Measurement of glycosaminoglycans in canine synovial fluid and its correlation with the cause of secondary osteoarthritis, age and body weight

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Abstract

Glycosaminoglycans are natural components of healthy joint cartilage and they also appear in healthy synovial fluid. An increased amount of glycosaminoglycans in synovial fluid is believed to be a marker of secondary osteoarthritis, regardless of its primary cause. The aim of our study was to define the relationship between glycosaminoglycans in the synovial fluid and joint disorders, age, and body weight. The samples of synovial fluid were obtained from dogs suffering from secondary secondary osteoarthritis (n = 35) and from control dogs (n = 18); control dogs had normal body weight. The results were compared among joints of dogs with secondary osteoarthritis divided into groups according to the criteria mentioned above and control dogs. Glycosaminoglycan concentrations in synovial fluid were measured using dimethylmethylene blue assay. The lowest mean value of glycosaminoglycans in synovial fluid was measured in the control group. Significantly higher glycosaminoglycan content ($P < 0.05$) was found in synovial fluid isolated from obese dogs compared to control dogs. Furthermore, we observed an age-related trend, in which the highest mean values were reached either in old dogs or pups. Despite the absence of significant differences in glycosaminoglycan values among dogs suffering from various types of secondary secondary osteoarthritis, the highest mean values were measured in fragmented coronoid processus group. Our data suggest that abnormally increased body weight has an impact on glycosaminoglycan concentration in synovial fluid which may imply faster degradation and turnover of joint cartilage. Such observation has not yet been published in veterinary medicine.

Dog, joint, obesity

Glycosaminoglycans (GAG) and proteoglycans form the amorphous part of cartilage matrix, contributing to 35% of total cartilage weight. These hydrophilic molecules help counteracting mechanical pressure, thus maintaining the space structure (Kempson et al. 1970); they regulate solution penetration into cartilage (Maroudas et al. 1980) and play an important role in differentiation and regeneration of cartilage tissue (Linsenmayer and Toole 1977).

Chondroitin-6-sulphate and chondroitin-4-sulphate represent the main group of GAG and are constituent parts of aggregated proteoglycans. Another GAG representative is keratan sulphate or dermatan sulphate (Uebelhart 2008).

Regarding physiological condition, GAG or chondroitin-6-sulphate a chondroitin-4-sulphate are found both in cartilage matrix and synovial fluid (SF) as they enter SF within normal cartilage turnover. Their concentration is elevated if the matrix is being degraded faster as in the case of certain pathological condition, e.g. osteoarthritis (OA). The OA development, pathophysiology, diagnostics and treatment are well reviewed by Renberg (2005). Primary OA is rarely seen in dogs; on the other hand, the secondary OA is quite common since 20% of dogs older than 1 year suffer from secondary OA (Johnston 1997). Secondary OA may result from many joint diseases such as developmental disorders (osteochondrosis - OCD, ununited anconeal process - UAP, fragmented coronoid process - FCP, hip dysplasia, patellar luxation), traumatic disorders (e. g. cranial cruciate ligament

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rupture - RCCL, intraarticular fractures) or other causes (e. g. chronic arthritis, aseptic necrosis of femoral head - Legg-Calvé-Perthes disease) (Pedersen and Poole 1978).

Changes in GAG concentration are thought to be a reliable marker of cartilage degradation level. An important factor that is necessary to objectify when comparing GAG concentration in SF is the chronicity of OA process. In early OA stages, the GAG amount may increase dramatically; however, GAG values drop slowly and approach nearly normal values in later OA stages (Innes et al. 1998). GAG can also be estimated in serum because they are filtrated through synovial membrane. Importantly, serum values may give false positive results as the half-time of GAG clearance in OA joints differs from healthy joints (Myers et al. 1996). Moreover, some authors believe GAG serum concentration is neither prognostic nor diagnostics marker of OA (Arican et al. 1994).

The physiological turnover of cartilage and its abnormal degradation during OA share some similarities. Metalloproteinases are primarily responsible for extracellular matrix degradation; also, they release proteoglycans from the complex with hyaluronic acid. Subsequently, free proteoglycans undergo proteolysis and their fragments of various size containing chondroitin sulphate, keratan sulphate, interglobular domains etc. enter SF and are further proceeded by synovial cells or removed by lymphatic vessels from the joint space. The majority of fragments enters the blood circulation and then is removed by the liver or kidneys.

The aim of our study was to prove the dependence of synovial GAG concentration on the primary cause of secondary OA as well as on the age and body weight of studied dogs.

