Journal of Proteomics 218 (2020) 103724

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Review

Some more about dogs: Proteomics of neglected biological fluids

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ARTICLE INFO

Keywords:
Amniotic fluid
Allantoic fluid
Seminal fluid
Saliva
Tears
Proteomics

ABSTRACT

We report in this manuscript what is known about the protein makeup of a selection of biological fluids in the domestic dog. The samples we review – amniotic and allantoic fluid, seminal fluid, saliva, bile, synovial fluid, tears – are still very poorly characterized in this species. For some of them we can present results from our own, mainly unpublished experiments.

Significance: The dog is one of the most widespread companion animals, and also of medical relevance as model species for some human diseases. Still, investigation of body fluids other than serum and urine is not so commonly undertaken, although – like in humans - also these sample types may have potential for diagnostic purposes. We compile published data about proteomes of fetal fluids, seminal plasma, saliva, bile, synovial fluid and tears, enriched by some yet unpublished data of our own (proteins of amniotic and allantoic fluid, tears). Closing gaps in our knowledge on dog proteins will further our understanding of (patho)physiological processes.

1. Introduction

Back in 2014, we first surveyed proteomic findings on dog biological fluids [1]. There are some obvious reasons for us to stick to this topic. The domestic dog (*Canis lupus familiaris* when considered a subspecies of the wolf or *Canis familiaris* when considered a distinct species) is the most widely spread terrestrial carnivore. In 2017, the year for which the latest figures are available (https://www.statista.com/statistics/), about 89.7 million dogs lived in households in the United States as pets - 11.5 millions, ca. 15%, more than at the time of our previous writing. The figures for the 10 'countries with the most dogs worldwide' total approx. 210 million animals (https://www.worldatlas.com/articles/). These impressive data emphasize the social relevance of the companionship provided by this animal species to our own. In PubMed, search string « dog OR canine » retrieves 362,184 entries while search string « (dog OR canine) AND (proteom* OR 2-DE - and synonyms - OR LC-MS) » yields 1411 entries; in UniProt, search term « dog » returns 1222 reviewed and 100,532 unreviewed entries. These data hint to a wide knowledgebase, from which information may be retrieved on a number of matters.

Accordingly, the present thematical issue in *Journal of Proteomics* could be the occasion for an update on the recent findings on plasma/serum and urine, definitely the most extensively investigated among the biological fluids, in this and in all animal species as well as in humans; or we could shift our attention to usually neglected biological samples. We took the second option, and in the following we are going to review literature data on a number of specimens that include amniotic and seminal fluid from the reproductive organs, saliva and bile from the digestive system, synovial fluid from the joints, and tears/tear film from the eyes. For some of these hardly characterized specimens we are also going to present some results from our own, mainly unpublished experiments.

2. An inventory of dog biological samples

2.1. Amniotic fluid and allantoic/allantoid fluid

Safe and healthy reproduction is the single step in dog's life on which breeders must concentrate most of their care. Out of this evidence one could expect that the reach of scientific investigation would match the economic relevance of the subject. However, this hardly seems to be the case.

Abbreviations: MCA, major canine allergen protein; NGAL, neutrophil gelatinase-associated lipocalin; SF, synovial fluid

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https://doi.org/10.1016/j.jprot.2020.103724
Received 30 January 2020; Accepted 28 February 2020
Available online 29 February 2020

Let's first recall the nature and variety of the biological specimens connected with pregnancy. The fetal membranes of amniotes are allantois, amnion, chorion and yolk sac; each of them encloses a liquid-filled cavity. Both development and contribution to placentation of the membranes vary greatly across mammals [2]. *Carnivora*, including *Canis familiaris*, are classified as *Laurasiatheria*. They have a zonary placenta, completely surrounding the fetus, and complex lamellar organization of maternal and fetal tissues. The amnion develops as folds of the extraembryonic somatopleure that meet and fuse dorsal to the embryo; it is avascular in early stages, but becomes vascularized by blood vessels of the internal allantoic membrane in later stages of pregnancy. The allantoic sac is large, filled with a clear yellow liquid, and surrounds almost completely the embryo; its central part forms the placental contacts of the endometrium. The yolk sac corresponds to a visceral pouch, with an extremely high number of blood vessels; it contains hemangioblasts that can differentiate into both hematopoietic and endothelial cells. In dogs, choriovitelline placenta features a temporary structure [2–4].

At a variant, in human development, there is an allantoic stalk, but no allantoic cavity; the yolk sac is a short-lived structure; and the exocoelom is obliterated by the end of the second trimester. In the mouse, there is no allantoic cavity; in contrast, the inverted visceral yolk sac supports the embryo until the chorioallantoic placenta is formed (around embryonic day 10) and acts as an accessory placenta through term [2]. All of these differences largely prevent the extension to dogs of knowledge gathered either on our own species or on mice as a model system.

Proteomic data about amniotic and allantoic fluid have been published for farm animals – samples were from pregnant cows [5–7], mares [8,9], sheep [10] and from fertilized hen's eggs [11,12] – but also for another pet, the cat [13]. Some of these reports analyze the composition of the fluids as a function of pregnancy/incubation time [5,12,13], one deals with the influence of assisted reproductive technologies [5] while the main pathological issues transversally addressed in all other investigations are bacterial infection and/or passive immunity from the mother to the conceptus.

No similar proteomic reports exist for dogs, but the concentration in the amniotic and allantoic fluids at term has been evaluated for a number of low [14–18] and high molecular weight substances. Among proteins, insulin-like growth factor-I [14], class G immunoglobulins [19], alkaline phosphatase, aspartate aminotransferase, creatine kinase, gamma-glutamyl transferase, lactate dehydrogenase and lipase [16] show significant differences in concentration between amniotic and allantoic fluids, on the background of a wide variability within and among litters. Variation is also noticed in creatinine, urea, bilirubin, total protein and globulin [16]. None of the above proteins could be recognized as marker of a pathological condition. Conversely, some indications seem to come from metabolites: for instance, the concentration of glucose in the amniotic fluid inversely correlates with the risk of puppies' death immediately after birth [17]. In turn, the concentration of glucose (as well as of β-hydroxybutyrate and of non-esterified fatty acids) in the fetal fluids directly correlates with that in the maternal circulation. Overall, the extent of the metabolic load of pregnancy in bitches depends on breed size and on the ratio of litter weight to dam's body weight [18].

Amniotic fluid is initially formed from maternal plasma passing through the fetal membranes. As the fetus develops, more liquid is produced by flow from fetal lungs and bladder, and is reabsorbed partly by fetal swallowing partly by transfer across the amnion to the fetal circulation. The composition of the fluid is dynamic; before skin keratinization (in humans, between week 10 and week 20 of gestation) it is similar to that of fetal plasma [20]. While – understandably – no data are available for human fetal plasma/serum proteome and only one publication deals with newborn specimens [21], some more has been done in rodents: the composition of fetal serum was compared to that in newborn rat by Wei et al. [22], while, back in 1998, we compared newborn to adult animals [23].

