# SYNOVIAL FLUID ANALYSIS, BIOMARKERS CRP (C-REACTIVE PROTEIN) AND COMP (CARTILAGE OLIGOMERIC MATRIX PROTEIN) IMPORTANCE IN DIAGNOSTIC OF CANINE JOINT DISEASES

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## SUMMARY

One of the main reasons dogs are going lame is a cranial cruciate ligament rupture (CCLR), leading to the tibiofemoral joint instability (Johnston et al., 2001). There is a reason to believe that the CCLR is a consequence of immune-mediated polyarthritis. Recently, many scientists have focused on biomarker researches, which can provide information about changes in the joints and efficiently monitor the progress of the disease, when treatment is started. This is particularly important for investigating the diagnostics of OA. The development of degenerative process can be prevented by the early taken necessary measures. Although there are explored and described some of osteoarthritis biomarkers, to date, none of them is in use in clinical practice (Tobias et al., 2012).

The aim was to evaluate changes in the canine synovial fluid physical properties and cells quantity and composition, when the cranial cruciate ligament rupture has been diagnosed. It was also investigated the concentration of biomarkers CRP and COMP in plasma and synovial fluid by the cranial cruciate ligament rupture and by clinically healthy dogs. In total there were 35 dogs tested, twenty-five with CCLR and ten healthy dogs from control group.

By researched group in canine synovial fluid there were found neutrophil and mononuclear cells more than usual should be. In synovial fluid there were found a small amount of red blood cells, although they are generally absent from synovial fluid. The study has been informative and has confirmed the assumption that in most cases the CCLR is a secondary disease caused by immune-mediated polyarthritis (IMP). Studies of biomarkers CRP and COMP from plasma and synovial fluid showed that by the cranial cruciate ligament rupture, the concentration of biomarkers was significantly increased compared with the results obtained from the control group samples.

The aim of our study was to show the importance of the synovial fluid composition and cells analysis by joint disease diagnostics and to promote the use of biomarkers in clinical practice.

**KEY WORDS:** dogs, synovial fluid indicators, cranial cruciate ligament rupture, biomarkers CRP and COMP.

## INTRODUCTION

The cranial cruciate ligament rupture gives the tibiofemoral joint instability. This is one of the main causes of dogs lameness (Johnson et al., 2001). Therefore, researchers focuses on the ligament rupture causes and consequences. It is considered that the joint instability allows the development of secondary joint pathology - osteoarthritis (Johnson et al., 2001). Osteoarthritis (OA) is a condition where the articular cartilage is being damaged and this is resulting pain, swelling, joint function limitations. It is known that 20 percent middle-aged dogs and 90 percent older dogs are suffering from OA. In the human population this percentage is even higher (Venable et al., 2008).

Some authors maintain that osteoarthritis is a secondary disease after CCLR, other authors propose that CCLR is a consequence of immune-mediated polyarthritis (IMP). The immune-

mediated arthritis can be indentified only by synovial fluid cells quantity changes, making synovial fluid cells analysis (Viliers et al., 2005).

Recently, there is very often diagnosed the cranial cruciate ligament rupture by the dogs. By most patients, arthrotomy reveals distinct articular cartilage defects, typical to OA. It was observed also that very often, after a certain period of time, the problem will recur in another limb. Because over the years, this problem takes on a huge scale, scientists are interested in finding a way to provide early diagnosis of changes in the joints and prevent their development. When OA of the joint is diagnosed, degenerative processes become unstoppable. Therefore, there is only one way left - to treat symptomatic by inhibiting pain. This makes concerns to the host, because it has to be regularly fed in drugs, the dog's quality of life is getting worse, activity is limited. The problem is that there is often failed to eliminate the sensation of pain completely, and the rest of the life a dog is suffering pain of some degree, which is increasing with age.

The synovial fluid physical properties tests of color, turbidity, viscosity provide valuable information. Turbid synovial fluid occurs by inflammatory joint diseases (Fossum et al., 2007). The reduced viscosity of the synovial fluid indicates a decrease in the concentration of hyaluronic acid (Slattery, 1995).

Under normal conditions, synovial fluid is not curdling, however, by patients with synovitis the blood clots are formatting (Cohen et al., 1975). The results of the synovial fluid cells research and evaluation of the cells quantity can be divided into several groups: corresponding to the normal range, the typical for osteoarthritis, rheumatoid, infectious arthritis, immune-mediated polyarthritis.