Materials and Methods

Animals

Our study included 36 privately owned dogs treated at the Small Animal Clinic of the University of Veterinary and Pharmaceutical Sciences Brno. Thirty-one of these dogs (35 joints) suffered from joint disease and 5 (18 joints) animals were control dogs that were euthanized for disorders not affecting joints (Table 1). These control animals had healthy weight and their age ranged from 6 to 12.5 years. The diagnosis of OA and its underlying cause was based on history, clinical examination, radiographic assessment and perioperative findings.

Dogs with joint disease were divided into several groups based on their disease, age, and body weight. Based on disease status, dogs were divided into four groups: control dogs (n = 5), dogs with rupture of CCL (RCCL) (n = 18), dogs with FCP (n = 7) and dogs with other causes of OA (OCOA), such as patellar luxation, OCD or hip OA (n = 6). Age clustering was the following: pups (up to 6 months for small breeds, 9 months for medium breeds and 12 months for large breeds) (n = 6), young dogs (age 6–12 months for small breeds, 9–18 months for medium breeds and 12–30 months for large breeds) (n = 8), and old dogs (9 years or older for small breeds, 8 years or older for medium breeds and 6 years or older for large breeds) (n = 4). The breed category (small, medium, and large breed) is used accordingly in Table 1. Animals exceeding Fédération Cynologique Internationale (FCI) standard for given breed by 10–19% were considered overweight (n = 4), dogs surpassing standards by 20% or more were rated as obese (n = 5). Overweight and obese dogs were pooled into one group. Obese dogs were also evaluated separately.

Sample collection

Synovial fluid samples were collected from all dogs by aseptic arthrocentesis and concentrations of GAG were examined. After assessing the sample volume, heparin solution (50 ml) was added and the sample was diluted sevenfold with phosphate-buffered saline (PBS), pH 7.0. The sample was then centrifuged at 200 *g* for 10 min at room temperature. The supernatant fluid was stored at -80 °C until assayed.

Dimethylmethylene blue assay for GAG concentration in SF

We used a modified colorimetric method that was based on the dimethylmethylene blue (DMMB) assay published by Arican et al. (1994). Briefly, samples of SF supernatant were sonicated (20 pulses q 0.5s, 80% power, Branson Sonifer 150, Branson Corp., Danbury, CT, USA), transfered to a 96-well plate and incubated with N-acetylcysteine and papain at 65 °C for 2 h. After digestion, iodoacetic acid, sodium chloride and DMMB were added and sample absorbance at 540 nm was determined by ELISA-reader iMS READER MF (LABSYSTEMS, Helsinki, Finland). Shark cartilage (BioChemika Fluka, Germany) chondroitin-6-sulphate solution was used to construct a standard curve. If not indicated otherwise, all chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic).

Statistical analyses

The normality of data distribution was tested first. The criterion of normality was not fulfilled hence the data were evaluated by the non-parametric Kruskal-Wallis test. The *P* values lower than 0.05 were considered significant.

Results

The GAG concentrations were measured in SF samples obtained from 53 joints (36 dogs) of which 18 joints were used as control samples. The results were compared among dog groups divided according to given criteria (disease status, age, body weight) and control dogs. The GAG found in SF together with other variables characterizing the dogs are shown in Table 1.

The GAG concentrations in SF compared among groups of dogs with different disease status are shown in Fig. 1. The mean values of GAG concentrations in SF were following: 1.14 ± 0.58 mg/ml, 1.43 ± 1.088 mg/ml, 1.81 ± 1.51 mg/ml, and 2.87 ± 2.43 mg/ml in the control group, RCCL, OCOA, and FCP groups, respectively.

The GAG concentrations in SF compared among age groups of dogs are presented in Fig. 2. The mean values of GAG concentrations in SF were following: 1.14 ± 0.58 mg/ml, 1.22 ± 0.51 mg/ml, 1.44 ± 1.42 mg/ml, 2.50 ± 2.59 mg/ml, and 2.71 ± 1.79 mg/ml in the control group, the group of young animals, adult dogs, pups, and old dogs, respectively.

The GAG concentrations in SF compared among body weight groups are presented in Fig. 3. The mean values of GAG concentrations in SF were following: 1.14 ± 0.58 mg/ml, 1.80 ± 0.73 mg/ml, 1.80 ± 0.58 mg/ml, and 2.85 ± 1.80 mg/ml in the control group, group of dogs with healthy weight, overweight group including obese dogs, and obese group, respectively. The mean value of GAG concentration in SF measured in the obese group was significantly higher compared to control dogs ($P < 0.05$).

