incomplete immune development [14–16]. In humans, a strong association between total IgG and gestational age has likewise been observed [16]. Given the invasiveness of blood sampling, non-invasive alternatives are preferable in neonatal diagnostics whenever possible.

In human medicine, AF serves as a valuable diagnostic medium for early detection of gestational disorders [17]. Recently, in canine studies, AF obtained at parturition has been shown to be a promising and ethically acceptable diagnostic sample [18]. AF includes compounds such as lecithin, sphingomyelin, cortisol, and surfactant protein A (SP-A), all of which are recognized as indicators of fetal lung maturity in humans [19–26] and have been similarly quantified and linked to gestational age in dogs [27]. Pentraxin-3 (PTX3), a soluble innate immunity mediator, has also been identified in AF [28–31]. In women, PTX3 serves as an early marker for placental dysfunction [32] and has been correlated with intrauterine growth restriction [33], miscarriage [34], preeclampsia [35], and gestational stage [36]. In veterinary fields, data on PTX3 remain limited, with one canine study revealing a positive association between AF PTX3 concentration and gestational age [27].

This study proposes that AF reflects prenatal immune status in puppies, influenced by maternal immunization, and could potentially indicate early pathological risks.

Accordingly, the first goal was to quantify total and specific IgGs against CPV-2, CAdV-1, and CDV in AF collected during elective cesarean delivery. These values were then compared with IgG levels in maternal plasma, taking into account each dam's vaccination history.

To deepen understanding of AF's immunologic role, total and pathogen-specific IgGs in both AF and maternal plasma were correlated with amniotic concentrations of lecithin, sphingomyelin, cortisol, SP-A, and PTX3. These biochemical markers—previously reported in a fetal maturity study [27]—were here analyzed alongside immune parameters.

Finally, all AF-derived markers were related to maternal traits (age, body weight), litter characteristics (size, sex ratio, birthweight), and neonatal outcomes, including Apgar score, viability, morbidity, and mortality during the first two postnatal months.

Materials and Methods

This investigation formed part of a broader research project on canine amniotic fluid (Linea 2 Groppetti_2016), authorized by the Ethics Committee of the University of Milan (OPBA_77_2017) and conducted in compliance with Italian regulations on animal welfare and experimentation. Each amniotic fluid (AF) specimen was divided into two parts. The first portion had already been analyzed for lecithin, sphingomyelin, cortisol, SP-A, and PTX3 gene expression in an earlier study on fetal development [27], where the data and correlations with fetal maturity were previously described. The remaining portion was reserved for the current analysis to assess the same biochemical indicators in conjunction with IgG titers. The current paper presents only the relationships between AF constituents, immune markers, and selected clinical findings.

Clinical records

A total of ten purebred female dogs, scheduled for elective cesarean delivery, were included. Both the surgical and anesthetic procedures followed standard clinical practice [37]. Only animals deemed healthy on the basis of clinical examination, ultrasonographic evaluation, and routine blood tests were enrolled.

Table 1 summarizes the main features of the bitches, including breed, age, body weight (BW), and litter size. Four of the ten animals (ID. 1, 4, 8, and 9) had up-to-date vaccinations with all core immunizations.

Table 1. Breed, age, body weight, and litter size of the bitches in the study.

ID	Breed	Age (ys)	BW * (kg)	Litter Size
1	German Shepherd	7	34.5	8
2	German Shepherd	5	28.3	6
3	American Bully	2	23.8	2
4	American Bully	3	23.3	4
5	American Bully	2.5	38.5	11
6	Rhodesian Ridgeback	7	40.5	11
7	American Bully	2.5	28.5	5
8	French Bouledogue	3	12.5	6
9	French Bouledogue	3.5	11.4	2
10	Bernese Mountain Dog	3	56.2	8
Mean ± SD		**3.9 ± 1.8**	**29.7 ± 13.4**	**6.3 ± 3.2**

*BW: body weight.

All dogs were monitored throughout their reproductive cycle—from the beginning of proestrus until delivery—following established procedures [38]. Background information and clinical data, such as age, body weight, breed, and vaccination status, were noted. Depending on the case, females were either naturally mated or artificially inseminated. They were fed commercial diets formulated for pregnant and lactating dogs (from mid-gestation through weaning).