Synovial fluid test can provide more accurate information about the pathology. Research includes microscopic evaluation, cell research, bacteriological and serological analysis. Results by having septic arthritis may significantly differ from the norm (Brunberg, 2001).

The diagnosis of joint disease only from synovial fluid test is not possible. However, the study results help to choose the appropriate additional analysis. For example, at cranial cruciate ligament rupture, by a lack of joint fluid tests the primary reason - immune-mediated polyarthritis may remain undetected.

Biomarkers' goal is to convey information on the physiological and pathological state of certain tissues and organs. Biomarkers can be used to achieve several goals:

1. as a diagnostic tool for the disease or pathological condition identification.

2. as a tool for determining the stage of disease and helping to classify the disease.

3. as an indicator of disease prognosis.

4. for monitoring of the clinical response to previous surgical intervention or medical treatment (Tobias et al., 2012).

The most commonly biomarkers are studied from serum and plasma. However, it may be measured from different body fluids - urine, saliva, synovial fluid, tears, cerebrospinal fluid, samples from biopsies (Tobias et al., 2012).

For the last three decades many studies have been conducted in order to discover the ways of early diagnosis of OA, monitoring the progress of the disease. The detection of cartilage defects in the early stage of osteochondritis is the aimed result for diagnostic, prognostic and therapeutic perspectives. Currently, diagnostic methods, such as arthroscopy, magnetic resonance, are expensive, can only be applied to the animal in the general anesthetic state and display the results only when a big area of the articular cartilage is injured or the cartilage fibrillation occurred (Tobias et al. 2012).

In our study there were analyzed two key biomarkers - COMP and CRP. COMP - pentametric non-collagenous glycoprotein - is an integral part of the articular cartilage. COMP is required for the normal function, development, structure of the matrix of articular cartilage. A large amount of COMP is found in articular cartilage, a small amount - in synovial fluid (Tobias et

al., 2012). In the early stage of OA metabolically active cartilage begins to decompose. A number of processes that aim to correct the defects associated with an increased amount of COMP in synovial fluid are proceeding in articular cartilage. In later stages of OA the cartilage becomes less metabolically active; amount of COMP reduces (Tobias et al., 2012). Up to date there have been few researches in order to assess the concentration of COMP in dogs' synovial fluid. Several studies, conducted with experimental canine OA models, confirmed the importance of the COMP marker and the connection with cartilage impairment (Tobias et al., 2012).

CRP is a protein produced by the liver. Its concentration is increased in the case of inflammation or the healthy tissues injury. The main benefit obtained from the measurement of CRP concentration is to assess the systemic inflammatory activity. Also, it is useful to verify the effectiveness of the treatment - by CRP concentration measurement before treatment, during it and after (Paul et al, 2011).

To date the monitoring of canine immune-mediated polyarthritis treatment with steroids has been based on the monitoring of clinical symptoms. Danish researchers decided to use CRP biomarkers for the assessment of inflammatory activity after initiation of treatment with steroids. It was found that CRP is an objective biomarker helping accurately assess the inflammatory processes occurring in the joints (Kjelgaard-Hansen et al., 2006).

The aim of the study is to perform joint fluid tests for dogs, which have been diagnosed with the cranial cruciate ligament rupture and surgical treatment was employed.

The objectives of the study. To evaluate the physical properties of synovial fluid in two dog groups - control and research's - to compare the results. To analyze synovial fluid cells changes in the canine control and research's groups. To evaluate the CRP and COMP biomarkers number in blood plasma and synovial fluid in the canine control and research's groups.

## MATERIALS AND METHODS

Work carried out in 2012 - 2014, in Lithuanian University of Health Sciences (LUHS) Veterinary Academy, dr. Leonas Kriaučeliūnas small animal clinic and Evaldas Diržinauskas private small animal veterinary clinic (Kaunas district).

Tested dogs were examined and treated for hind limb lameness, by the cranial cruciate ligament rupture. All of these dogs suffered from joint instability caused by cranial cruciate ligament rupture (CCLR). The patients were treated surgically (with DeAngelis method), using the lateral parapatellar arthrotomy and extracapsular joint stabilization by implanting an artificial ligament. During the operation the degree of manifestation of osteoarthritis was estimated.