Discussion

Secondary OA is one of the most common orthopaedic disorders in dogs. Despite the fact that secondary OA is a common result of abnormal development of joint structures (elbow or hip dysplasia, FCP, UAP etc.), the extending lifespan, increasing number of

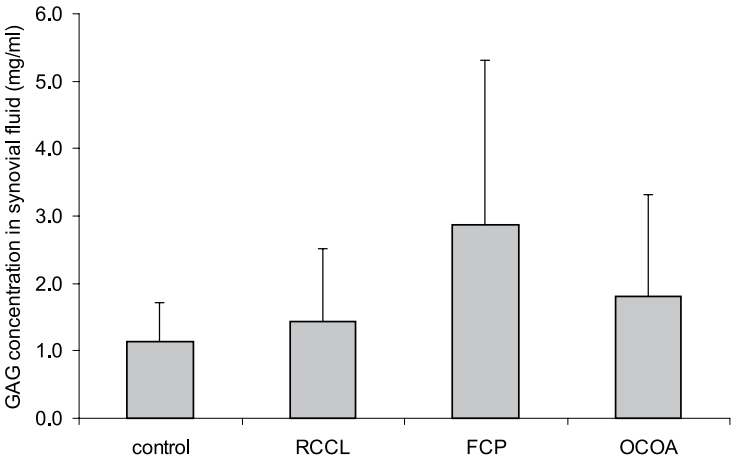

Fig. 1. Comparison of glycosaminoglycan (GAG) mean values among groups of dogs based on disease status. Control - dogs euthanized for disorders not affecting joints, n = 18 (joints); RCCL - dogs with ruptured cranial cruciate ligament, n = 18 (joints); FCP - dogs with fragmented coronoid process, n = 10 (joints); OCOA - dogs with other causes of osteoarthritis, n = 7 (joints).

Table 1. Glycosaminoglycan (GAG) concentration in synovial fluid and basic characteristics of patients and control dogs used in the study.

Breed	Breed category	Sex	Age	Age category	Weight (kg)	Weight category	Diagnosis	Joint	OA changes by rads	GAG (mg/ml)
Dogs with joint disease										
Labrador Retriever mix	large	F	6 y	adult	35	overweight	RCCL dx. chronic	stifle dx.	N/A	0.784
American Cocker Spaniel	medium	M	7 y	adult	14.7	overweight	RCCL sin.	stifle sin.	mild	1.788
Yorkshire Terrier	small	M	8 y	adult	4	obesity	RCCL sin. and medial patellar luxation III/IV grade	stifle sin.	mild	5.404
Pug	small	M	4 y	adult	11	obesity	bilateral medial patellar luxation II/IV grade	stifle dx.	severe	4.081
Beagle	medium	M	5 y	adult	22	obesity	RCCL sin.	stifle sin.	N/A	1.946
Dogue de Bordeaux	large	F	5 y	adult	45.5	healthy w.	RCCL sin.	stifle sin.	moderate	1.085
Boxer	large	F	3.5 y	adult	38	obesity	RCCL dx chronic and RCCL sin acute	stifle dx.	severe	1.267
Leonberger	large	M	5.5 y	adult	69.5	healthy w.	FCP sin.	elbow sin.	none	1.09
Pit Bull	medium	F	4 y	adult	21	healthy w.	RCCL dx.	stifle dx.	mild	1.155
Pit Bull	medium	M	4 y	adult	27	healthy w.	RCCL dx. chronic	stifle dx.	severe	0.917
Bernese Mountain Dog	large	M	5 y	adult	50	healthy w.	RCCL sin.	stifle sin.	severe	0.637
Tibetan Mastiff	large	M	4 y	adult	60	healthy w.	partial RCCL sin.	stifle sin.	N/A	0.658
Tibetan Mastiff	large	M	4 y	adult	60	healthy w.	partial RCCL dx.	stifle dx.	N/A	0.945
Labrador Retriever	large	M	3 y	adult	36	healthy w.	RCCL dx.	stifle dx.	moderate	0.679
Tosa Inu	large	F	19 m	young	45	healthy w.	bilateral hip OA	hip sin.	mild	2.058
Newfoundland Dog	large	M	1 y	young	73	healthy w.	bilateral FCP	elbow sin.	severe	0.707
Newfoundland Dog	large	M	1 y	young	73	healthy w.	bilateral FCP	elbow dx.	severe	0.876
Newfoundland Dog	large	F	18 m	young	50	healthy w.	RLCC dx.	stifle dx.	mild	1.631
Bernese Mountain Dog	large	F	16 m	young	36	healthy w.	bilateral FCP and elbow dysplasia	elbow dx.	severe	0.525
Hovawart	large	M	2.5 y	young	52	overweight	shoulder OCD dx.	shoulder dx.	none	1.19
Labrador Retriever	large	F	N/A	young	N/A	healthy w.	partial RCCL sin.	stifle sin.	none	1.79
American Bulldog	large	M	2 y	young	32	healthy w.	RCCL sin.	stifle sin.	mild	1.03
Newfoundland Dog	large	F	2.5 y	young	64	healthy w.	part. rupture of brachial biceps tendon dx. and OCD	shoulder dx.	mild	1.19
Boxer	large	F	11 y	old	29.2	healthy w.	RCCL sin.	stifle sin.	N/A	1.596
Labrador Retriever	large	M	8 y	old	35	healthy w.	bilateral FCP and bilateral hip dysplasia	elbow sin.	severe	4.452
Labrador Retriever	large	M	8 y	old	35	healthy w.	bilateral FCP and bilateral hip dysplasia	elbow dx.	severe	4.684
Beagle	medium	F (sp.)	8.5 y	old	18.5	obesity	RCCL sin. acute	stifle sin.	mild	1.57
Golden Retriever	large	F	10 y	old	40	overweight	RCCL dx. and gonarthrosis	stifle dx.	moderate	1.24
Labrador Retriever	large	F	6 m	pup	16	healthy w.	bilateral FCP	elbow sin.	N/A	5.544