This scattered setting is the background to our recent pilot work on dog fetal fluids. Fig. 1 shows a 2-DE DIGE experiment in which amniotic fluid and allantoic fluid from a healthy puppy and serum from its dam at the time of giving birth are run together, after individual labeling with different Cy dyes; the overlay of the 3 channels is shown in the first panel of the figure, the individual channels are displayed in the following, after converting color to gray levels. As it may be expected from the data on human and rat samples, there are both similarities and differences between serum and fetal fluids. The largest spot seen in the 2-DE map is that of albumin; its color approaching white implies that its load is balanced among the three types of samples. Conversely, three main spot rows stain red as a result of a noticeably higher concentration in serum than in either fetal fluid. From their p*I*-M_r data and by comparison with the map in [1], the ones with lowest and highest size may be identified as haptoglobin alpha and beta chain, respectively, the intermediate one as apolipoprotein A-I. The concentration of immunoglobulins is also higher in serum than in the fetal fluids. A spot row (marked *), in blueish as a result of a higher concentration of the associated proteins in both fetal fluids, was identified by MS as a mixture of alpha-fetoprotein and alpha-1-antitrypsin (data in Supplemental Table 1). Finally, a protein highly heterogeneous both in charge and in size, in green, is more concentrated in allantoid fluid; no easy identification may be guessed for this peculiar protein, which prompted further investigation. Fig. 2 shows in the top panel the pattern of a DIGE experiment in which amniotic and allantoic fluids are run together, with a different labeling vs Fig. 1: in this case, the unknown protein, ca. 25 kDa in size, is displayed in red. The middle panel shows the pattern of the same allantoic fluid sample after silver impregnation: the protein of interest, though, fails to stain in healthy animals. The unknown protein, however, turned out to be especially abundant in an allantoic fluid sample from a diseased puppy (a puppy with anasarca, a rare, but severe and generalized form of edema, with subcutaneous tissue swelling throughout the body): its pattern is shown in the bottom panel. Analysis of the material in the most prominent spots lead to the identification (Supplemental Table 2) of the attending protein as lipocalin 2 (https://www.uniprot.org/uniprot/E2RSM4), a close homolog, with 68% identity, to human neutrophil gelatinase-associated lipocalin, or NGAL in short (https://www.uniprot.org/uniprot/P80188).

In the samples we analyzed by 2-DE DIGE, the NGAL spot chain appeared in both types of fetal samples and was usually present in higher concentrations in anasarca animals. Measurement by ELISA of NGAL concentrations in a selection of amniotic fluids (overall from 18 healthy and 4 anasarca puppies of three different breeds) confirmed these findings, with, however, large individual differences and a possible influence of breed (Supplemental Fig. 1).

In previous proteomic reports using the 2-D electrophoretic approach, this protein has been detected as a single, faint alkaline spot in human urine [24] and bronchoalveolar lavage fluid [25]. Conversely, NGAL has been identified in a train of prominent spots in the 2-DE maps of ^{35}[S]-labeled proteins secreted from rat hepatocytes [26]. Comparing panels of Fig. 1 in the latter report, apolipoprotein A-I, with p*I* and M_r similar to NGAL, gives rise to spots of comparable intensity when the serum pattern is stained with SYPRO-Ruby (panel A) and when the hepatocyte supernatant is phospho-imaged (panel D). Conversely, NGAL spots may be observed only in the latter conditions, an evidence that seems to fit with our failure to detect the protein after electrophoretic fractionation and standard staining procedures.

As known for humans, NGAL is found at high levels in bone marrow and in tissues prone to exposure to microorganisms (including uterus). The protein has a strong affinity for iron complexes, thus depriving bacteria of a vital nutrient; through its bacteriostatic effect NGAL contributes to innate immunity. NGAL is released from neutrophil granules and thought to be an acute phase protein. The factors controlling NGAL expression range from pro-inflammatory cytokines to growth factors. Elevated NGAL levels have been observed in different