The control group consisted of dogs brought to veterinary clinics for target sterilization or castration operations. Survey of patients' owners and orthopedic study did not reveal any orthopedic diseases.

Investigational arthrocentesis for tested dogs was performed in general anesthesia, following asepsis-antisepsis requirements, before starting joint arthrotomy surgery.

Physical properties of the resulting synovial fluid were evaluated - the color, viscosity, turbidity, mucinous clot formation. After the assessment of physical properties the sample residue was sent to the medical clinic of the LUHS Medical Academy Laboratory for cells research.

*Research of synovial fluid's physical properties*. Synovial fluid color was determined by visual assessment of synovial fluid taken without additives. Viscosity of the synovial fluid was assessed by placing a drop on the thumb, putting indicating finger on it, slowly separating the fingers and monitoring generated strings (Baker et al, 2000). Synovial fluid was divided to: the low-viscous, when there were no strings or the strings were up to 1cm long, medium

viscosity - the string length was 1 cm to 2 cm, and viscous - the strings longer than 2 cm. Synovial fluid's turbidity was determined visually, dividing the synovial fluid to clear or turbid.

*Cells' number and composition research methods.* For analysis of cell's number and composition synovial fluid was taken to a vacuum tube with anticoagulant K2EDTA. The sample was stored in vacuum tube with K2EDTA salt at room temperature (15-25ºC), and studies have been carried out at least 30 minutes and no longer than 2 hours from arthrocentesis operation.

The total number of white blood cells and red blood cells was calculated using a microscope, in disposable plastic counting chambers FAST-READ, using acetic acid dye solution, which has been poured on synovial fluid to visualize leukocytes and assess their total amount. Leukocytes are differentiated by looking through microscope to synovial fluid smear. A drop of synovial fluid was taken with a Pasteur pipette and spread on a slide within 1-2 cm$^2$ area in order to prepare the smear. Smear was dried at room temperature, then fixed and stained using HEMACOLOR paint set. After dyeing and drying, leukocytes differentiation was performed using the Olympus CX41 microscope (*Olympus, Japan*).

*Biomarkers CRP and COMP research methods.* CRP concentration was analyzed using reagents kits of *Dog C-Reactive Protein (CRP) ELISA test kit* (*Life Diagnostics; USA*). COMP concentration was analyzed using reagents kits of *Canine Cartilage Oligomeric protein (COMP) ELISA Kit* (*TSZ ELISA; USA*). Investigations were carried out in accordance with the manufacturer's instructions.

*Statistical data analysis.* The survey data were estimated using the "R" statistical package. In order to analyze data the arithmetic means ($\bar{x}$), their bias ($m_x$), trusted intervals (PI) of tested dogs (n) properties were evaluated. The reliability of control and test groups arithmetic mean's difference was determined by t-test. The Pearson correlation was estimated to assess linear links elasticity between the quantitative variables. Categorical variables influence to normally divided quantitative properties was investigated by the method of dispersive analysis (ANOVA). Nonparametric frequency tables were analyzed using Fisher and $\chi2$ tests. Data were considered as statistically reliable, when $p \leq 0.05$.


**RESULTS**

By the evaluation of joint fluid color from the patients, diagnosed with CCLR, synovial fluid samples was found that most were of the gray-yellow color (n = 17), a bit less of the pink (n = 5) and red (n = 3) color samples (Figure 1.).

Of these patients synovial fluid samples were obtained mainly non-viscous synovial fluid samples (n = 21).

