MEDICAL UNIVERSITY "PROF. DR. PARASKEV STOYANOV" - VARNA

FACULTY OF MEDICINE

DEPARTMENT OF GENERAL MEDICINE

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RESEARCH PARTICIPATION OF HUMORAL FACTORS OF CONGENITAL IMMUNITY IN THE PATHOGENESIS IN ACTIVATED OSTEOARTHRITIS

ABSTRACT

of a dissertation for the award of the educational and scientific degree of "Doctor" in the specialty of "General Medicine"

Supervisor: Assoc. Prof. Zhenya Ruseva, MD

Varna, 2024

The dissertation consists of 120 typed pages and is illustrated with 24 tables, 18 figures, and 6 histograms. The list of cited literature includes 237 titles, with 4 in Cyrillic and 233 in Latin script.

Note: The numbering of tables, figures, and histograms in the abstract does not correspond to those in the dissertation.

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Materials for the defense are published on the website of the Medical University "Prof. Dr. Paraskev Stoyanov" - Varna and are available at the Department of General Medicine at the Medical University "Prof. Dr. Paraskev Stoyanov" - Varna.

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ABBREVIATIONS USED:

Latin Abbreviations:

ACR - American College of Rheumatology
CRP - C-reactive protein C - complement
CR - complement receptors
C5aR - complement receptor C5a
C3aR - complement receptor C3a
DAMPs - damage-associated molecular patterns
ECM - extracellular matrix
IL - Interleukin
MAC - membrane attack complex
MMP - matrix metalloproteinases
NF-κB - nuclear factor kappa beta
PG - prostaglandin
PAMPs - pathogen-associated molecular patterns
SM - synovial membrane
TNF - Tumor Necrosis Factor
TLR - toll-like receptor

Cyrillic Abbreviations:

АХ - arterial pressure
ГК - glucocorticoids
Ео - eosinophils
ИК - immune complexes
Ма - macrophages
Мо - monocytes
мл - milliliter
мкл - microliter
ОА - osteoarthritis
ОК - osteoclasts
РА - Rheumatoid Arthritis
СЗСТ - connective tissue diseases
ЧД – liver

1. LITERATURE REVIEW

Osteoarthritis (OA) is a disease of the synovial joint characterized by three main pathogenetic elements:

• Cartilage loss

• Remodeling of the adjacent bone

• Associated low-grade local inflammation.

Formerly classified as a non-inflammatory condition due to the absence of systemic inflammation and neutrophils in the synovial fluid, it is now considered that low-grade local inflammation is the driving force behind the degenerative process.

The destruction of cartilage, a distinctive feature of OA, is attributed to the activity of matrix metalloproteinases and aggrecanases such as MMP1, MMP2, MMP13, and ADAMTS5. Following the initial proliferative phase, hypocellularity occurs in degenerating hyaline cartilage with reduced synthesis of extracellular matrix. The chondrocyte count significantly decreases with the progression of joint degeneration due to premature cell death through necrosis and apoptosis of chondrocytes. Disruption of the collagen network occurs due to the breaking of links between collagen fibrils. Proteoglycan degradation begins, reducing the size of their aggregates. They absorb water, becoming more accessible to enzymatic attack by metalloproteinases. The production of metalloproteinases increases, and the production of tissue inhibitors of metalloproteinases decreases. Changes in hyaline cartilage result in alterations in the cellular and molecular composition of the synovial fluid.

Inflammation of the synovial membrane, clinically most commonly manifested as effusion, is a characteristic feature of activated OA. Under conditions of low-grade inflammation (OA activation), the synovium hyperplasias, primarily infiltrated by macrophages and a smaller number of B and T lymphocytes and natural killer cells. In OA, synovitis is more focal, unlike in rheumatoid arthritis (RA). Synovitis can show significant changes even before visible cartilage degeneration occurs. Synovitis is associated with pain, functional deficit, and can even be an independent factor in the onset of OA and accelerate the structural progression of the disease.

Products of cartilage degradation released into the synovial fluid are phagocytosed by synovial cells, maintaining synovial inflammation. In turn, activated synovial cells in the inflamed synovium produce catabolic and pro-inflammatory mediators leading to excessive production of proteolytic enzymes responsible for cartilage degradation, creating a positive feedback loop. The inflammatory response is intensified by activated synovial T cells, B cells, and infiltrating macrophages. Cartilage fragments can play a role as neoantigens, inducing aseptic inflammation.

Low-grade inflammation progresses through the production of cytokines by chondrocytes, type A synoviocytes, mononuclear cells, and subchondral osteoblasts: IL-1β,

IL6, IL8, IL15, IL17, IL18, and TNF-alpha, contributing to the catabolic cascade characteristic of OA.

OA is not associated with a pronounced adaptive immune response. Currently, there is evidence from numerous studies regarding the leading role of the innate immune system in OA (Figure 1). The innate immune system is the first line of immune defense.

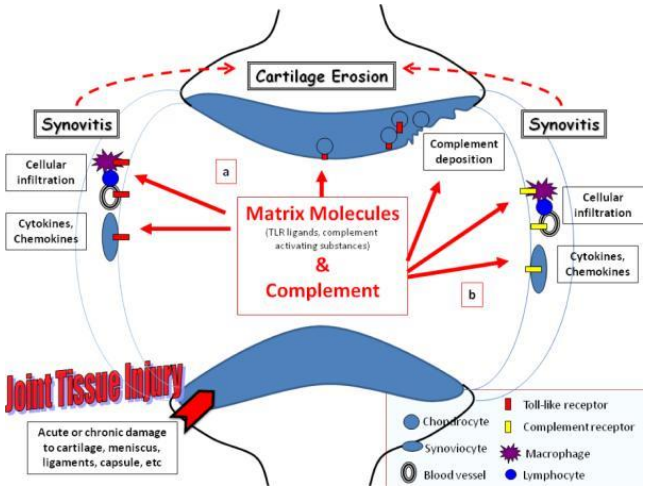


Figure #1. Innate immune system - first line of immune defense. Activation model of complement in OA.