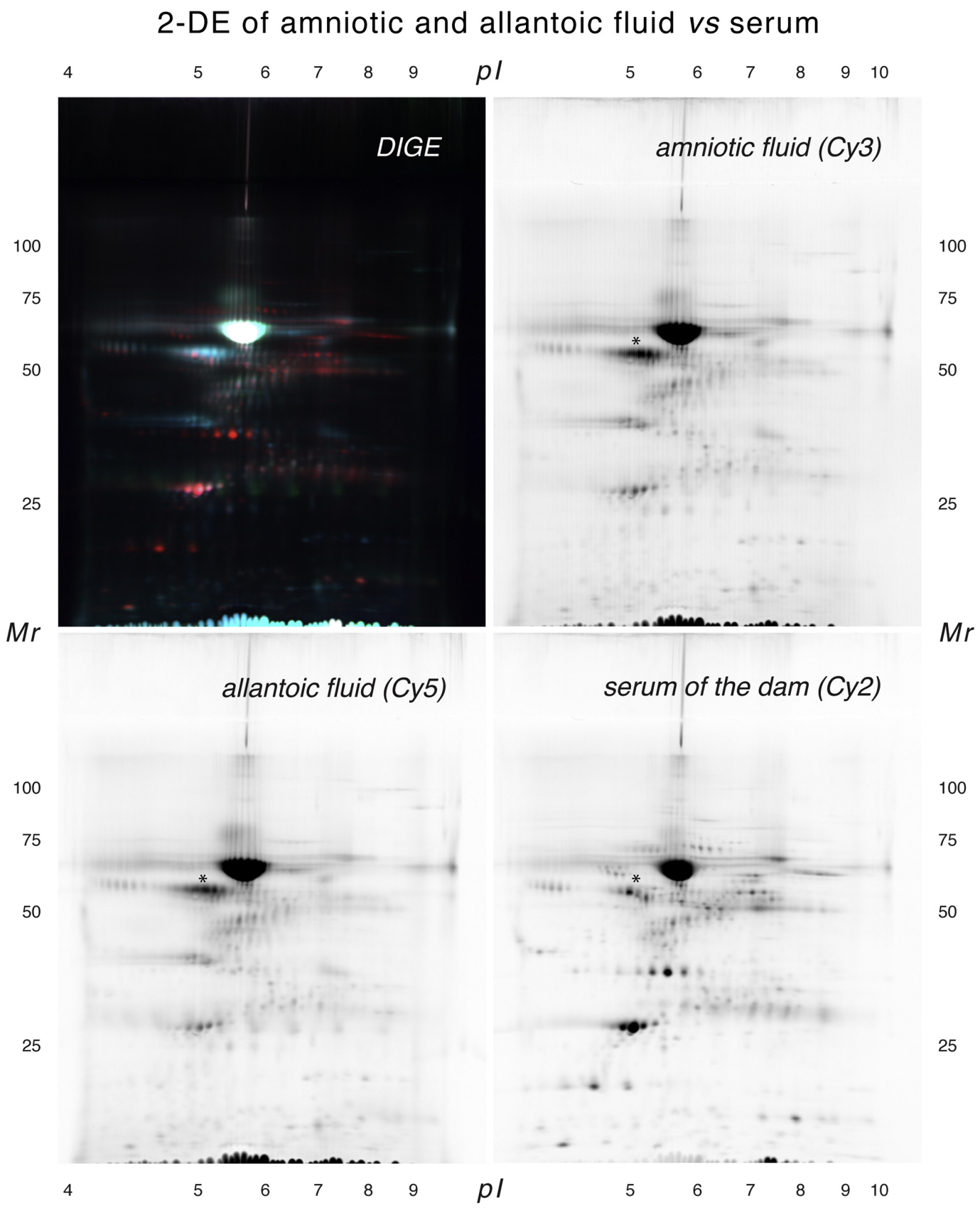

Fig. 1. 2-DE of amniotic and allantoic fluid vs serum.
2-DE DIGE gel of amniotic and allantoic fluid from a puppy and of the serum from its dam (breed: Maremma sheepdog). The individual patterns are shown, as rendered in gray scale, in separate panels (marked with the name of the fluid and the fluorescent dye used for protein prelabeling), while their overlay is shown in the top left panel (marked DIGE), in false colors: serum, red; allantoic fluid, green; amniotic fluid, blue. Experimental details: IPG-DALT was run with pH 4–10 NL in 1std and 10–15% T PAA in 2ndd [38], the MS results for the spot marked with asterisk are presented in Supplemental Table 1. Our unpublished results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

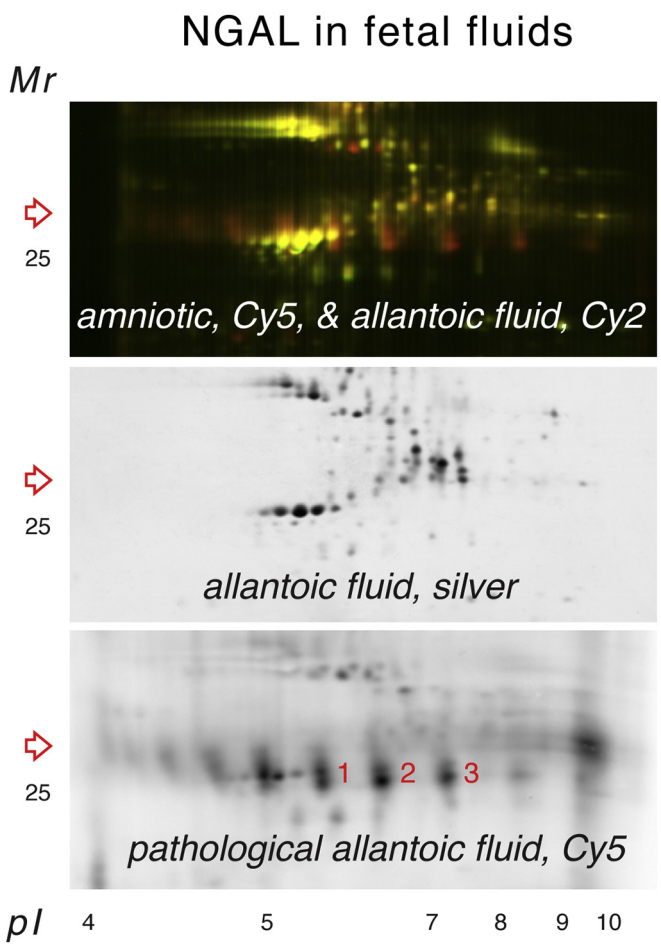

Fig. 2. NGAL in fetal fluids.
Close-up on the low $M_r$region in the 2-DE map of allantoic fluid from a healthy (top and middle panel; a Maremma sheepdog) and a diseased puppy (bottom panel; the puppy, an English bulldog, had anasarca), run alone (middle and bottom panel) or together with amniotic fluid (top panel). Staining was by fluorescent prelabeling (top and bottom panel: amniotic fluid, green; allantoic fluid, red) or with silver (middle panel). Experimental details: as inFig. 1[38]. Spots 1–3 in the bottom panel were excised for protein identification; the results are presented in Supplemental Table 2. Our unpublished results.

human carcinomas [27–30].

The protein has been investigated in dogs as well as in humans [31] and in other animal species, e.g., [32] as a marker of renal disease; the most recent report on dogs is by Kim et al. [33]. The involvement of NGAL in the complications of pregnancy is connected partly with either subclinical or overt kidney injury in intrauterine growth restriction [34] and pre-eclampsia [35,36], partly with infections in pregnancies complicated by preterm birth [37].

2.2. Seminal fluid, or seminal plasma

Seminal plasma collects the secretions produced by testicles, epididymis and prostate, the only accessory sex gland in dogs that contributes with its fluid approx. 95% of the ejaculate [39]. Contrary to pregnancy-related fluids, seminal plasma has long been investigated in dogs. A ground-breaking investigation was devoted in 1983 [40] to soluble (cytosolic) proteins extracted from slices of dog prostate after metabolic labeling with [^{3}H]leucine or [^{35}S]methionine. The authors had to resort to two types of 2-DE set-ups, with isoelectric focusing and non-equilibrium pH gel electrophoresis (NEpHGE) in the 1std, in order to resolve both acidic and basic sample components. Due to the limited amount of material being involved, only a handful of spots could be resolved and, because of technical limitations, no identification could be attempted. No protein was synthesized by the tissues of castrated animals but treatment with 5 alpha-androstane-3 alpha-17 beta-diol (10 mg/day for 2 weeks) restored the pattern of protein synthesis to that of intact animals. Among other early achievements on the topic, both a review on proteins secreted by dog prostate [41] and a comparison by electrophoresis among soluble proteins in seminal fluid from various animal species [42] were published back in 1985.

Table 1
Proteins of dog seminal plasma (updated from [39]).

Protein	Reference
Acid phosphatase	[43]
Alkaline phosphatase	[43,44]
Arginine esterase (canine prostatic specific esterase CPSE, *zinc-binding proteins*)	[45–50]
Catalase	[51]
Glutathione peroxidase	[52]
Heparin-binding proteins	[53]
Lactoferrin	[54]
Matrix metalloproteinases (−2 and −9)	[55,56]
Osteopontin	[57]
Superoxide dismutase	[52]

Individual proteins, or protein groups, have been purified and/or characterized in seminal fluid; a list is provided in Table 1, expanded and updated from the compendium by Aquino-Cortez et al. [39].