Cesarean sections were performed once fetuses were judged mature, based on gestational age as outlined in previous literature [38–40]. Data, including litter size, sex, neonatal weight, vitality, illness, and deaths within the first two months of life, were recorded. Each puppy received standard neonatal care or resuscitation immediately after delivery according to established recommendations [41]. Within five minutes after birth, an Apgar score ranging from 0 to 14 was used to evaluate vitality, classifying each neonate as normal, moderately stressed, or severely distressed [42]. All newborns were allowed to nurse colostrum and maternal milk, with supplemental formula provided for large litters if necessary.

Maternal blood sampling

Prior to the cesarean operation, 1 mL of blood was collected from the cephalic vein under general anesthesia. Samples were drawn into K_2EDTA tubes, centrifuged at 1500× g for 10 minutes at room temperature, and plasma aliquots were stored at −20 °C until immunoglobulin analysis.

Collection of amniotic fluid

Amniotic fluid samples were taken during cesarean delivery following the previously described method [27]. A 20 mL sterile syringe was used to gently puncture the amniotic sac wall while holding the puppy in a vertical position, head upward, and aspirating from the lowest area of the sac to avoid needle injury. Each AF sample was portioned into two 15 mL tubes, centrifuged at 500× g for 15 minutes at room temperature, and the supernatant was kept at −80 °C for subsequent evaluation.

Amniotic fluid evaluation

Determination of total IgGs

Total Immunoglobulin G concentrations were quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Dog IgG Quantitation Set, Bethyl Laboratories, Montgomery, TX, USA). Ninety-six–well plates were coated with 100 µL per well of affinity-purified sheep anti-dog IgG antibody diluted 1:100 in 0.05 M carbonate–bicarbonate buffer (pH 9.6) and incubated for 1 hour at ambient temperature. Plates were then washed four times with Tris-buffered saline containing 0.05% Tween 20 (TBST). Blocking was carried out with 200 µL per well of TBST for 30 minutes, followed by another washing cycle.

Serial dilutions of standard canine antibodies (500 to 7.8 ng/mL) were prepared for calibration. Based on optimization tests, AF samples were diluted 1:5000 in TBST. 100 µL of each sample or standard was added in

duplicate wells and incubated for 1 hour at room temperature. After washing, 100 µL per well of HRP-conjugated sheep anti-dog IgG (dilution 1:100,000) was added and left for another 1 hour. Plates were washed five times, followed by 100 µL per well of substrate solution (H_2O_2 and TMB), and incubated for 15 minutes in the dark. The reaction was halted using 100 µL of 0.18 M sulfuric acid, and absorbance was measured at 450 nm using an ELISA reader (Thermo Fisher Scientific, Tokyo, Japan). The coefficients of variation for intra- and inter-assay precision were 3.08% and 4.38%, respectively.

Specific IgG—VacciCheck

Each plasma and amniotic fluid (AF) specimen was evaluated using the Canine VacciCheck test kit (Biogal, Kibbutz Galed, Israel; distributed in Italy by Agrolabo, Scarmagno, Italy), following the manufacturer's protocol. This assay is a dot-ELISA-based, semi-quantitative rapid test authorized for determining specific IgG antibody titres against canine parvovirus type 2 (CPV-2), canine adenovirus type 1 (CAdV-1), and canine distemper virus (CDV). The VacciCheck system provides high analytical sensitivity and specificity for all three viruses and is validated for both diagnostic and research purposes.

Antibody concentration is interpreted based on the intensity of colored dots, compared with a six-point reference scale (1–6). The S0 reference corresponds to titres below detection thresholds: <1:20 for CPV-2, <1:4 for CAdV-1, and <1:8 for CDV. Conversely, a value of S3 represents titres of 1:80 (CPV-2), 1:16 (CAdV-1), and 1:32 (CDV). Dogs showing titres equal to or greater than S3 were classified as having protective immunity against the respective viruses.