Mucin clot test is directly dependent on quality and quantity of the hyaluronic acid in the synovial fluid. After mucin clot test from synovial fluid samples of the patients with the cranial cruciate ligament rupture, following results were obtained: mucin clot had formed (n = 4), had not formed (n = 21). All synovial fluid samples, from which a mucin clot had not formed, were non-viscous. The data presented in Figure 2.
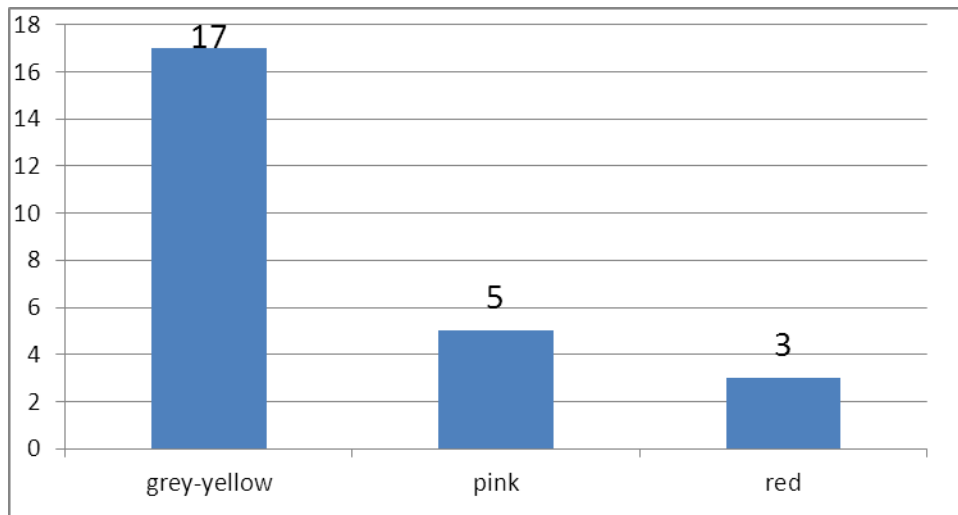
Figure 1. **The joint fluid color from the patients, diagnosed with the cranial cruciate ligament rupture, samples.**
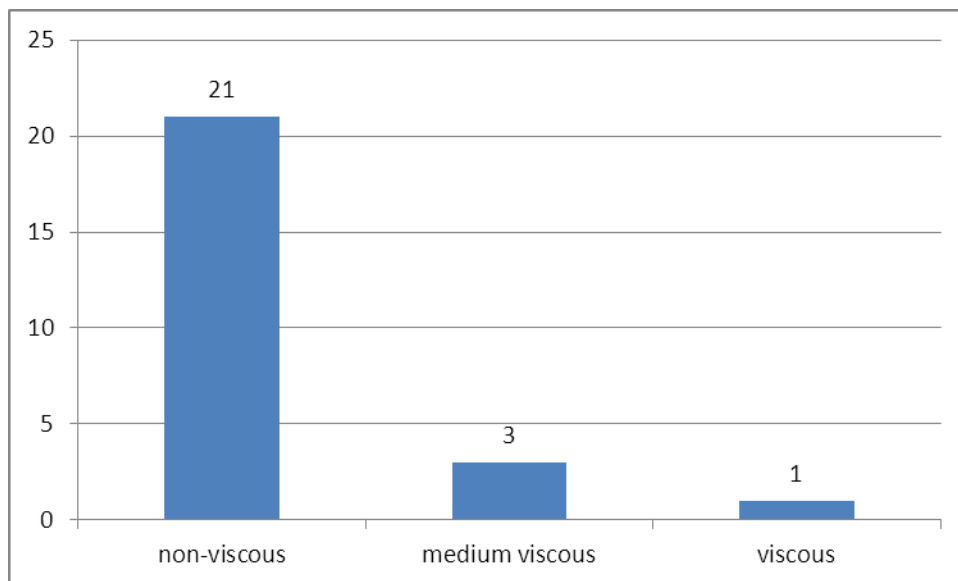


Figure 2. **Viscosity study from patients, diagnosed with the cranial cruciate ligament rupture, samples.**

All dogs with the cranial cruciate ligament rupture limped. For this reason the owners turned to veterinary clinics.

In all cases synovial fluid from the joints of control group dogs was of gray-yellow color, medium viscosity, clear and formed mucin clot.