Source: Editor-In-Chief C. Michael Gibson, M.S., M.D. Osteoarthtitis pathophysiology (Internet open access)

Activation of the innate immune response begins with the stimulation of cell membrane receptors that recognize molecules secreted by pathogens - PAMPs (pathogen-associated molecular patterns). Outside of infection, the same receptors recognize and activate in response to cell damage and extracellular matrix. In conditions of degenerative processes in the joint, components of the extracellular matrix (ECM), fibronectin isoforms, and fragments of hyaluronic acid are presumed ligands for PAMPs. In these cases, PAMPs can be activated through endogenous damage-associated molecular patterns (DAMPs) rather than microbial ligands.

In the group of DAMPs are toll-like receptors (TLRs), constitutively expressed on the cell membrane of various cells, as well as intracellularly located. Different ligands activate different TLRs. Disruption of matrix homeostasis in osteoarthritis (OA) is an example of the activation of these receptors during chronic damage. The subsequent cellular response involves the activation of specific transcription factors, with a key role played by nuclear factor κB (NF-

κB). TLR7 is linked to the X chromosome, partially explaining the higher frequency of gonarthrosis in females. Chondrocytes can also serve as targets for TLR activation. Numerous matrix metalloproteinases produced by cartilage in OA are dependent on NF-κB activation. Despite the unclear role of TLRs in OA, targeting these signaling pathways in osteoarthritic pathogenesis is a potential new approach to modulating the balance between anabolic and catabolic processes in hyaline cartilage. Recent studies have further clarified that systemic inflammation, through inflammatory mediators, may reprogram chondrocytes towards hypertrophic differentiation and catabolic responses via the NF-κB pathway and autophagy mechanisms.

The humoral factors of the innate immune system include CRP and the complement system. When an exogenous or endogenous trigger activates the complement system, proteases cleave complement proteins, activate them, and initiate its cascade. The three pathways of complement activation (classical, alternative, and lectin) share a common key activation step - the C3 convertase. It cleaves and activates complement component C3, generating C3a and C3b. The products of C3 degradation can serve as an assessment of complement system activation. Unlike the mentioned C3 fragments with a short half-life, C3c is a reliable stable biomarker for assessing plasma C3 levels. The conversion of C3 to C3c occurs over approximately an hour at body temperature. There are complement receptors (CR) on the surface of many cells, whose function is to adhere to objects targeted for phagocytosis. C3R activates the complement cascade itself, as well as the cell's oxygen metabolism, which is crucial for phagocytosis. Each of the complement cascade pathways has activation triggers. The ultimate result of complement activation is lysis of target cells, opsonization, followed by chemotaxis of macrophages and neutrophils, phagocytosis, and transport of immune complexes to Kupffer cells in the liver and to the spleen for clearance. The complement system, as part of the innate immune system, is one of the first lines of defense against invading pathogens. Apart from this evolutionarily determined protective function, it also plays a role in removing cell debris and immune complexes, opsonization, and stimulating B- and T-lymphocytes. Products of cartilage degradation released during joint degeneration, apoptotic chondrocytes, represent a distinct class of powerful complement modulators. Various components of the extracellular matrix (ECM) and their fragments in degenerative joints can activate complement. Complement activation in the joint can occur directly due to mechanical stress and through local production of complement factors by type A synoviocytes during osteoarthritic disease activation. In OA, the main cells in synovial fluid are chondrocytes and synoviocytes. Complement fractions bind to complement receptors on the surface of their cell membranes via TLR. The complement system is involved in multiple processes during osteoarthritic disease: chondrocyte degeneration, ECM degradation, low-grade inflammation in the arthritic joint, cell lysis, synovitis, unbalanced bone remodeling, osteophyte formation, and reparative processes such as neoangiogenesis. Data from multiple studies indicate that the role of the complement cascade in OA is still not fully understood; it remains uncertain whether the complement cascade acts only as a cleansing system or as a leading pathogenetic factor in activated OA. Synovial inflammatory reactions involve the synthesis and release of a variety of cytokines and chemokines. The humoral factors of inflammation are a potent hepatostimulating factor for the synthesis of CRP and other acute-phase proteins. IL6,

produced by macrophages, monocytes, dendritic cells, and adipocytes, is one of the main hepatostimulating factors for acute-phase protein synthesis to maintain homeostasis. Native CRP undergoes calcium-dependent binding to specific protein molecules such as phosphorylcholine, nuclear chromatin, and plasma very low-density lipoproteins. These aggregates activate the complement, which, through the MAC, stimulates platelets and phagocytic cells, opsonizing and eliminating foreign cells, products of cell destruction, and apoptosis. The concentration of proteins in synovial fluid depends on their concentration in plasma, molecular weight and shape, permeability of the synovial membrane, and local production or consumption of that protein. Proteins in synovial fluid are up to 20% of their content in blood plasma. The accepted norm for complement levels in synovial fluid is about 10% of serum levels. Due to complement consumption in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), bacterial arthritis, crystal-induced arthritis, these levels in specific cases are about 30% lower. Current studies on OA indicate higher levels compared to healthy individuals.

3. Objectives and Tasks of the Dissertation

The hypothesis of this study aims to provide evidence for the direct involvement of humoral factors of the innate immune system in the pathogenesis of osteoarthritic disease. This involves removing destructive and apoptotic products from the degenerative process within the joint space and addressing the maintenance of low-grade inflammation in the arthritic joint.

The main objectives of the study are to examine the levels of certain humoral factors of the innate immune system to establish their clinical significance in patients with activated osteoarthritis.