Lecithin, sphingomyelin, cortisol, SP-A, and PTX3 determination in amniotic fluid

Following previously established protocols [27], lecithin and sphingomyelin levels were analyzed using HPLC–MS (Thermo Q-Exactive Plus, Thermo Scientific). Cortisol was measured via a quantitative enzyme-linked fluorescent assay (ELFA) (MiniVidas, bioMérieux, Bagno a Ripoli, Italy). The SP-A concentration was determined through a commercial sandwich ELISA kit (LifeSpan BioSciences, Seattle, WA, USA), while PTX3 mRNA expression in the amniotic cell fraction was quantified by qPCR.

Statistical analysis

Maternal parameters, such as age and body weight (BW), were analyzed as both continuous and categorical variables: age (≤3 years or >3 years) and BW (≤30 kg or >30 kg). According to the WSAVA vaccination guidelines [7], dogs receiving all core vaccines as recommended were labeled "regularly vaccinated", while those deviating from the prescribed schedule were classified as "irregularly vaccinated."

Neonatal mortality was documented at birth, within 7 days, and at 2 months of age. Puppies displaying any clinical symptoms—specifically diarrhea, the only condition observed during the monitoring period—were identified as "pathological", whereas those remaining asymptomatic were considered "healthy."

All analyses were performed in GraphPad Prism 6 (La Jolla, CA, USA). Statistical significance was set at $p < 0.05$. Descriptive data were presented as mean ± standard error. The Shapiro–Wilk test verified normality. Depending on data distribution, correlations were examined using parametric (two-tailed Pearson) or non-parametric (two-tailed Spearman) tests. Group comparisons were conducted using either the Student's t-test (parametric) or the Mann–Whitney test (non-parametric).

Experimental design

The retrospective analysis covered three major variables: (1) maternal vaccination protocol, (2) maternal antibody titres for each core antigen, and (3) puppy health status within two months postpartum.

Regarding vaccination protocol, females were categorized into regularly vaccinated (core vaccines administered at standard intervals—annually or every 2–3 years, as per manufacturer's recommendations) and irregularly vaccinated (missing or delayed boosters).

For maternal IgG titres, dogs were divided into low-titre (below S3) and high-titre (≥S3) groups based on VacciCheck readings. The S3 benchmark was considered protective, equating to titres of 1:80 (CPV-2), 1:16 (CAdV-1), and 1:32 (CDV).

Puppy health outcomes were grouped as healthy (no clinical signs such as diarrhea) or pathological (presence of diarrhea during the observation period).

Results and Discussion

Clinical findings

A total of 63 neonates were delivered—34 males and 29 females—all alive at birth. Birth weights ranged from 236 g to 770 g (mean 438.5 ± 140.5 g), and Apgar scores spanned 4–14 (mean 10.9 ± 2.3). Unfortunately, eight puppies died within the first 48 hours postpartum: one with anasarca survived only briefly due to severe illness, two were accidentally crushed by their mothers, and five deaths were of undetermined causes, possibly linked to improper neonatal care.

The remaining 55 puppies survived through the two-month follow-up. Among these, 18 puppies from five bitches exhibited diarrhea of varying severity and were classified as pathological. Since no definitive etiological diagnosis (infectious or nutritional) was reached, all diarrheic cases were grouped together. Notably, three bitches—two French Bulldogs sharing the same household and one Bernese Mountain Dog—had litters entirely affected (totaling 16 puppies). The other 37 neonates remained clinically normal throughout the study.

Immune status

Specific and total IgG concentrations were quantified in all maternal plasma and amniotic samples, except for two puppies from litter ID.5, where amniotic fluid collection was insufficient.

Maternal plasma total IgG values ranged between 5.6 and 14.1 mg/mL (mean 10.2 ± 2.8), while amniotic IgG levels varied from 0.02 to 0.5 mg/mL (mean 0.1 ± 0.09), with no significant correlation observed between them.

Regularly vaccinated and irregularly vaccinated females exhibited comparable plasma IgG levels—10.0 ± 4.1 mg/mL and 10.4 ± 2.0 mg/mL, respectively—showing no statistical difference **(Figure 1)**. However, amniotic IgG values at delivery tended to be higher (p = 0.07) in litters from regularly vaccinated mothers (0.18 ± 0.1 mg/mL) compared with those from irregularly vaccinated bitches (0.13 ± 0.08 mg/mL) **(Figure 2)**.