In assessing the results, it was found that from synovial fluid samples of the patients diagnosed with the cranial cruciate ligament rupture were obtained more than twice the number of leukocytes, as compared with the samples from the control group (Figure 3.).
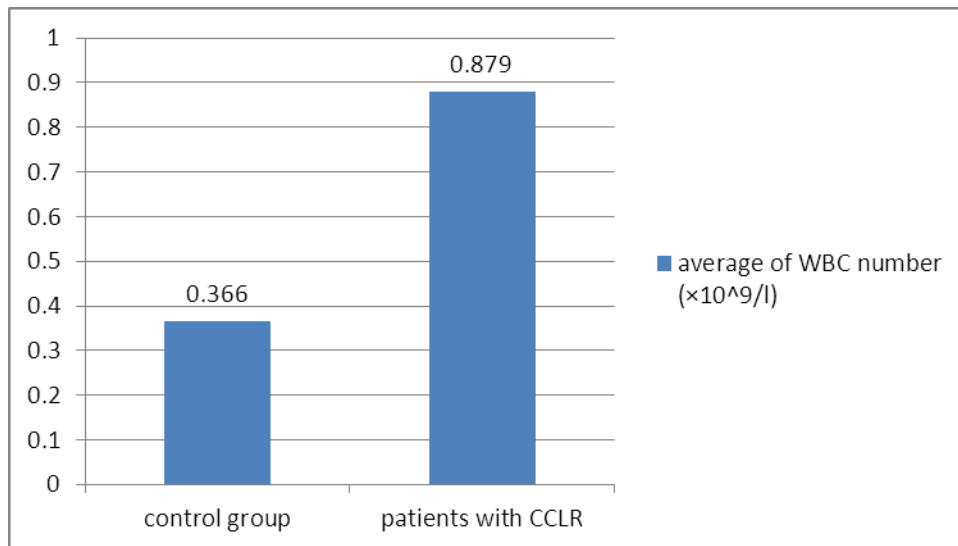
Figure 3. **The total leukocytes number in synovial fluid samples of the control group and the patients with CCLR.**

A small amount of erythrocytes was found in the control group samples. From synovial fluid samples of patients diagnosed with the cranial cruciate ligament rupture total erythrocytes number was more than 20 times higher (Figure 4.).
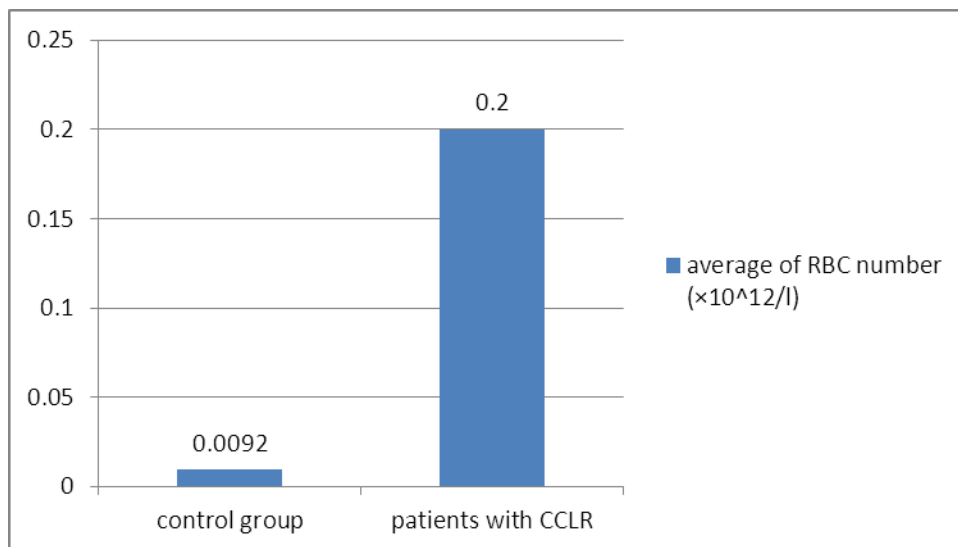


Figure 4. **The total erythrocytes number in synovial fluid samples of the control group and the patients with CCLR.**