To achieve this goal, the following tasks were outlined:

1. Analyze scientific literature regarding the involvement of humoral factors of the innate immune system in the inflammatory process and formulate a model of low-grade local inflammation in osteoarthritis.
2. Select patients with activated gonarthrosis according to ACR criteria (1991) and the absence of other conditions that could cause effusion.
3. Verify the radiological stage according to the Kellgren-Lewrence scale.
4. Use ultrasonography to confirm synovial effusion and during controlled arthrocentesis.
5. Investigate the humoral immune indicators CRP, C3, and C4 complement fractions in blood plasma and synovial fluid.
6. Conduct a comparative analysis of their levels in both bodily fluids and search for correlation dependencies based on the radiological stages of gonarthrosis according to Kellgren-Lewrence and the gender of the patients.

4. Materials and Methods

4.1. Clinical Material:

Patients with activated knee osteoarthritis were selected for the study, ensuring the absence of anamnestic, clinical, and laboratory data for other conditions that could cause effusion. The diagnosis of "gonarthrosis" was made based on ACR (1991) clinical and radiographic criteria. A total of 156 participants, aged between 43 and 90 years, meeting ACR criteria for knee osteoarthritis, were included in the study, with 121 women and 35 men. All participants signed an informed consent form. The study was approved by the Ethics Committee of UMBAL Burgas AD.

4.2. Criteria:

4.2.1. Inclusion Criteria:

The sole inclusion criterion was the presence of effusion in the knee joint (a sign of activated OA) in patients with osteoarthritic disease, regardless of the radiological stage according to Kellgren-Lewrence.

4.2.2. Exclusion Criteria:

- Comorbidity with other rheumatic diseases, including RA, psoriatic arthritis, spondyloarthritis, SLE, vasculitis, gout, fibromyalgia, etc.
- Anticoagulant use, blood circulation disorders.
- Previous intra-articular fracture or documented high-energy trauma to the lower limb.
- Severe decompensated comorbidities; very low ANA values.
- Immunocompromised patients.
- History of vasovagal shock, pregnancy, breastfeeding.
- Local skin infection, psoriatic plaque in the area of potential arthrocentesis.
- History of treatment with systemic corticosteroids in the last 3 months or intra-articular depot steroids in the last 6 months.
- NSAIDs intake in the last 7 days.
- Patient refusal for arthrocentesis.

4.3.1. Clinical Methods

After obtaining informed consent and consent for the provision of personal data, detailed medical histories were collected from all study participants, and their objective condition, including a complete joint status, was assessed. Inclusive and exclusive criteria were evaluated, and the approved protocol was implemented, including venipuncture and arthrocentesis.

A venipuncture was performed to collect 3 ml of venous blood in EDTA-monovettes. After plasma separation, it was analyzed for levels of CRP, C3, and C4 complement fractions.

Arthrocentesis was performed with an extended knee joint using lateral access to the suprapatellar bursa. Synovial fluid obtained through arthrocentesis was also placed in EDTA-monovettes. After centrifugation, the supernatant (A) was separated from the "pellet" (B), and both samples were analyzed for soluble and cell-bound fractions of CRP, C3, and C4.

4.3.2. Laboratory Methods

The collected blood plasma and samples A and B from synovial fluid, obtained as described above, were sent to a certified clinical laboratory. Each sample was examined for CRP, C3, and C4 complement fractions. Plasma and synovial concentration values were then compared, along with the values in fractions A and B of synovial fluid. The results underwent statistical analysis, followed by discussion and conclusions.

The tests were conducted at the certified "Lina" laboratory in Burgas on a Cobas c501 instrument with Roche reagents and calibration for C3, C4, CRP - c.f.a.c. Proteins. The method is standardized against an internal method traceable to CRM 470 (RPPHS – reference preparation for proteins in human serum). Quality control has been performed using PCCC Level 1 and Level 2.

CRP was examined using an immunoturbidimetric test with particle enhancement. The method is based on human CRP, which agglutinates with latex particles coated with monoclonal anti-CRP antibodies. Aggregates are determined turbidimetrically. The reference values are below 6.0 mg/l.

C3 and C4 were examined using an immunoturbidimetric analysis, a laboratory-standardized method. The method is based on the reaction where human C3 and C4 form a precipitate with specific antisera, determined turbidimetrically at 340 nm.

Table #1. Investigated Indicators and Their Reference Range

Indicators	Reporting scope	PU
1. CRP	0,3 – 350 mg/l	< 6,0 mg/l
2. C3	0,3 – 5,0 g/l	0,9 – 1,8 g/l
3. C4	0,06 – 1,0 g/l	0,1 – 0,4 g/l

Reference values for C3 are: 0.90 – 1.8 g/l, and for C4: 0.1 – 0.4 g/l.

In our study, we adhered to the following **Work Protocol:**

1. **Blood Sample Collection for Analysis:** Blood was collected in EDTA monovettes, and within 20 minutes of venipuncture, it was centrifuged at 4000 rpm for 10 minutes. The plasma was separated from the formed elements, and the obtained plasma was stored in transport monovettes. The same was frozen at -20 degrees Celsius for a maximum of 8 days. After thawing, the samples were sent to the laboratory for analysis.
2. **Synovial Fluid Collection for Analysis:** Arthrocentesis of the knee joint was performed with the patient's knee in an extended position. The obtained synovial fluid was placed in EDTA monovettes. After centrifugation, the supernatant (A) was separated from the "pellet" (B), and both samples were analyzed for soluble and cell-bound fractions of CRP, C3, and C4. One milliliter of synovial fluid, aspirated simultaneously from each patient, was placed in an EDTA monovette, to which 400 IU of hyaluronidase was added. It was then centrifuged at 4000 rpm for 10 minutes. In the next step, we divided it equally into: a sample with supernatant A and a sample with centrifugate (or the so-called "pellet" B). Both synovial fluid fractions were placed in separate transport monovettes, and 10 µl of a protease inhibitor cocktail was added to sample B, following the manufacturer's protocol. The samples were frozen at -20 degrees Celsius for a maximum of 8 days. After thawing, they were sent to the laboratory for analysis.