Few reports on the contrary have addressed the composition of the fluid as a whole. Protein fractionation by size in two SDS-PAGE set-ups, with 13 and 22%T PAA, yielded a total of 37 bands, 19 and 18 in the low and high %T gels and with a protein load of 80 and 16 µg, respectively. In the 13% gel, molecular weights ranged from ca.100 to ca. 17 kDa, with 4 bands present in all dogs. In the 22% gel, molecular weights ranged from ca. 16 to ca. 4 kDa, with 9 bands present in all dogs. Considering both gels, the majority of proteins (85%) had molecular weights below 17 kDa, with the band at ca. 6 kDa being the most prominent in all dogs. Of these components, two (ca. 67 and ca. 59 kDa) appeared to significantly ($P \leq .01$) correlate with the properties of the semen, e.g., sperm mobility (with r ranging from 0.46 to 0.76). At the time of paper preparation, 2007, no attempt to arrive at the identification of either the common or of the differential proteins was made by de Souza et al. [58].

Similar protein patterns of unfractionated seminal plasma were obtained at that time in our own studies [59] (Fig. 3). The main protein with a non-reduced M_r of about 30 kDa split into several bands upon reducing treatment (one intense band at 17 kDa and multiple slightly less prominent ones at 12–14 kDa). This protein, at that time named PSP (prostate specific protein), was also found as the main protein in urine of entire (non-castrated) male dogs and proved age- and health-dependent, correlating well with the health status of the prostate [59].

Extensive identification of seminal plasma proteins was instead obtained years later by Aquino-Cortez et al. in the so far most detailed proteomic investigation on this specimen. Two fractions were separately collected from the ejaculate of each dog, a sperm-rich fraction and a prostatic fraction, while the pre-sperm fraction was discarded. After centrifugation, 30 µg of protein were run per lane in SDS-PAGE with an 8–16%T PAA gradient. In keeping with the lower sample load, a lower number of bands could be detected by Coomassie stain vs the above report: 7–9 bands in the sperm-rich fraction, 4–6 bands in the prostatic fraction. Analysis by tandem MS on 6 gel slices identified a total of 268 proteins; 251 were common to both fractions; a large share of the hits (133 among the common and 13 among the differential proteins) were in fact 'predicted' proteins (https://www.uniprot.org/help/protein_existence). Some of these proteins had already been described in the seminal plasma of other species. Arginine esterase and lactotransferrin were the most abundant among the common proteins, UPF0764 protein C16orf89 homolog and epididymal-specific lipocalin-

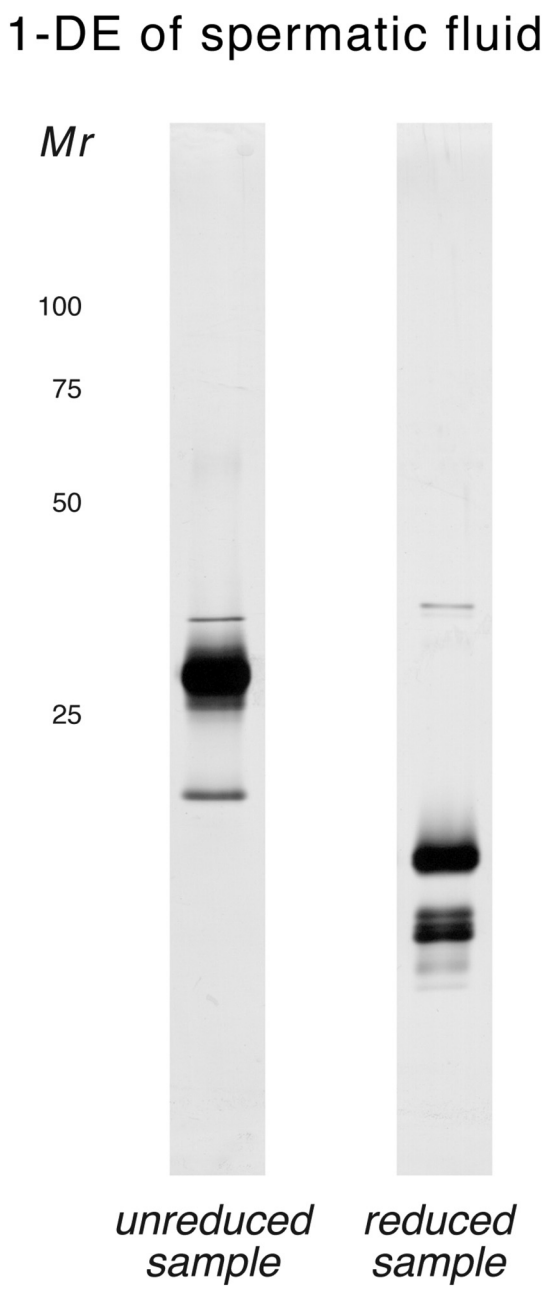

Fig. 3. Spermatic fluid.
1-DE pattern of spermatic fluid from an entire male dog, run both under non-reducing and reducing conditions. Experimental details: separation in SDS-PAGE, with or without adding DTT to the sample and short-time boiling, on a slab with a 10–20% PAA gradient in the upper half and a homogenous 20% PAA gel in the lower half, as in [61]; staining with silver.

9 the most abundant among the components specific to the sperm-rich fraction [60].

While the above investigations were based on 1-DE set-ups, 2-DE was run on a fraction purified by affinity procedures. Zinc-binding proteins from metal chelate chromatography were run in the 1std both by native gel electrophoresis [49] and by denaturing IEF [50], followed by SDS-PAGE in the 2ndd, to demonstrate aggregation into high M_r complexes on the one side and heterogeneity of the components (approx. 50 protein species per sample) on the other. MS analysis on the major (monomeric) spots identified all of them as arginine esterase (EC:3.4.21.35). The catalytic properties of the enzyme, as listed under UniProt entry P09582, ESTA_CANLF, involve the preferential cleavage of Arg-|-Xaa bonds in small molecule substrates, with high selectivity in releasing kallidin (lysyl-bradykinin) from kininogen; kallidin can then be converted to bradykinin by aminopeptidase clipping. However, its exact physiological function in dog seminal plasma is not defined. In veterinary literature this protein is known as canine prostatic specific esterase (CPSE). In a way similar to prostate-specific antigen (PSA) in human medicine, CPSE has been identified as a suitable biomarker for several conditions; a higher concentration was indeed reported in dogs suffering from several prostatic diseases, such as prostatic hyperplasia, bacterial prostatitis, or prostatic carcinoma [62]. Thus, CPSE seems to be identical to the above described main protein PSP in male dogs' urine [59].

In post-vasectomy samples, bands around 43 and 29 kDa cannot be observed any longer; the relative abundance of band ca. 73 kDa decreases, that of bands ca. 8 and ca. 4 kDa increase [63].

Some proteins in biological fluids of dogs cause allergy in humans; among them, Can f 5 corresponds to prostatic kallikrein, and as such it is present in dog urine and eventually in dog dander (skin flakes shed from the body of the animals) [64]. Cross-reactivity between Can f 5 and PSA is the root cause for allergy to human seminal fluid in patients with allergy to male dogs with sensitization to Can f 5 [65–67]. Can f 5 is bound by IgE antibodies from 70% of subjects with dog allergy.