Neutrophil level from samples of the patients with the cranial cruciate ligament rupture was 13 percent higher than in the control group samples. Mononuclear amount was 12 percent larger in group of the patients with the cranial cruciate ligament rupture compared with the results obtained from the control group samples (Figure 5.).
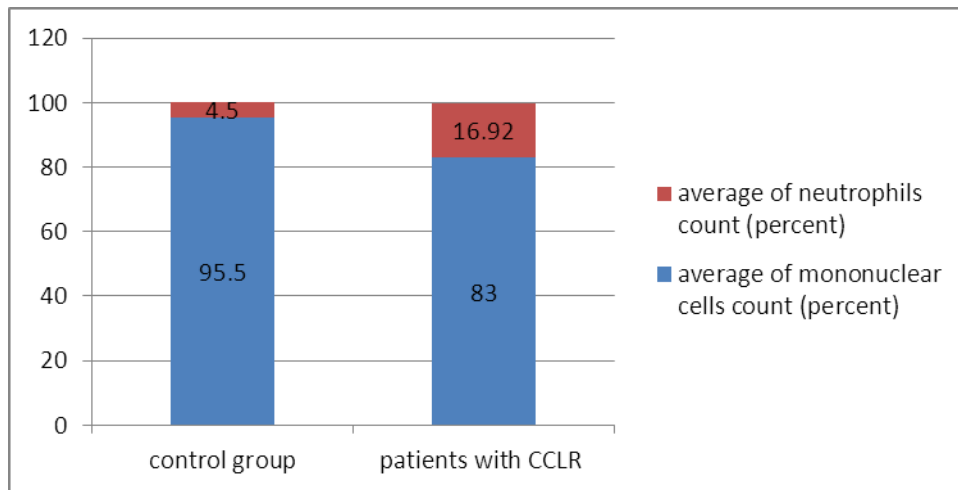
Figure 5. **Neutrophil and mononuclear cells amount in the control group and the patients with CCLR.**

In reference to mononuclear and neutrophil level changes, synovial fluid samples were divided into three groups: normal, specific to NIMP and specific to OA.

All synovial fluid samples from the control group were normal. The results of the patients diagnosed with the cranial cruciate ligament rupture were various: the most were specific to NIMP (n = 11), the same number specific to OA (n = 7) and normal (n = 7). The rating is given in Figure 6.
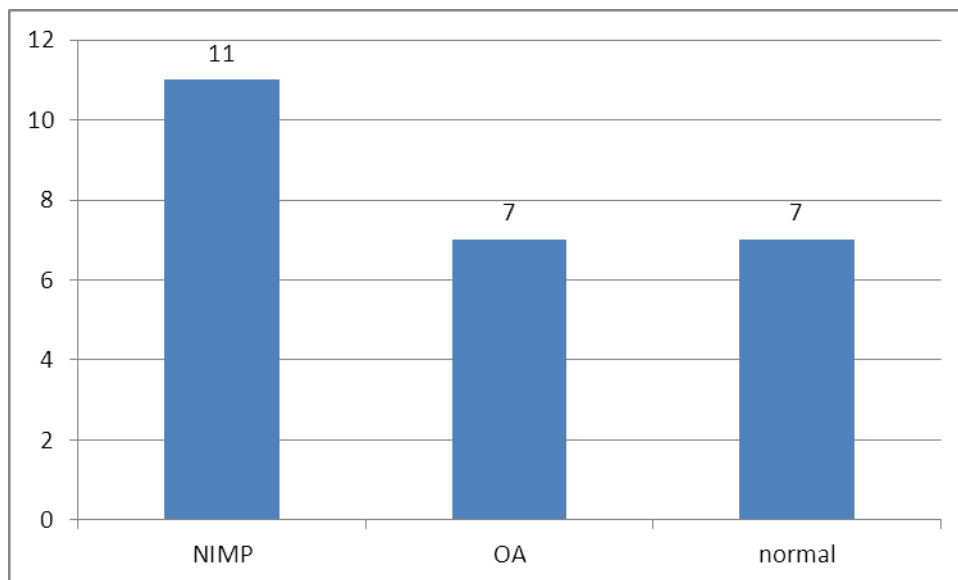


Figure 6. **The assessment of synovial fluid cells changes in group of the patients with CCLR.**

Unstable joint of all of the mentioned dogs who have been diagnosed with the cranial cruciate ligament rupture was treated surgically with DeAngeli method performing lateral parapatellar arthrotomy and extracapsular joint stabilization by implanting an artificial ligament (Figure 7).