We divided the study into two stages.

1. The first stage was conducted with 50 patients, where we examined the levels of CRP, C3, C4 in blood plasma and synovial fluid without fractionating synovial fluid. The aim was to compare the obtained ratios with the generally accepted values. These results from the representative sample of the Bulgarian population were compared with data from global studies.
2. The second stage, with the remaining 106 patients, involved centrifuging the synovial fluid, separating fractions A and B, and examining each of them separately for levels of the mentioned proteins. The goal was to investigate both soluble and cell-bound C3 and C4. Subsequently, we performed a comparative analysis of synovial and plasma concentrations of CRP and C3 and C4, as well as differences in the values of these indicators in the different fractions of synovial fluid – A and B. We sought a correlation between the levels of humoral factors and different radiographic stages according to Kellgren-Lewrence, as well as the presence of a correlation with the gender of the patients. The obtained result was discussed according to the hypothesis presented above.

3.3.3. **Instrumental Methods:** To verify the radiographic stage according to the Kellgren-Lewrence scale for all study participants, conventional radiography was performed in an upright position using the "Schuss" position on the Agfa DX – D500 X-ray machine. For diagnosing the activity of gonarthrosis, we conducted ultrasound examination of the knee joint,

using the Chison Model Q9 ultrasound with a linear transducer D12L40L with a frequency range of 7-18 MHz, for instrumental confirmation of synovial effusion and assisted arthrocentesis, which was necessary for some of the patients.

4.3.4. **Statistical Methods:** The software used for statistical data processing was IBM SPSS Statistics 20.

Other statistical methods used included:

- Descriptive Statistics: Calculating mean values, standard deviation, coefficient of variation, skewness, and kurtosis.
- Correlation Analysis: Determining the strength and direction of the relationship between the examined indicators: CRP, C3, C4 in blood serum and synovial fluid A and B, as well as correlation coefficients. All stages of correlation analysis were performed: prior analysis with graphical presentation of the data in the correlation field as a scatter plot; measuring the connection using a specific formula; checking the statistical significance of the correlation coefficient, and interpreting the results obtained.
- Graphical Analysis: Using graphical analysis to visualize the obtained data.
- Analysis of Variance: Establishing statistically significant differences in the examined indicators in different age groups, gender, radiographic stage, etc.
- The main statistical method used in our work was testing the statistical hypothesis for dependent samples or the so-called Student's t-test. The alpha level of significance we set was 1% or 0.01, guaranteeing a 99% probability of the obtained results in our assumed hypothesis. We denoted the null hypothesis as H0 and the alternative hypothesis as H1.

5. RESULTS

After the statistical processing of the data obtained from the scientific study, the following results were obtained:

5.1. Target Patient Group Characteristics:

The covered target patient group is in the age range between 43 and 90 years, with an average age of 64.18 years. The average age for females is 65.68 years, and for males, it is 61 years, corresponding to statistical data for the predisposing age for knee osteoarthritis.

5.2. Analysis of CRP Levels in Synovial Fluid vs. Blood Plasma:

The analysis of CRP levels in synovial fluid compared to those in blood plasma for the first group of 50 patients is presented in Figure No. 2. The statistical method used is "Testing of Statistical Hypotheses." The following hypotheses were formulated:

- Ho: $\mu \leq 20\%$ - null hypothesis, i.e., μ in synovial fluid for the general population is $\leq$ 10% of the value in blood plasma.
- H1: $\mu > 20\%$ - alternative hypothesis, i.e., μ in synovial fluid for the general population is > 10% of the value in blood plasma. μ is the parameter of the general population, the mean value. Since it became clear from the introductory part that the average values of CRP, C3, and C4 in synovial fluid are normally around 20% for CRP and 10% for C3 and C4 of the plasma values, μ is assumed to be respectively below and above these percentages in the two hypotheses. Using the method of Testing of Statistical Hypotheses, the obtained results for the first 50 patients in our sample were compared with the generally accepted norms.

The average percentage of CRP levels in synovial fluid for the sample is 42.14% of plasma levels. p-value = 0.0000 => Reject Ho in favor of H1 at a significance level of $\alpha = 1\%$ => With 99% certainty, we can say that the average for the general population is greater than 20% (the norm according to the literature) in patients with activated knee osteoarthritis.

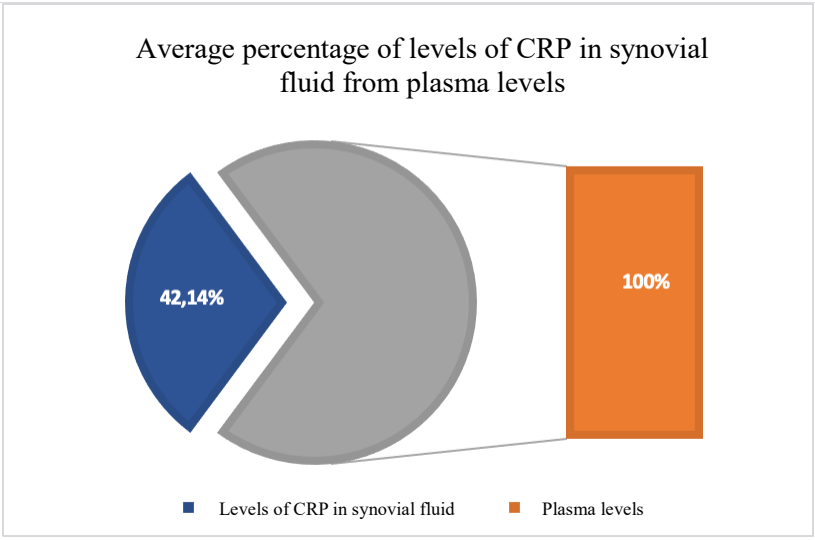


Figure No. 2: Average % of CRP Levels in Synovial Fluid vs. Plasma Levels

5.3. Analysis of C3 Levels in Synovial Fluid vs. Plasma

The average percentage of C3 values in synovial fluid for the sample is 34.90% of plasma levels.