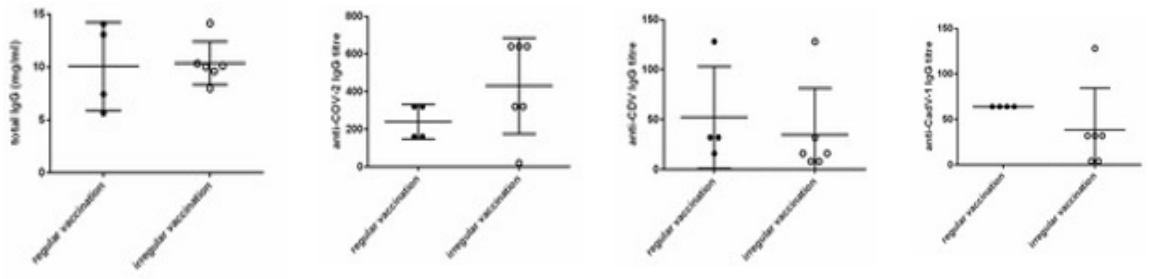

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Figure 1. Effect of consistent (n = 4) versus inconsistent (n = 6) vaccination protocols on maternal serum IgG concentrations and antibody titres.

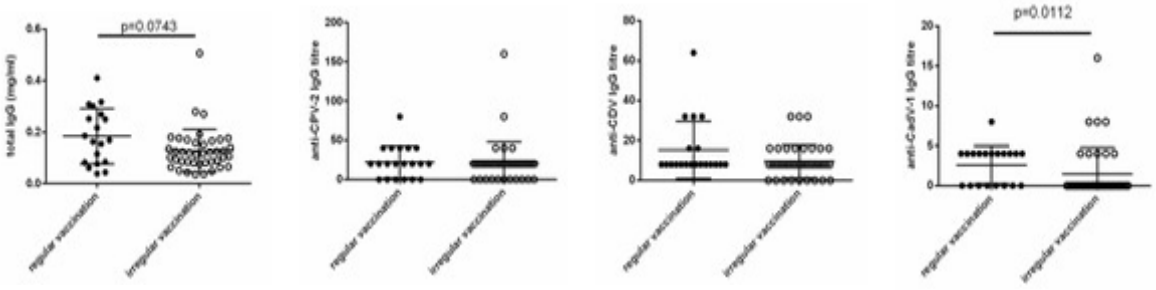

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Figure 2. Effect of consistent (n = 20) versus inconsistent (n = 43) vaccination schedules on IgG concentrations and titres in the amniotic fluid.

The titres of IgG antibodies specific to CPV-2, CAdV-1, and CDV measured in both maternal plasma and amniotic fluid are summarized in **Table 2**.

Table 2. Antibody titres against CPV-2, CAdV-1, and CDV in maternal plasma and amniotic fluid.

Virus	Sample	n (dams)	n (puppies)	min–max	≤1:20 / ≤1:4 / ≤1:8	1:160–320 / ≤1:32 / ≤1:16	>1:320 / >1:32 / >1:16	Negative	≤1:20 / ≤1:4 / ≤1:16	>1:20 / >1:8 / >1:16	1:20–1:640 / 1:4–1:128 / 1:8–1:128
CPV-2[1]	Plasma	10	—	—	1	4	5	—	—	—	—
	AF	—	61	negative —1:40	—	—	—	17	33	11	—
CAdV-1[2]	Plasma	10	—	—	2	2	6	—	—	—	—
	AF	—	61	negative —1:4	—	—	—	39	16	6	1:4–1:128
CDV[3]	Plasma	10	—	—	2	3	5	—	—	—	—
	AF	—	61	negative —1:16	—	—	—	9	45	7	1:8–1:128

1 CPV-2: canine parvovirus; 2 CAdV-1: canine adenovirus type 1; 3 CDV: canine distemper virus.

As shown in **Figure 1**, antibody titres against CPV-2, CAdV-1, and CDV in the plasma were comparable between regularly and irregularly vaccinated mothers. However, in the amniotic fluid, levels of CAdV-1–specific IgG were significantly higher ($p = 0.01$) in the litters of regularly vaccinated bitches, while no such difference was detected for CPV-2 or CDV **(Figure 2)**.