**60**

Figure 7**. Lateral parapatellar arthrotomy of the knee joint.**

Synovial fluid and blood plasma biomarkers CRP tests were analyzed in the canine control and study groups. It was found that the amount of CRP from synovial fluid and blood plasma samples of the cranial cruciate ligament rupture group was higher than from the control group (Figure 8.).
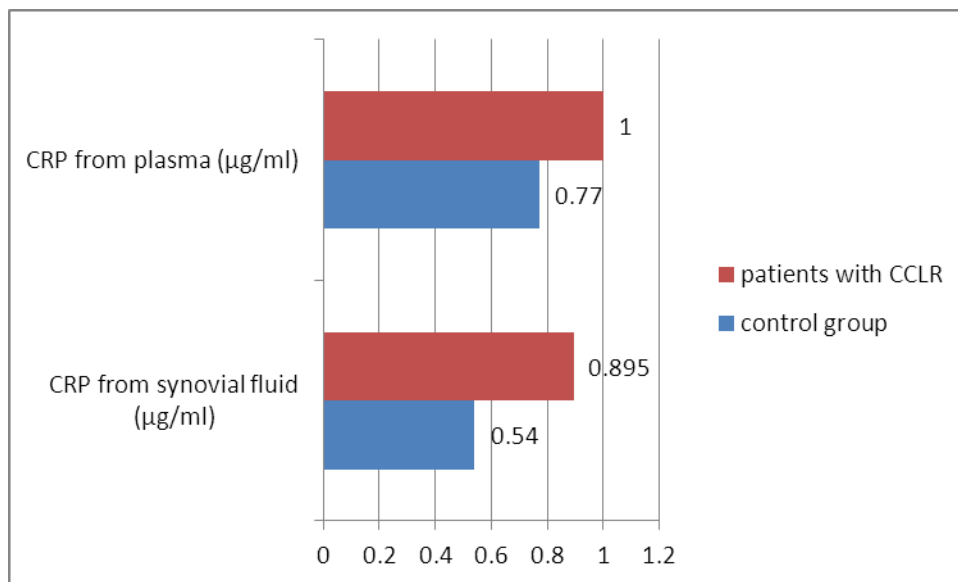


Figure 8. **Biomarker CRP level from synovial fluid and blood plasma samples of the control and the patients with the cranial cruciate ligament rupture groups.**

COMP biomarker from synovial fluid and blood plasma studies in the control and the cranial cruciate ligament rupture groups showed that higher COMP amount was from synovial fluid and blood plasma samples of the cranial cruciate ligament rupture group compared to the

results obtained from synovial fluid and blood plasma samples of the control group (Figure 9.).
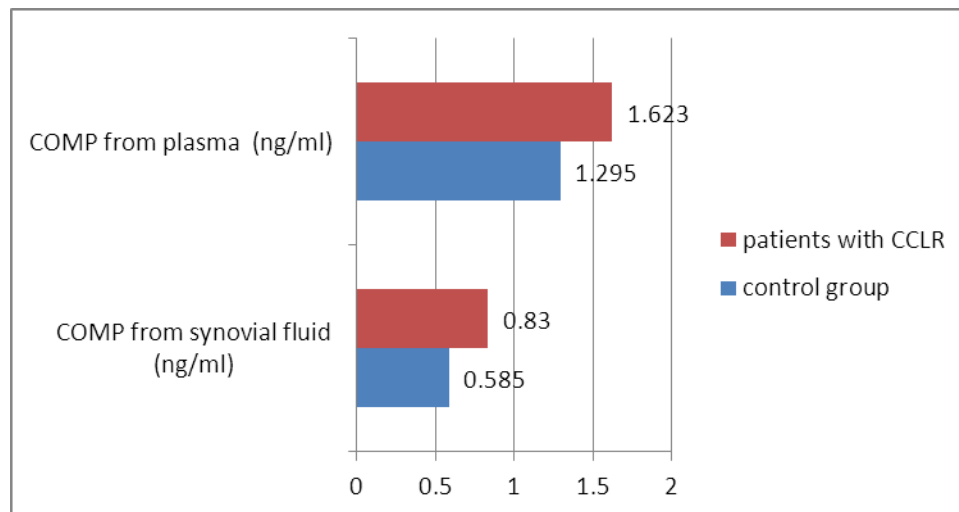


Figure 9. **Biomarker COMP level from synovial fluid and blood plasma samples of the control and the patients with the cranial cruciate ligament rupture groups.**

Our research results showed that the tests of the physical properties, cells number and composition, biomarkers of synovial fluid provide a lot of information, analyzing the causes and consequences of lameness. In reference to the research results, we can say that a lot of information about inflammation extent, articular cartilage damage can be obtained from biomarker studies.