- p-value = 0.0000 => Reject Ho in favor of H1 at a significance level of $\alpha = 1\%$ => With 99% certainty, we can say that the average for the general population is greater than 10% (norm according to the literature) in patients with activated knee osteoarthritis.

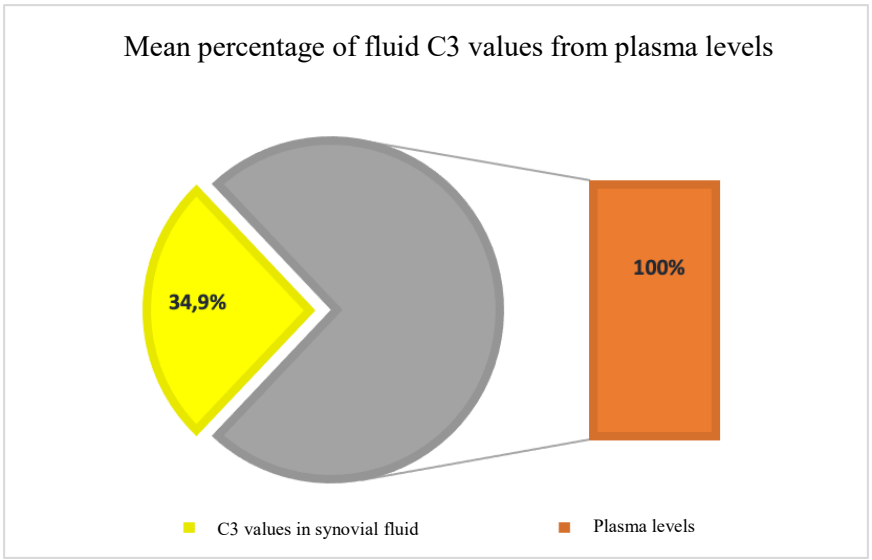


Figure No. 3: Average Percentage of C3 Values in Synovial Fluid vs. Plasma Levels

5.4. Analysis of C4 Levels in Synovial Fluid vs. Plasma

The average percentage of C4 levels in synovial fluid for the sample is 30.97% of plasma levels.

- p-value = 0.0000 => Reject Ho in favor of H1 at a significance level of α = 1% => With 99% certainty, we can say that the average for the general population is greater than 10% (norm according to the literature) in patients with activated knee osteoarthritis.

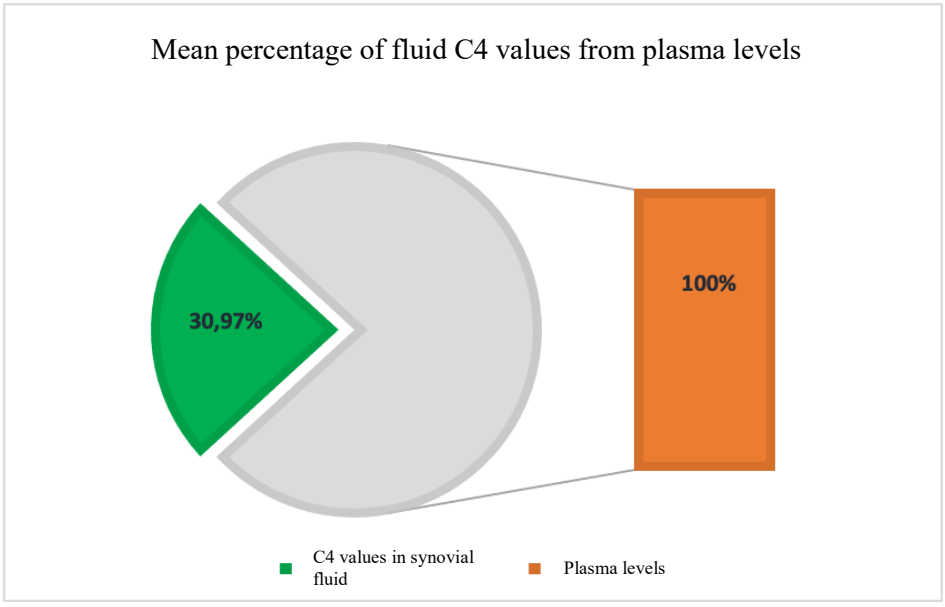


Figure No. 4: Average Percentage of C4 Levels in Synovial Fluid vs. Plasma Levels

5.5. Analysis of the Difference Between Values of the Three Investigated Proteins in Synovial Fluid A and B

Again, using the "Testing of Statistical Hypotheses" methodology:

- CRP: p-value = 0.1635 => No basis to reject Ho at any significance level: α = 1%, α = 5%, or α = 10% => No basis to claim that CRP values in fluid B are statistically larger than those in fluid A for the general population.
- C3: p-value = 0.0000 => Reject Ho in favor of H1 at a significance level of α = 1% => With 99% certainty, the difference B – A in the general population is positive, or C3 values in fluid B are larger than those in fluid A.
- C4: p-value = 0.0045 => Reject Ho in favor of H1 at a significance level of α = 1% => With 99% certainty, the difference B – A in the general population is positive, or C4 values in fluid B are larger than those in fluid A.