Table 3 provides the defined threshold titres (S3) considered protective for each virus in canine plasma [43], along with the number of bitches exhibiting titres above those levels. Bitches were divided according to antibody titre: those below the threshold (S3) were designated low-titre, while those equal to or above S3 were classified as high-titre.

Table 3. Percentage of bitches with protective antibody titres against CPV-2, CAdV-1, and CDV.

Protective Threshold (S3)	CPV-2 ≥1:80 *	CAdV-1 ≥1:16 *	CDV ≥1:32 *
Percentage of regularly vaccinated (4) protected bitches	100	100	75
Percentage of irregularly vaccinated (6) protected bitches	83.3	66.7	33.3
Percentage of protected bitches out of the total (10)	90	80	50

Bold entries indicate protective IgG values.

Among the ten bitches evaluated, four (three regularly vaccinated and one irregularly vaccinated) displayed seroprotective titres for all three pathogens. Comparing high- and low-titre dams, higher amniotic IgG levels against CPV-2 were noted in offspring of high-titre mothers ($p = 0.003$; **(Figure 3)**). Although not statistically significant, there was a tendency for greater amniotic IgG titres against CAdV-1 ($p = 0.08$) and CDV ($p = 0.09$) in litters from high-titre dams.

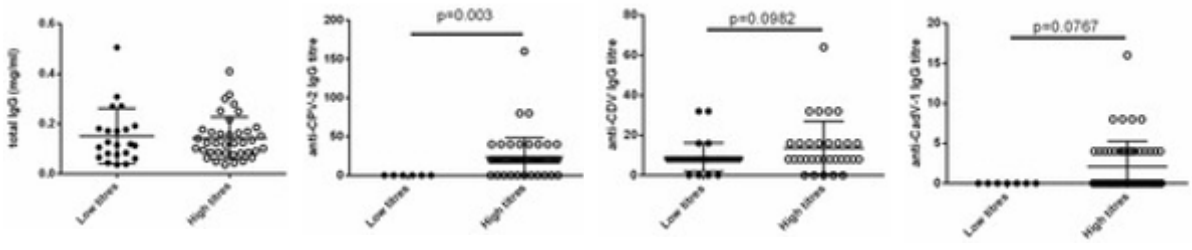

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Figure 3. Amniotic antibody titres to CPV-2, CAdV-1, and CDV in relation to high and low maternal antibody titres.

Among measured amniotic biomarkers (lecithin, sphingomyelin, cortisol, SP-A, PTX3), only sphingomyelin

demonstrated a negative correlation with total amniotic IgG (p = 0.0057). Conversely, cortisol in amniotic fluid was positively correlated with anti-CDV IgG titres (p = 0.0358), while PTX3 showed an inverse correlation with anti-CAdV-1 titres (p = 0.0136).

Associations with maternal and neonatal variables

Certain maternal traits—age, weight, and litter size—were associated with IgG concentrations in plasma or amniotic fluid and with amniotic markers. Maternal age showed a positive association with amniotic SP-A (p = 0.0101), while both maternal weight and litter size correlated negatively with amniotic cortisol levels (p = 0.0004 and 0.0055, respectively). Additionally, maternal age, body weight, and litter size correlated with anti-CPV-2 IgG titres (p < 0.0001 for all), showing negative correlation with age and positive ones with the other two factors.

When comparing neonatal sex, no significant variation was seen in either total or specific amniotic IgG concentrations **(Figure 4)**.

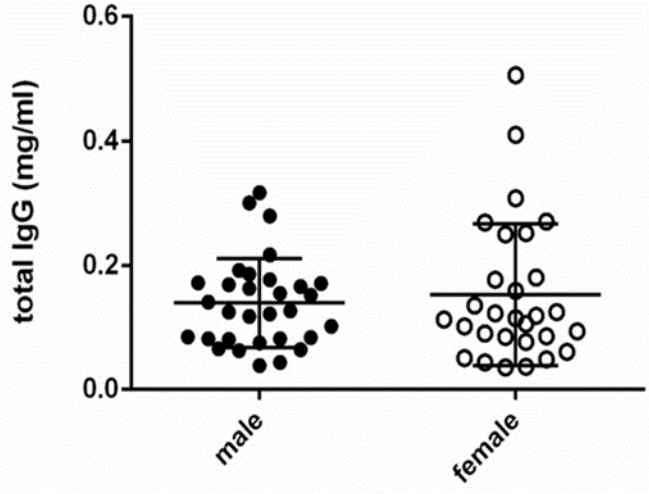

Figure 4. Comparison of total IgG levels in amniotic fluid between male (n = 34) and female (n = 29) neonates.