**CONCLUSIONS**
1.  It was assessed, that 68 percent of joint fluid samples from dogs with the cranial cruciate ligament rupture were of the gray-yellow color, 20 percent of the pink and 12 percent of the red.
2.  84 percent of synovial fluid samples from the joints of patients who have been diagnosed with CCLR were non-viscous, 12 percent - medium viscous, 4 percent - viscous. Mucin clot test in 84 percent of samples was negative, in 16 percent samples – positive, all samples were clear.
3.  Compared with the synovial fluid samples of the control group, in samples of the patients with orthopedic diseases were evaluated twice as high leukocytes (WBC) and almost 20 times higher erythrocytes (RBC) level.
4.  Mononuclear cells count from the tested patient group's synovial fluid samples was 12 percent higher, and the neutrophil count 13 percent higher compared to the control group.
5.  Neutrophil and mononuclear cells count in the canine control group's samples was within the mark, in 44 percent of cases of tested patients group these cells corresponded to changes specific to nonerosive immune-mediated arthritis, 28 percent - changes specific to osteoarthritis and 28 percent were within the mark.
6.  Biomarker's CRP level was higher in blood plasma, but not in synovial fluid. Biomarker's CRP level in blood plasma and synovial fluid samples of tested patients group was almost twice as high as compared to the corresponding samples of the control group.

7. Biomarker's COMP level was twice higher in blood plasma. In synovial fluid samples of the control group and tested patients this marker was similar, but it's level in blood plasma was higher of tested group patients samples.

**REFERENCES**
1. Bahr, T., Preisinger, R., Kalm, E. (1995) Untersuchungen zur Zellzahl und Melkbarkeit beim Rind. Mitteilung: Genetische Parameter der Melkbarkeit. Ziichtungskunde 67. P.105-116.
2. Baker R., Lumsden J. H. (2000) Color atlas of cytology of the dog and cat. USA, Mosby, P. 209-215.
3. Bansal, B. K., J. Hamann, N. T. Grabowski, and K. B., Singh (2005) Variation in the composition of selected milk fraction samples from healthy and mastitic quarters, and its significance for mastitis diagnosis. J. Dairy Res. Vol. 72, P.144–152.
4. Brunberg L. (2001) Diagnosing lameness in dogs. Vienna-Berlin, Blackwell Sciences, P.229.
5. Cohen A.S., Brandt K.D., Krey P.K. (1975) Synovial fluid in laboratory diagnostic procedures. JAV, Brown and Co, P.1154 – 1195.
6. Cowell R. L., Tyler R. D. (1989) Diagnostic cytology of the dog and cat. USA, American veterinary publications, P.121-136.
7. Davidson M., Else R.W., Lumsden J.H. (1998) Manual of small animal clinical pathology. UK, BSAVA, P.135-136.
8. Fossum T.W., Duprey L.P., O'connor D. (2007) Small animal surgery. USA, Mosby Elsevier, P.930-1333.
9. Houlton J.E.F., Cook J.L., Innes J.F., Bobbs S.J.L. (2006) Manual of canine and feline musculosletal disorders. UK, BSAVA, P.21-26.
10. Johnson K. A., Hart R. C., Kochevar D., Hulse D. A. (2001) Concentrations of chondroitin sulfate epitopes 3B3 and 7D4 in synovial fluid after itra-articular and extracapsular reconstruction of the cranial cruciate ligament in dogs. AJVR; Vol. 62, Nr.4, P.581-587.
11. Radin R.E., Meyer D.J. (2010) Canine and feline cytology a color atlas and interpretation guide. USA, Saunders Elsevier, antras leidimas, P.309.
12. Slatter D. (1995) Textbook of small animal surgery. USA, W.B. Saunders company, P.618-622.
13. Villiers E., Blackwood L. (2005) BSAVA Manual of canine and feline pathology. UK, BSAVA, P.355-362.
14. Kjelgaard-Hansen M.(2004) Canine C-reactive protein – a study of applicability of canine serum C-reactive protein. PhD thesis, Frederiksberg, Denmark, P.119.
15. Paul C., Hansson L.O., Seierstad S.L., Kriz K. (2011) Canine C-reactive protein – a clinical guide. Life assays, Lund, Sweden, T. 4030, P.10.
16. Tobias K.M., Johnston S.A. (2012) Veterinary surgery small animal. UK. Elsevier Saunders, P.1078-1111.