2.3. Saliva

Saliva is a mixture of secretions from various major and minor salivary glands. Its main functions are maintenance of homeostasis in the oral cavity, protection against pathogens and the first steps in food digestion [68]. Apart from inorganic substances, different cells (blood cells, epithelial cells) and microorganisms, the fluid contains a variety of proteins and peptides including immunoglobulins, enzymes and cytokines, some of them deriving from serum or nasal-bronchial secretions [69]. The composition of saliva is variable, reflecting nutritional status and (patho)physiological state of the individual. As it can be collected in a non-invasive and comparatively stress-free way, it has great potential as a diagnostic sample and has already been employed in diagnostic tests for cancer as well as oral and systemic diseases, esp. in human medicine [70,71].

Like in other fields, investigations in animals lag behind and collection of proteomic data on saliva started later than in humans. However, by applying LC-MS/MS, distinct differences between salivary proteome compositions in various species have been shown, depending on their type of feed/nutrition [72]. One of the species selected for this investigation was dog, as representative of the carnivores. In its saliva 2555 proteins were identified in homology searches (compared to 571 (horse, lowest number) and 3128 (rat, highest number of hits)), but these numbers may reflect also the quality of the databases. Still, a remarkably large share of them were reported being metabolic proteins. An in-depth canine salivary protein catalogue has been established by nanoscale liquid chromatography-tandem mass spectrometry from healthy dogs without evidence of periodontal disease (N = 36) [73]. Dogs represented 4 breed phylogeny groups, based upon single nucleotide polymorphism haplotypes (ancient, herding/sighthound, and two miscellaneous groups). This resulted in 2491 proteins and endogenous peptides, and all breeds showed a high degree of proteins and peptides with antimicrobial functions. However, the ancient breeds group was distinct in that it contained unique proteins and was missing many proteins and peptides present in the other groups [73]. Also another study, comparing dog and human salivary proteomes, reported for dogs higher levels of proteins related to regeneration and healing processes such as fibroblast growth factor and epidermal growth factor [74]. This study has been performed in Thai village dogs, thus there might be an influence of living conditions or some breed-specific differences. Influence of breed was investigated by Pasha et al. [75], comparing Labrador retrievers and Beagles (8 animals per group, of different sex), and by Lucena et al. [76], for 4 different purebred dogs

(Portuguese Podengo, Greyhound, Rafeiro Alentejano, and Beagle). Proteomic methods applied differ: LC-MS/MS on an LTQ-Orbitrap following TMT labeling quantified up to 60 proteins, detecting higher variation between species than within animal groups [75] and noting a much lower alpha-amylase level than in human saliva (influencing onset of food digestion). The second study was performed on dog saliva subjected to 2-DE with CBB-G250 stain [76]. Seven protein spots were listed to differ, but only a few could be identified by MALDI-TOF/TOF. PLS-DA was able to cluster the 4 breeds, with the largest differences between Portuguese Podengo and Beagle. The method used in the latter manuscript has been set up in a previous study and comprised TCA-precipitation of saliva, allowing detection of 83 spots in the CBB-stained gel, i.e. more spots than with the other protocols tested by the authors [77].

There are different ways to collect saliva and the method to obtain this sample may alter both, protein concentration and fluid composition, as well as the contribution of the different glands (collection exemplified for humans in [78]). Two options used in dogs were tested by [76], namely collection of unstimulated saliva or stimulation with lemon juice. Without stimulation, protein concentration was roughly twice as high, and nine spots differed in the 2-DE profile depending on the collection method (thereof 4 belonging to the immunoglobulin family).

Recently, proteomic investigations in canine saliva have switched to the study of different diseases, taking advantage of the easy sample collection and the possibility to collect specimens frequently and at short intervals. This includes for instance both systemic viral (parvovirus [79]) and parasitic infections (experimental leishmaniosis [80]), all these studied by the same investigator groups and using TMT-analysis. In the first case, natural parvovirus infection brought about 190 proteins with differential abundance, whereof 90 differed between survival and control groups. Specifically, 10 proteins seemed to have an influence on survival of the animals (ezrin, allergen Dog 1, lactoperoxidase, L-lactate dehydrogenase A, cystatin-M and macrophage-capping protein, alpha-actinin-1, peptidyl-prolyl cis-trans isomerase A, ribosyl dihydronicotinamide dehydrogenase, lactoylglutathione lyase). Antimicrobial humoral immune response was found as a pathway playing a major role [79]. Experimental leishmaniosis, caused by the protozoan *Leishmania infantum*, can be fatal for dogs and humans and is not easy to diagnose, as antibody titer and clinical signs need not correspond. Differential comparison of animals before and after infection (6–8 months post-infection) revealed 47 proteins with differing representation, and the created interactome network displayed an important role of carbohydrate metabolic processes [80]. A clearly metabolic disease is the so-called obesity-related metabolic dysfunction (ORMD), which is similar to the human metabolic syndrome (MetS). A study including two sets of dogs, both with control and ORMD-affected animals, set out to further explore the syndrome and find potentially useful diagnostic markers. Nine proteins were of potential interest, as differentially abundant, involved in glycolysis, immune reaction and oxidative stress. More detailed investigations seem advisable, as all dogs involved in the study were of different breed, with authors claiming that this corresponds to the situation in a veterinary clinic [81].

2.4. Bile

Bile is produced by the liver to then be drained through biliary ducts into gallbladder and from there, after cholecystokinin stimulation, into the duodenum [82]. Its major functions are in promoting the digestion of alimentary fats with the amphipathic bile acids it contains, and in providing an excretory pathway for many endogenous and exogenous compounds. Bile is a challenging biological sample in its modes of both collection and analysis [82]. Due to the deep anatomical location of gallbladder, it must be collected by endoscopic (i.e. retrograde cholangiopancreatography) or surgical (i.e. cholecystectomy) procedures; in humans, this implies that virtually all specimens are obtained from diseased individuals, which makes it hard to define a reference composition. The solutes in bile are ca. 60% biliary acids, 3% phospholipids, 9% unesterified cholesterol, 3% bilirubin, 5% inorganic ions vs up to 7% proteins [83]. The many non-protein components strongly interfere with protein fractionation and identification procedures, and need to be removed as extensively as possible by physical means before protein analysis is attempted; this includes sequential flotation by ultracentifugation and fractionation either by size (through gel filtration) or by differential solubility (through extraction of non-protein components or through protein precipitation).