Table 1. Glycosaminoglycan (GAG) concentration in synovial fluid and basic characteristics of patients and control dogs used in the study. *(Continued)*

Breed	Breed category	Sex	Age	Age category	Weight (kg)	Weight category	Diagnosis	Joint	OA changes by rads	GAG (mg/ml)
Bullmastiff	large	F	6 m	pup	25	healthy w.	tarsal OCD sin.	tarsus sin.	N/A	4.606
German Shepherd	large	M	9 m	pup	20	healthy w.	bilateral FCP	elbow sin.	N/A	6.678
Beauceron	large	F	9 m	pup	30	healthy w.	shoulder OCD dx.	shoulder dx.	none	1.036
Labrador Retriever	large	F	5 m	pup	18	healthy w.	avulsion of the origin of the CCL sin.	stifle sin.	N/A	0.329
Labrador Retriever	large	F	6 m	pup	17.5	healthy w.	bilateral elbow dysplasia (bilateral FCP suspected)	elbow sin.	none	1.41
Labrador Retriever	large	F	6 m	pup	17.5	healthy w.	bilateral elbow dysplasia (bilateral FCP suspected)	elbow dx.	none	0.952
Control dogs										
Flat Coated Retriever	large	M (neu.)	7 y	old	34.8	healthy w.	bone fibroma (fibrous cortical defect)	shoulder dx.	mild	1.232
Doberman Pincher	large	F	12.5 y	old	28	healthy w.	euthanasia - pulmonary metastasis	stifle sin.	N/A	1.267
Doberman Pincher	large	F	12.5 y	old	28	healthy w.	euthanasia - pulmonary metastasis	stifle dx.	N/A	1.442
Doberman Pincher	large	F	12.5 y	old	28	healthy w.	euthanasia - pulmonary metastasis	elbow sin.	N/A	0.917
Boxer	large	M (neu.)	6 y	adult	38	overweight	euthanasia - prostate carcinoma	stifle sin.	N/A	0.196
Boxer	large	M (neu.)	6 y	adult	38	overweight	euthanasia - prostate carcinoma	stifle dx.	N/A	0.329
Labrador Retriever	large	F	6 y	adult	22	healthy w.	euthanasia - jejunal foreign body and invagination	stifle sin.	N/A	1.33
Labrador Retriever	large	F	6 y	adult	22	healthy w.	euthanasia - jejunal foreign body and invagination	stifle dx.	N/A	0.31
Labrador Retriever	large	F	6 y	adult	22	healthy w.	euthanasia - jejunal foreign body and invagination	elbow sin.	N/A	1.43
Labrador Retriever	large	F	6 y	adult	22	healthy w.	euthanasia - jejunal foreign body and invagination	elbow dx.	N/A	1.29
Labrador Retriever	large	F	6 y	adult	22	healthy w.	euthanasia - jejunal foreign body and invagination	shoulder sin.	N/A	1.52
Labrador Retriever	large	F	6 y	adult	22	healthy w.	euthanasia - jejunal foreign body and invagination	shoulder dx.	N/A	0.29
Labrador Retriever	large	F	6 y	adult	22	healthy w.	euthanasia - jejunal foreign body and invagination	carpus sin.	N/A	0.99
Central Asian Shepherd Dog	large	M	8 y	old	46	healthy w.	euthanasia - spinal cord compression	stifle sin.	N/A	2.39
Central Asian Shepherd Dog	large	M	8 y	old	46	healthy w.	euthanasia - spinal cord compression	stifle dx.	N/A	1.19
Central Asian Shepherd Dog	large	M	8 y	old	46	healthy w.	euthanasia - spinal cord compression	elbow sin.	N/A	1.29
Central Asian Shepherd Dog	large	M	8 y	old	46	healthy w.	euthanasia - spinal cord compression	elbow dx.	N/A	1.02
Central Asian Shepherd Dog	large	M	8 y	old	46	healthy w.	euthanasia - spinal cord compression	shoulder dx.	N/A	2.02