Birthweight, Apgar scores, and early mortality had no measurable influence on IgG content. Interestingly, pups categorized as pathological displayed elevated amniotic lecithin (p = 0.0001), sphingomyelin (p = 0.0004), and cortisol (p = 0.0006), but reduced SP-A levels (p = 0.0107) relative to healthy counterparts **(Figure 5)**.

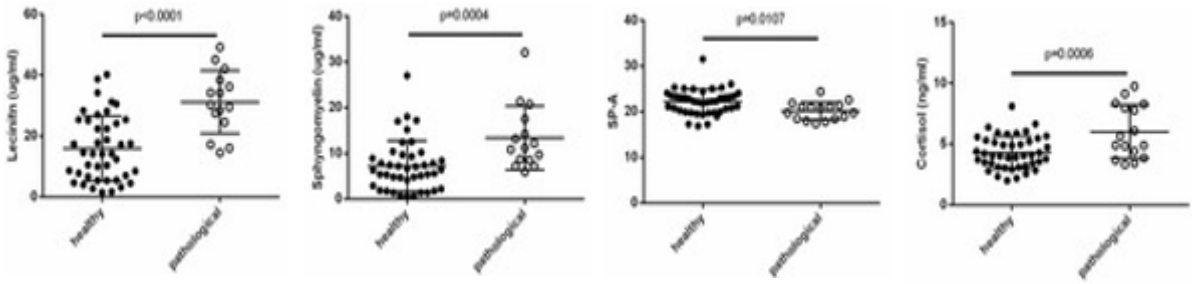

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Figure 5. Variations in amniotic fluid molecule expression between healthy (n = 37) and pathological (n = 18) neonates.

Moreover, pathological puppies showed lower anti-CAdV-1 IgG titres (p = 0.035) compared with healthy ones **(Figure 6)**.

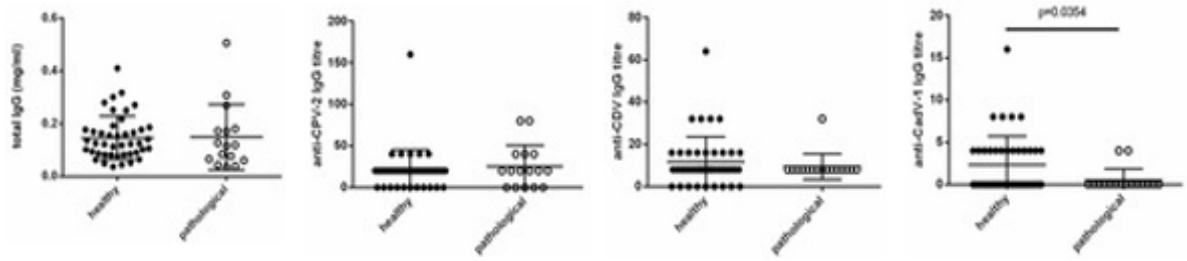

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Figure 6. Specific and total IgG titres in healthy (n = 37) versus pathological (n = 18) neonates.

Of particular interest, the occurrence of neonatal pathologies appeared linked to the dam's immunization history. The proportion of affected puppies was significantly higher among litters from dams that had not undergone regular vaccination (p = 0.049; **(Figure 7)**).

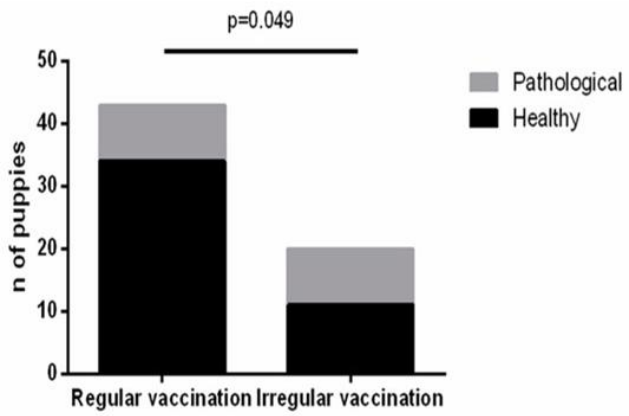

Figure 7. Relationship between maternal vaccination status and puppy health condition.