As early as in 1960, bile proteins were characterized by paper electrophoresis in control and pathological human samples, as well as in animal specimens, including dogs [84]. For humans the identification, and quantitation, of the main bile protein components was then readily possible using immunological reagents directed against normal plasma components (by such techniques as crossed immunoelectrophoresis, rocket immunoelectrophoresis and radioimmunoassay) [85]. The ratio between concentration in bile and concentration in blood was found for most proteins to be inversely proportional to the molecular size, hinting to a passive diffusion mechanism letting molecules pass through the tight junctions between hepatocytes or bile duct cells. For a few proteins, however, this relation does not hold and both some small proteins, namely thyroxine binding globulin, GC globulin and alpha 2HS-glycoprotein, and some large ones, namely secretory IgA, IgM, hemoglobin and ceruloplasmin, are found in bile at higher than expected concentrations [86]. The cross-reactivity between human and dog proteins is high enough for the latter to be effectively bound by antibodies produced after immunization of a foreign species with their human homologs: as a result, in our paper about the proteome of dog serum, as many as 12 out of 27 proteins were identified via immunoblot together with/instead of via MS [1]. No immunological reagent, however, was exploited during early investigations on animal bile, including dog's, and no short list of components could thus be provided for this species [87]. The discrepancy between investigations on human and non-human samples continues to date. Dog is used in preclinical studies but not as an animal model for liver disease; collection during medical procedures on diseased subjects is seldom performed due to the already mentioned difficulties in sample procurement. Possibly for these reasons, also in recent years the study of dog specimens lags behind; specifically, no systematic proteomic investigation was carried out on bile. Conversely, for humans, a review published in 2009 [88] already could attempt a consensus between available literature, as provided by four large proteomic analyses. According to this survey, including single-hit identifications, a total of 283 proteins may be found in human bile (44% of which common to two or more studies). Rather than aiming at extending the list of the identified components, more recent papers deal with technical improvements in the procedures [89,90]. At a difference from proteomics, metabolomics has been used to characterize the low molecular weight substances in dog bile. Reversed-phase gradient UPLC-ESI-MS, in both positive and negative ionization modes, has been applied to the analysis of untreated control bile obtained from cannulated dogs, with thousands of ions resolved and quite a few compounds identified [91]. A recent publication dealt with a pathological condition, mucocele, characterized by secretion of abnormally thick mucus by the gallbladder epithelium and resulting in bile duct obstruction or gallbladder rupture [92]; the abnormalities in bile composition could be tracked to likely pathogenic mechanisms.

2.5. Synovial fluid

Synovial fluid (SF) is a viscous fluid found in many joint cavities; its main function is lubricating the articular surfaces. Besides decreasing friction during movements, it transports nutrients and waste. SF can be regarded as an ultrafiltrate from plasma additionally containing

proteins produced by the cells of the joint tissues and of the cartilage matrix (e.g., hyaluronan, lubricin). The permeability of the synovial membrane increases in disease, leading to a change in protein composition; locally produced constituents may give rise to further compositional changes depending on the condition. These features make it an ideal target for studies on joint diseases, in search for biomarker candidates aiding diagnosis. Quite a number of recent reviews summarize the findings accumulated about human joint diseases, like osteoarthritis, through proteomics or other omic methods [93]. Most of these investigations start from SF, often after hyaluronidase treatment to decrease its viscosity, sometimes also including a depletion step to remove the high-abundance proteins albumin and IgG (see, for instance, later in [94], for canine samples).

For dogs, there is only a small number of studies on SF that deserve the attribute *proteomic*, although earlier studies investigating single proteins or protein families exist (for instance, on serum amyloid A (SAA) isoforms [95], different metalloproteinases [96,97], glycosaminoglycans (GAG) [98]). Among them is one investigation with broader scope, employing immunoelectrophoresis to quantify immunoglobulins and several acute phase proteins in SF of dogs with degenerative joint disease [99].

Our literature search resulted in two studies applying 2-DE. The first was a hip-joint model for coxofemoral joint osteoarthritis [100]. It identified seven main synovial proteins and presented their location in a 2-DE map with 1std in a pH 4–7 range; among them, vitamin D-binding protein and two spots of kininogen 1 increased in intensity during a follow-up period of 14 weeks after surgery. The second is a case study on SF from a dog diagnosed with idiopathic immune-mediated polyarthritis, causing lameness in the animal [101]. Compared to healthy dogs, the sample showed increased concentrations of immunoglobulins, complement C4b-binding protein alpha chain, actins and keratin type II. Unfortunately, the report gives no gel images.

A GeLC-MS/MS study on dogs with stifle joint arthritis compared to the same animals 8–12 weeks after surgery or to healthy controls failed to detect any difference among serum samples (overall 68 nonredundant proteins identified). For SF, a list of 76 nonredundant proteins was established. Concentrations of eleven proteins (complement component 3 precursor, complement factor I precursor, apolipoprotein B-100 precursor, serum paraoxonase and arylesterase 1, zinc-alpha-2-glycoprotein precursor, serum amyloid A, transthyretin precursor, retinol-binding protein 4 precursor, alpha-2-macroglobulin precursor, angiotensinogen precursor, and fibronectin 1 isoform 1 preproprotein) differed by at least a factor of 2 in comparison with the control group. Findings suggested an important role of the complement system and of proteins involved in lipid and cholesterol metabolism (besides acute phase reaction) [94]. A similar study was undertaken in [102], but using a less sensitive approach by MALDI-TOF/TOF for protein identification following SDS-PAGE. Authors focused on their main finding, increase of apolipoprotein A-I, whose upregulation they validated by ELISA; they propagated this protein as potential marker candidate for this type of disease.

Only two papers, already published in 1999 and from the same group, deal with metabolomic investigations in SF. The first one studied the SF metabolome of denervated knee fluid by NMR [103]. Glycerol, hydroxybutyrate, glutamine/glutamate, creatinine/creatine, acetate and *N*-acetyl-glycoprotein concentrations were statistically significantly increased. These findings, together with significant trends towards elevated lactate, alanine and pyruvate levels in the denervated knee fluids, were consistent with NMR spectroscopy metabolic profiles of normal and osteoarthritic canine synovial fluids [104]. The latter study showed, in addition, reduced levels of glucose, and elevated levels of *N*-acetyl-glycoproteins, acetate, and acetamide as well as higher alanine and isoleucine concentrations in SF of arthritic dogs. Authors draw the conclusions that the intraarticular environment in canine osteoarthritis may be more hypoxic and acidotic than in a normal joint, lipolysis is an important source of energy, and fragmentation/degradation of the *N*-acetyl-glycoprotein polymer component of synovial fluid (mostly hyaluronan) appears to increase in progressive osteoarthritis.

2.6. Tears and tear film

Tears cover the epithelial surface of the eye as an extracellular fluid; they form a film, consisting of an outer lipid layer, a middle aqueous phase and an inner glycocalyx in contact with the corneal surface [105]. Tears are non-invasively accessible, with samples most often collected either with glass capillaries or with Schirmer strips (sterile filter paper strips) [106].