5.6. Investigation of the Noted Dependencies (B/A) According to the Ro Stage

Patients from the four radiological stages according to Kellgren-Lewrence are included: I Ro stage - 3, II Ro stage - 45, III Ro stage - 38, and IV stage - 20 patients (Figure No. 5).

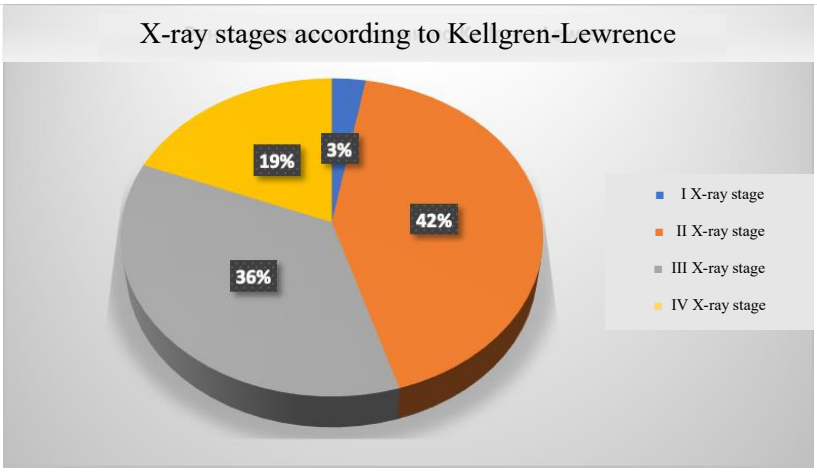


Figure No. 5: Distribution of Patients According to Radiological Stage

In this study, the samples are independent. μ1 is the mean for the I group, which includes patients in Ro stages I and II (due to the low number of patients in I Ro stage). μ2 is the mean for the II group, which includes patients in Ro stages III and IV (due to the low number of patients in IV Ro stage).

Hypotheses:

- Ho: $\mu 1 - \mu 2 \leq 0$ - null hypothesis
- H1: $\mu 1 - \mu 2 > 0$ – alternative hypothesis or
- Ho: $\mu 1 \leq \mu 2$
- H1: $\mu 1 > \mu 2$

5.6.1. CRP B/A Values Depending on the Radiological Stage (Figure No. 6)

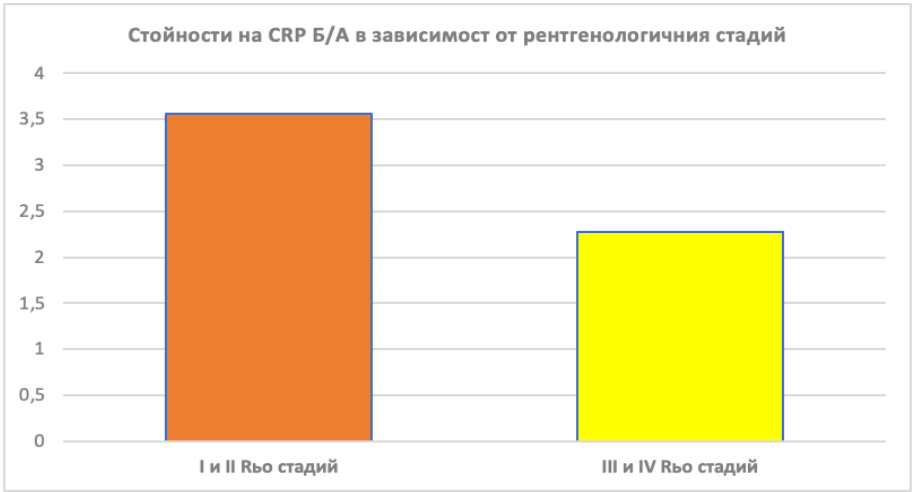


Figure No. 6: CRP B/A Values Depending on the Radiological Stage

Conclusion: With 99% certainty: p-value = 0.0805 => No basis to reject Ho in favor of H1 at a significance level of $\alpha = 1\%$ => Insufficient information to claim that the difference $\mu 1 - \mu 2$ is positive (>0) or $\mu 1 > \mu 2$ at a significance level of 1%. Thus, at a 1% significance level, there is not enough reason to accept that CRP values in synovial fluid depend on the radiological stage.

5.6.2. C3 Values Depending on the Radiological Stage (Figure No. 7)

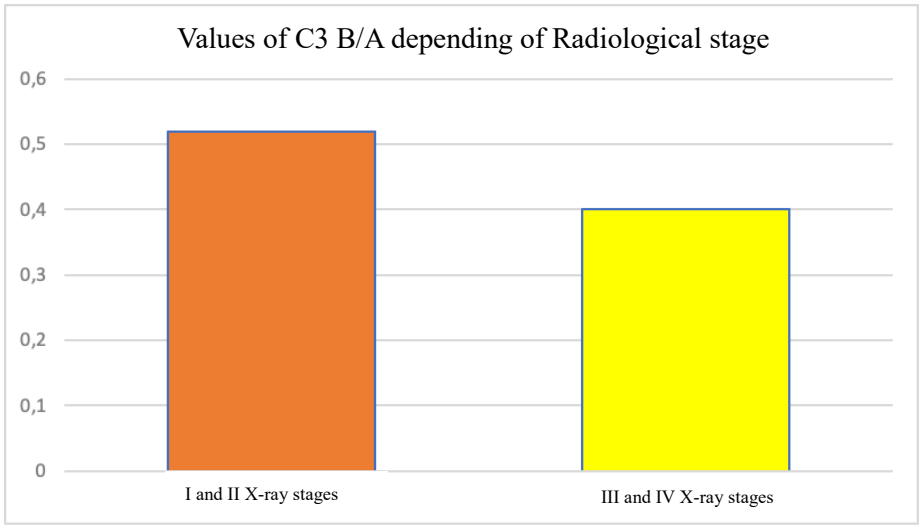


Figure No. 7: C3 B/A Values Depending on the Radiological Stage

Conclusion: p-value = 0.0030 => Reject Ho in favor of H1 at a significance level of $\alpha = 1\%$ => With 99% certainty, the difference $\mu 1 - \mu 2$ is positive, or $\mu 1 > \mu 2$. Practically, this means that C3 values in synovial fluid are higher at earlier radiological stages, i.e., I and II, compared to advanced disease – III and IV radiological stages.