Amniotic fluid (AF) fulfills multiple vital physiological roles, serving as a protective buffer that shields the fetus from mechanical impacts and temperature fluctuations, while also supporting the development and maturation of both respiratory and gastrointestinal systems [44]. In humans, AF is also widely utilized in early diagnostic testing for infections, chromosomal abnormalities, and congenital defects [45], and has recently gained attention as a potential tool in regenerative therapies [46].

In canines, AF collection during cesarean delivery represents a rapid, non-invasive, and low-risk sampling procedure that does not interfere with neonatal extraction. In the present study, amniotic samples were successfully collected from all 63 newborn puppies, though two samples were insufficient, likely due to the large litter size (11 pups) typical of a medium breed. This observation may indicate a negative correlation between litter size and AF volume, a relationship not yet documented but warranting further exploration.

The current findings confirm the presence of both total and virus-specific immunoglobulin G (IgG) antibodies against CPV-2, CAdV-1, and CDV in canine AF at birth. As the fetus ingests AF containing immunoglobulins—and considering possible intestinal absorption—there may be a positive association between IgG levels in AF and neonatal blood, as similarly described in humans [47].

Determining the neonatal immune profile is critical for evaluating infection susceptibility and optimizing vaccination timing. The immune response following vaccination is known to vary significantly and depends on several intrinsic and extrinsic factors [11, 48–51]. Maternally derived antibodies (MDAs) are transmitted transplacentally and through colostrum intake immediately after birth. During the initial postnatal period, these antibodies protect the puppy, although their titres differ widely among individuals [11, 50, 51]. Despite their protective benefits, MDAs can inhibit effective vaccine-induced immunity as long as their concentrations remain high [6, 11, 51, 52].

Pregnancy represents a unique immunological condition in which adaptive responses are downregulated to ensure fetal tolerance [53], while innate immunity remains active to maintain equilibrium [54]. Interestingly, our analysis showed no direct relationship between total or specific IgG titres in maternal plasma and vaccination history.

Hence, evaluating vaccine antibody titres before breeding is advisable, even in routinely immunized bitches [55]. In our population, protective antibody titres were found in 90% versus 86% of dogs for CPV-2 and 80% versus 71% for CAdV-1, consistent with prior data [56]; however, protection against CDV was lower (50% vs. 72%). These variations may arise from factors such as population size, sex, breed, age, health, stress, or environmental conditions [2, 11, 56, 57].

Transplacental transfer of immunoglobulins plays a key role in early immunity for newborns with immature immune systems [53]. Recent studies in dogs highlight the importance of passive immunity in reducing neonatal infections and mortality [51]. While direct evidence for antibody passage across the canine placenta is limited, the mechanism is recognized in humans [58]. Our data did not reveal a correlation between total maternal and amniotic IgG concentrations, implying either partial fetal IgG production or variable efficiency of transplacental transfer among littermates. Although not statistically significant ($p = 0.07$), total AF IgG levels were slightly higher in litters from consistently vaccinated mothers.

Regarding specific antibodies, CAdV-1–specific IgG levels were significantly higher ($p = 0.01$) in the AF of regularly vaccinated dams, while CPV-2–specific IgGs were elevated ($p = 0.003$) in the AF of high-titre bitches. CAdV-1 and CDV antibodies followed a similar, though nonsignificant, pattern. These observations may indicate distinct placental transport efficiencies or antibody half-lives for different viral antigens following immunization [7, 11, 48, 59].

AF appeared to provide valuable insight into neonatal immune competence, potentially serving as a diagnostic indicator for assessing early immune protection. Notably, puppies that later developed gastrointestinal symptoms within two months exhibited lower CAdV-1–specific IgG titres than healthy controls ($p = 0.04$).