Tears contain proteins, peptides, lipids, small molecular metabolites and electrolytes. In humans, their composition has been monitored both in physiological conditions (e.g., influence of aging [107]) and in ocular and systemic diseases, in search for biomarker candidates (e.g., dry eye syndrome [108,109], glaucoma medication [110]; Parkinson's disease [111]).

One of the first investigations in the animal field was a comparative study of normal tears from cat, dog, koala, mouse and rat, both by size-exclusion chromatography (on Superose 12) and by SDS-PAGE, concluding that patterns were species-specific and quite different to the human tear protein profile [112]. De Freitas Campos et al. established the first 2-DE map of canine tears, starting from a pooled sample obtained from several healthy dogs ($N = 14$) of different breeds. Comparing this with a pool from dogs bearing various kinds of cancer, protein abundance differences were found in actin, albumin and a protein analogous to human lacryglobulin [113]. The dominant protein chain in both sample types, and unaffected by cancer, was identified as major canine allergen protein (MCA), an analogue of lipocalin (a protein present also in human tears). More recently, an investigation combining 1-DE SDS-PAGE and MALDI-TOF/TOF analysis (GeLC-MS) identified 125 proteins in 17 CBB-stained bands. Out of these, 25 are known to be present also in human tears. Starting material were tears from healthy animals ($N = 6$) of various breeds (German Shepherds, Doberman, Labrador, mixed breeds) and different age (2–6 years) [114].

We have recently performed some experiments on tear film proteins of pugs. This breed is characterized by a short, round face and large prominent eyes, and these anatomical features give rise to a high number of eye diseases, known under the term ‘brachycephalic ocular syndrome’ [115,116]. As part of a study on eyelids and tear film [117], also tear samples of selected animals were collected. According to the severity of associated corneal disease, animals ($N = 9$) were categorized into ‘mildly’, ‘moderately’, or ‘severely’ affected. As ocular diseases start in early life of pugs, healthy cross breeds of similar age served as a control group. 2-DE protein patterns of single samples were captured in two ways: by working with pre-labeled samples (fluorophore T-Red310) and by silver staining of the gels (details in Suppl. file - Tear film protein experiment). Fig. 4 displays two silver stained gels representative for mildly and severely affected animals, pointing out spots/proteins that are either specific for tear film or characteristic for the disease severity of the respective sample group (for protein names, see Table 2). Most of these proteins showed a clear trend in abundance from healthy or mildly affected to severely affected animals, with a few that seemed breed-specific (healthy controls not matching; e.g., enolase, 14–3-3, alpha-lactalbumin). The most prominent protein in the 2-DE pattern is MCA (spot 8A identified as a representative of this chain); similar to the findings reported by [113] for cancer-bearing animals, its concentration is stable across the groups. Nevertheless, some regulated minor spots above or below this chain could also be attributed to MCA (8B, 8C, 8D), representing presumably fragments or PTM species. Details about spot regulation and identification can be found in Supplemental Fig. 2 and Supplemental Table 3. Distribution of the identified proteins confirmed the duality of origin (serum/blood origin or specificity for tear film), though all of them (except SFN, stratifin or 14-3-3 sigma) were combined in one cluster in STRING pathway analysis

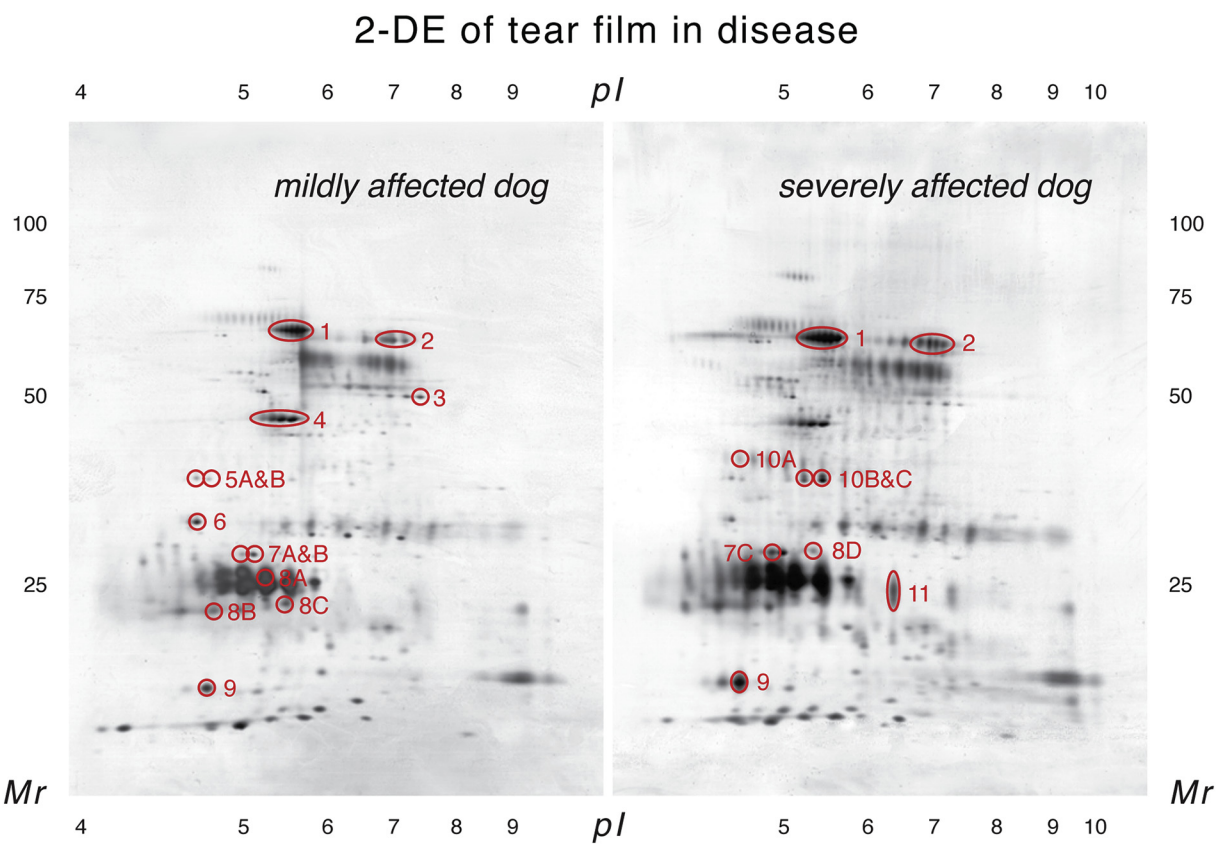

Fig. 4. 2-DE of tear film in disease.
2-DE map of tear film, from two pugs with either mild or severe ocular disease. Experimental details: IPG-DALT as inFig. 1, but with only one sample per gel; staining with silver. Spots 2–11 were excised for protein identification; the results are presented inTable 2and, in extended form, in Supplemental Table 3. Spot 1 was identified due to its position in the gel and with reference to previous results [1].