M	male	RCCL	cranial cruciate ligament rupture	dx.	dexter = right
F	female	FCP	fragmented coronoid process	sin.	sinister = left
M (neu.)	male (neutered)	OA	osteoarthritis	GAG	glycosaminoglycans
F (sp.)	female (spayed)	OCD	osteochondrosis	y	year
N/A	not analyzed	CCL	cranial cruciate ligament	m	month

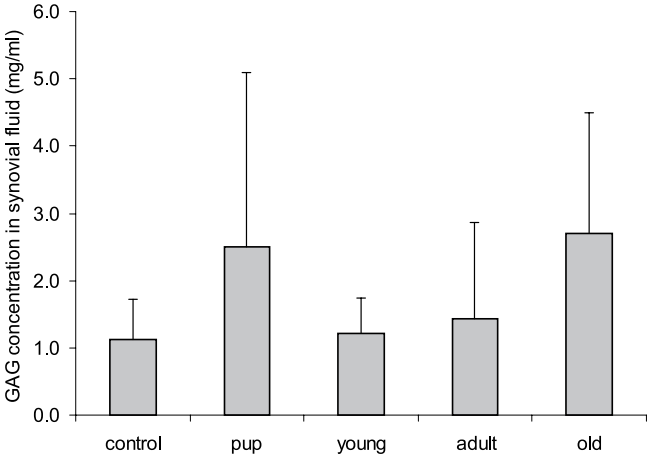

Fig. 2. Comparison of glycosaminoglycan (GAG) mean values among age groups of dogs. Control - dogs euthanized for disorders not affecting joints, n = 18 (joints); pup - dogs with joint disease (6 months and younger for small breeds, 9 months and younger for medium breeds, and 12 months and younger for large breeds), n = 7 (joints); young - dogs with joint disease with the ages of 6 - 12 months for small breeds, 9–18 months for medium breeds, and 12–30 months for large breeds, n = 9 (joints); adult - dogs with joint disease between the ages of 1–9 years for small breeds, 1.5–8 years for medium breeds, and 2.5–6 years for large breeds, n = 14 (joints); old - dogs with joint disease with the age of 9 years and older for small breeds, 8 years and older for medium breeds, and 6 years and older for large breeds), n = 5 (joints).