5.6.3. C4 Values Depending on the Radiological Stage (Figure No. 8)

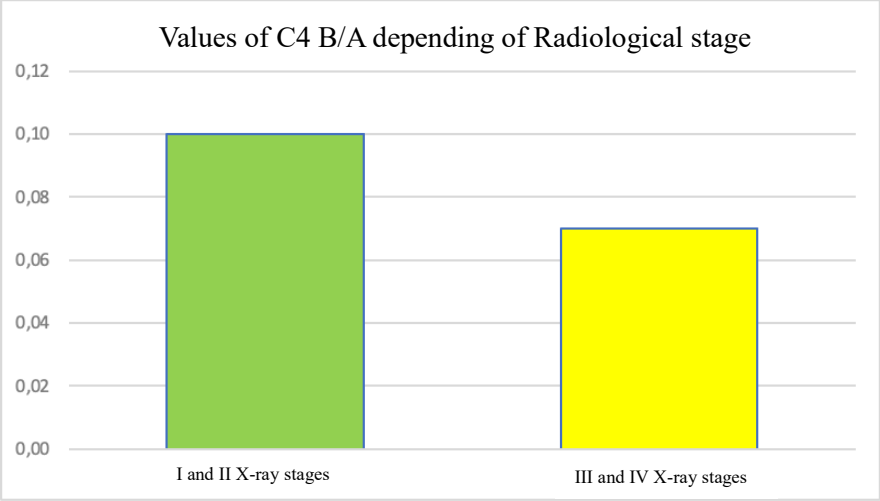


Figure No. 8: C4 B/A Values Depending on the Radiological Stage

Conclusion: p-value = 0.0005 => Reject Ho in favor of H1 at a significance level of α = 1% => With 99% certainty, the difference μ1 - μ2 is positive, or μ1 > μ2. Practically, this means that C4 values in synovial fluid, similar to the results for C3, are higher at earlier radiological stages, i.e., I and II, compared to advanced disease – III and IV radiological stages.

5.7. Graphical Analysis of the Averaged Values of the Three Investigated Indicators in the Three Investigated Samples (Figure No. 9)

	blood plasma	SF A	SF B
avarage CRP	5,9	2,86	2,72
avarage C3	1,15	0,39	0,44
avarage C4	0,29	0,09	0,09

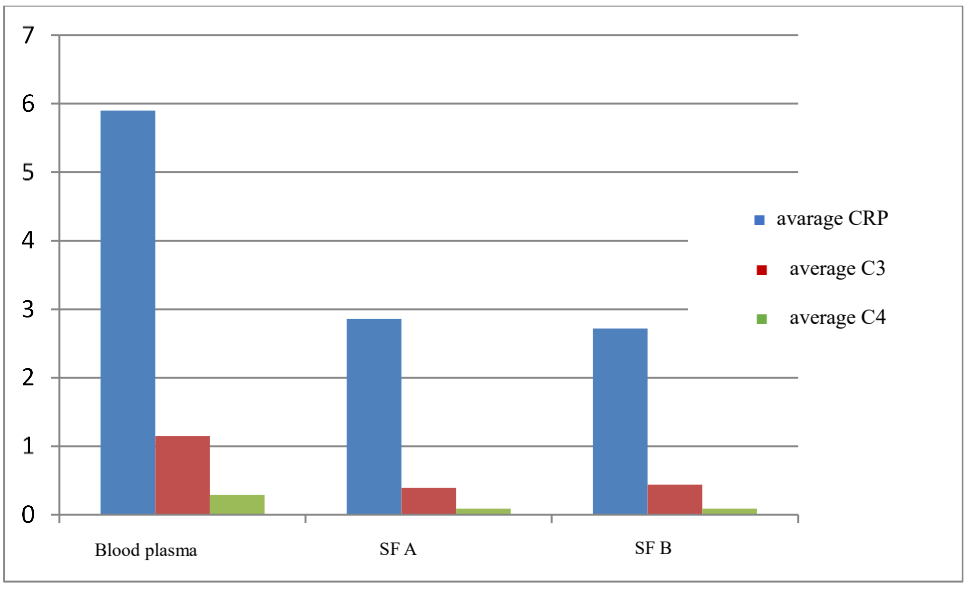
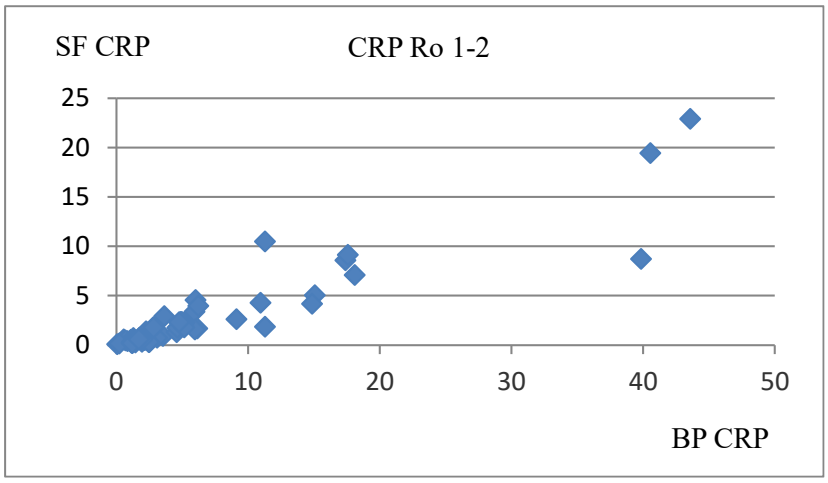


Figure No. 9. Graphical view of mean values of CRP, C3 and C4 in blood plasma, synovial fluid A and synovial fluid B.