(Supplemental Fig. 3). Proteins abundant in serum (albumin, apolipoprotein A-I, haptoglobin) increased with severity of ocular lesions; possible reasons may be augmented vascularization or permeability of the blood vessels, inflammation or other imbalances (e.g., changes in the lipid layer of the tear film).

The preliminary results reported here are derived only from a small number of animals, and individual variation among patients seems considerable, despite categorization. Nevertheless, findings display similar trends as detected in human ocular disorders for several of the mentioned proteins: increased albumin, haptoglobin, apolipoprotein A- and zinc-alpha-2-glycoprotein, as well as decreased lacritin (all by [118]) and lowered lactoferrin levels [119]. Lacritin is an extracellular glycoprotein produced by the lacrimal gland and known to promote tear secretion, proliferation and survival of epithelial cells as well as corneal wound healing [120]. Lactoferrin, in contrast, is produced by different glandular epithelial cells and is present in multiple exocrine fluids such as breast milk and different mucosal secretions (tears, nasal fluids, saliva, pancreatic, gastrointestinal and reproductive tissue secretions). It is a member of the transferrin family, known as iron-binding and multi-functional [121]. Due to its immunomodulatory and antimicrobial activity and consistent association with dry eye disease, it is one of the three translational biomarkers (i.e., translation of proteomic biomarkers into clinical practice) in ophthalmology reported in [122]; the other two are MMP-9 as marker for inflammation and IgE as indication of allergy.

Table 2
Identification of the proteins in the tear film spots (as marked in Fig. 4).

Spot #	Accession number	Protein identification
1	ALBU_CANLF	Albumin
2	NP_001274005.1	Lactotransferrin precursor
3	F1PCH3_CANLF	Enolase 1
4	J9NXE2_CANLF	Actin, cytoplasmic 1
5A, B	F1PDJ7_CANLF	Alpha-2-glycoprotein 1, zinc-binding
6	F1PQ93_CANLF	Stratifin
7A, B	F1PDJ5_CANLF	Apolipoprotein A-I
8A	J9P0B5_CANLF	Lipocln_cytosolic_FA-bd_dom domain-containing protein
8B, C	J9P0B5_CANLF	Lipocln_cytosolic_FA-bd_dom domain-containing protein
	XP_022267068.1	Extracellular glycoprotein lacritin
8D	J9P0B5_CANLF	Lipocln_cytosolic_FA-bd_dom domain-containing protein
9	F1PTQ7_CANLF	Alpha-lactalbumin
10A-C	HPT_CANLF	Haptoglobin
11	XP_022267068.1	Extracellular glycoprotein lacritin

3. Conclusions

'Neglected markers' was the topic of a previous review from our group [123], in which we discussed altered levels of serum proteins in mice as clues to the outcome of experimental procedures in these model animals: an approach few researchers are taking, despite some obvious pros. We are back with the current writing to comment on the attitude at neglecting: this time at neglecting, in proteomics studies, biological fluids other than plasma/serum, urine and CSF. Recalling anatomy basics, and clinical practice in humans, one can write a long list of such fluids, which collect their components from more or less restrained body compartments and are collected themselves with more or less ease from the body.

A disproportion exists, in humans, as for the number of

investigations on different biological samples: search string « human plasma proteomics » in PubMed yields 25 times more hits than « human tear proteomics » and 30 times more than « human amniotic fluid proteomics ». An even larger disproportion exists as for the interest devoted to our own and to any other animal species: considering the one of our current interest, the number of hits for « human plasma proteomics » is more than two orders of magnitude larger (140 times larger, to be exact) than for « dog plasma proteomics ». We need to recall that the biology of every species has its own peculiarities: for instance, as our several past publications have made clear, the proteome of plasma/serum varies extensively even when comparing closely related species (e.g., rats [124] and mice [123]); under 2.1 in this review, we detailed the differences between the fetal annexes in humans on the one side and in mice on the other. Overall, not always knowledge gathered on one species can be directly stretched to another.

From all the above, it does not come to a surprise that we were able to retrieve only partial and scattered literature data about the 'neglected' dog specimens we meant to review. As scientists carrying out proteomic investigations at the University of Veterinary University Vienna, however, we have little attitude to neglect any animal specimen. Over the years, we, in various collaborations, have produced many interesting data about a number of dog biological fluids, in some case complementing, in other pioneering the construction of a proteome database. Their results are presented here, often for the first time. These data are usually spin-offs from clinical routine, as they arise from the comparison between dogs treated as patients at the vet clinic and other animals without signs of disease. Enrolled this way, the number of both cases and controls seldom have the chance to grow really large, mostly if the dogs are sorted according to breed – a precautionary move that becomes a necessity when a correlation between breed and disease incidence is known or suspected from epidemiology. In the examples reported in our review, this is the case for the occurrence of eye disorders in pugs, in connection with the peculiar shape of their skull. The pathological condition is so widespread that no tear sample from healthy adult pugs could be tested and a trend for the concentration of various proteins was analyzed across a range of disease severity. Conversely, the number of anasarca cases vs the number of healthy puppies is low in all the breeds and statistical treatment of the data about NGAL is meaningful only if animals from different breeds are combined (Supplemental Fig. 1), even if the baseline values of NGAL seem breed-dependent. So, while we could take advantage of the especially high concentration of NGAL in the pathological samples to obtain sufficient material for MS identification, we must leave to future investigations on a larger number of animals any firm conclusion about the correlation between anasarca condition and elevated levels of this protein in the fetal fluids.

We searched PubMed for information on further biological samples, i.e., colostrum and milk. It is known that the differences in overall composition among species are large enough to prevent the direct use of milk from one to feed the young of another. Despite some obvious veterinary interest, however, we could not retrieve any proteomic data about milk or colostrum of dog, and found only studies on a few (mainly milk-specific) individual proteins. This compares not only with the 41 reports on colostrum plus the 383 on milk from our own species but also with the 33 *plus* 355 on bovine and the 7 *plus* 84 on ovine items. These figures highlight the weight economical relevance connected with human nutrition has on orienting veterinary research more towards livestock than towards pets.

Our review is contributed to *Journal of Proteomics* as part of a thematical issue devoted to "Farm and companion animal proteomics". The limited number of reports we could retrieve in literature when preparing our previous survey on dog biological fluids [1] and again on this occasion hints to a still insufficient attention of the vet community to the latter in comparison with the former. As such, our review is often an assessment of what evidence is lacking and could/should be gathered in the next future.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.103724.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

This research was supported in part by grants from Progetto Eccellenza, Miur, Italy.

The authors wish to thank Barbara Nell and Marion Krecny from the Ophthalmology Service at the University of Veterinary Medicine Vienna for their collaboration in providing staged tear samples and additional support.

We associated all of our recent reviews with some musical piece; we continue this tradition: this time the main accompaniment to the writing was Beethoven's Third Symphony.

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