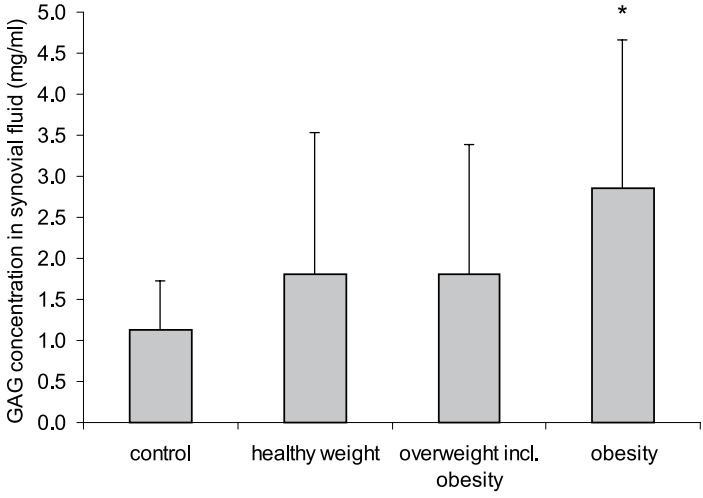

Fig. 3. Comparison of glycosaminoglycan (GAG) mean values among weight groups. Control - dogs euthanized for disorders not affecting joints with healthy weight, n = 18 (joints); healthy weight - dogs with joint disease meeting the breed standards of the Fédération Cynologique Internationale (FCI), n = 26 (joints); overweight - dogs with joint disease exceeding FCI breed standards by 10–19%, n = 4 (joints); obesity - dogs exceeding with joint disease FCI breed standards by 20% and more, n = 5 (joints). *significant difference ($P < 0.05$).

overweight dogs and changed social status of dogs in general have a gross impact on increasing the incidence of secondary OA among world canine population including the Czech Republic.

The OA is a chronic degenerative joint disorder involving joint cartilage, underlying bone structures and the synovial membrane that interact together during degradation and reparation process (Owens and Biery 1999). The OA is considered a non-inflammatory arthropathy; its typical features are fragmentation and loss of joint cartilage, narrowing or even collapsing of joint space well seen on radiographs, increased subchondral density (sclerosis) and newly formed bone structures at the edge of joint surfaces (osteophytes) (Pedersen and Poole 1978). In this study, we focused on cartilage degradation products in SF, which are believed to be elevated during certain stages of OA.

In spite of the relatively low number of evaluated joints along with high data variability, some interesting conclusions could be drawn. The GAG concentrations in SF samples seem to be higher in dogs suffering from OA than in control dogs, regardless of their actual body weight. However, obese dogs reached a significant difference compared to control dogs. Although the finding supports the general idea of a relationship between the body weight and OA development in joints, there are no studies available specifically discussing the body weight versus GAG concentration in SF.

Also, there is a certain age influence seen as the old dogs and pups have the highest mean values and on the other hand, the lowest mean value was measured in young dogs group. A similar conclusion was made by authors evaluating the correlation of GAG concentration in SF and age in horses; the highest values were measured in a group of newborn foals and those values kept decreasing over the lifetime (van den Boom et al. 2004). Later, the same authors cast doubts on GAG measurement in early stages of cartilage damage when no obvious changes could be seen since those values were lower compared to values of horses with at least minimal visible OA changes (van den Boom et al. 2005). Negative correlations between GAG values in SF and cartilage damage stage were reported by other authors in horses, too (Fuller et al. 2001). The GAG concentration and severity of radiographic changes correlated negatively even in dogs (Innes et al. 1998) and there was no correlation between radiographic changes and GAG in human patients (Belcher et al. 1997). These indicators (severity of radiographic changes and GAG content in SF) did not correlate in our study either (data not shown). The elevated GAG and keratan sulphate concentrations were observed in the patients' SF samples during the acute process compared to the chronic disease. The authors explained this as a result of higher metabolic rate and final GAG depletion in joint cartilage content (Ratcliffe et al. 1988). The same fact was also confirmed in dogs throughout the early and late OA stages (Innes et al. 2005).

It may be considered that high GAG values are caused with a high metabolic rate in pups when anabolic processes significantly outnumber catabolic processes during the intensive phase of their growth; on the other hand, in the old dogs, catabolic processes that degrade cartilage tissue may also increase GAG in SF. Another explanation for this phenomenon can be an increased glycosylation of cartilage proteins in older animals, which leads to higher GAG concentration in SF (DeGroot et al. 2001).

The variability in age of dogs included in our study might explain the differences among groups based on primary joint disorder due to the fact that the FCP group, which achieved the absolutely highest mean value of GAG concentration in the whole study, was dominated by pups and old dogs (66% of dogs diagnosed with FCP) but the RCCL group with the lowest mean GAG value was mainly formed from adult and young dogs (81%) who had low GAG values. Nevertheless, the high FCP values in FCP group can be influenced by the fact that all samples were obtained from one compartment, the elbow joint exclusively. The GAG concentration may significantly differ among joints of a healthy animal (Fuller et al. 1996).

Finally, our study supports the widely-accepted dogma that obesity is a negative factor contributing to joint degradation process, manifested by an increased level of GAG released

into samples of canine SF. Further evaluation is needed in order to answer the question how soon the obesity-dependent degradation starts and whether the process can be significantly slowed down after the individual has lost the abundant body weight.

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