In our earlier investigation, we identified several biochemical components—lecithin, sphingomyelin, cortisol, SP-A, and PTX3—in canine AF [27]. Some of these molecules demonstrated associations with both total and specific IgG concentrations and neonatal clinical variables.

A positive correlation between AF cortisol and CDV-specific IgG was observed, though its biological significance remains unclear. The relationship may align with the concept that moderate, transient stress (leading to elevated cortisol) enhances immune function and IgG production [60], whereas prolonged stress negatively impacts neonatal immunity [61].

We also detected an inverse association between AF sphingomyelin and total IgG, possibly reflecting sphingomyelin's modulatory effect on CD1d-mediated antigen presentation to T and NK cells [62]. The negative correlation between PTX3 and anti-CAdV-1 titres likewise requires further clarification.

The influence of maternal age on AF constituents such as SP-A, lecithin, and sphingomyelin has been infrequently explored, even in human studies. In women, ageing has been linked with decreased SP-A and inconsistent trends for blood lecithin levels—patterns opposite to those observed in dogs [63, 64]. Therefore, additional research is needed to elucidate the physiological and clinical relevance of these findings in canine reproduction.

A marked elevation in the concentrations of lecithin ($p = 0.0003$), sphingomyelin ($p = 0.0008$), and cortisol ($p = 0.01$) was observed in the amniotic fluid (AF) of puppies showing pathological signs compared with healthy littermates. Previous studies in both humans [65] and dogs [27] have described a positive association among these three AF components. It can be hypothesized that increased amniotic cortisol, indicative of fetal distress, may predispose neonates to disorders such as diarrhea, aligning with the fetal programming hypothesis, which links intrauterine events with later-life diseases [66]. Maternal stress is also known to elevate neonatal vulnerability to illness [67]. Furthermore, insufficiency in surfactant constituents has been implicated in respiratory and renal dysfunction, with SP-A serving as a recognized biomarker for disease in humans [68]. Consistent with this, the pathological group in our study displayed lower SP-A concentrations than the healthy group ($p = 0.005$). Nevertheless, since the precise causes of these disorders were undetermined and factors like maternal and neonatal nutrition can influence diarrheal onset, more comprehensive studies are needed to clarify these mechanisms.

The possible effects of breed differences and gestational age on AF composition variations in dogs should also be further explored. Studies in women of diverse ethnic origins demonstrate distinct amniotic immunomodulatory profiles [69, 70], suggesting that comparable variability could exist in dogs. Such differences may impact neonatal immunity, as certain canine breeds are genetically more susceptible to infectious conditions [71]. Moreover, a recent investigation revealed changes in AF composition throughout gestation in both dogs and cats [72].

Genetic and breed-related effects on cortisol concentrations have been reported in various livestock species [73]. The negative correlation between amniotic cortisol and both maternal weight and litter size found in our results

could be related to this phenomenon, given that larger breeds tend to be heavier and produce bigger litters compared with smaller breeds.

Another noteworthy finding was the positive relationship between maternal body weight and CPV-2-specific IgG titres in AF. Body size is recognized as a factor influencing immune responsiveness, with larger dogs often showing reduced vaccine efficacy due to a greater amount of subcutaneous fat at injection sites, which may trap vaccine antigens and reduce immune activation [74, 75]. However, two recent studies by Dall'Ara *et al*. reported that larger dogs exhibited higher CPV-2 antibody titres than medium or small breeds. This stronger response may result from increased environmental exposure, as CPV-2 can persist for long periods outside the host. Because larger dogs are typically more active and spend more time outdoors, they may have a greater likelihood of encountering the virus [76].

These observations support the potential of amniotic fluid as a diagnostic medium for evaluating neonatal immunity and overall health in dogs. They also underline the necessity for expanded research to determine how AF assessment can aid in breeding management and veterinary preventive care.

Conclusion

Collecting amniotic fluid (AF) at birth offers a non-invasive and reliable method to evaluate neonatal immune status and provides valuable information on the maternal antibody (MDA) transfer process in dogs. In the future, this approach could serve as an early indicator of health risks in newborn puppies. Further investigation into breed- and gestation-related differences in AF composition may improve our understanding of neonatal immune development and its influence on puppy health outcomes.

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