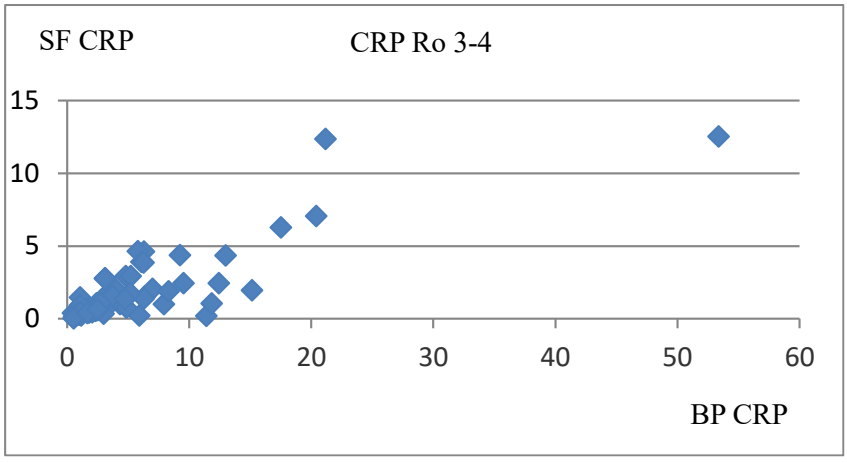
It is noteworthy that the average values of the three indicators in blood plasma are within the limits of reference norms. This confirms that these patients show no evidence of either systemic inflammation (elevated values) or autoimmune (decreased values) in these patients.

5.8. By applying variance analysis, it was again concluded that the established dependencies are more distinctly expressed in the earlier stages of the disease, but persist, albeit to a lesser degree, in the III and IV radiological stages, evident from histograms № 1, 2, 3, 4, 5, 6.



Histogram No. 1: CRP at Radiological Stages I and II
A histogram depicting CRP levels at radiological stages I and II.

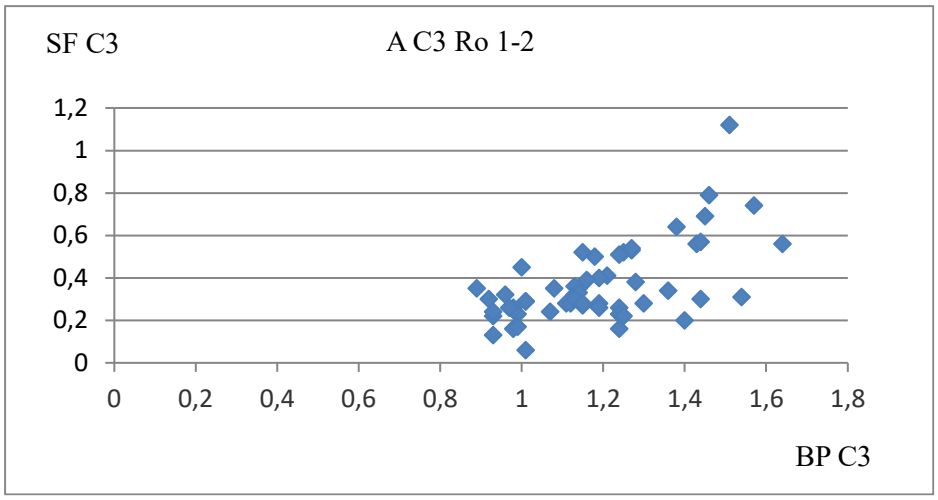
There is a very strong correlation observed, with a correlation coefficient of r = 0.92.



Histogram No. 2: CRP at Radiological Stages III and IV

A histogram illustrating CRP levels at radiological stages III and IV.

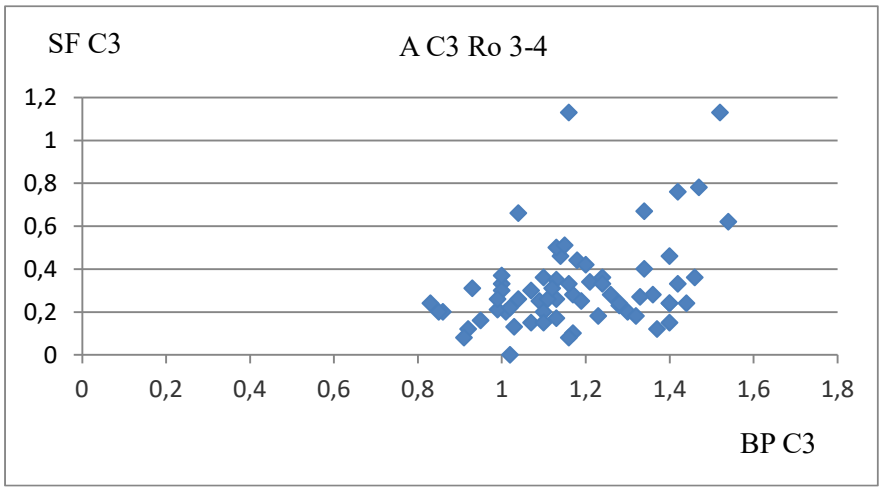
A strong correlation is observed with a correlation coefficient of r = 0.83, albeit weaker compared to the early radiological stages.



Histogram No. 3: C3 at Radiological Stages I and II

A histogram depicting C3 levels at radiological stages I and II.

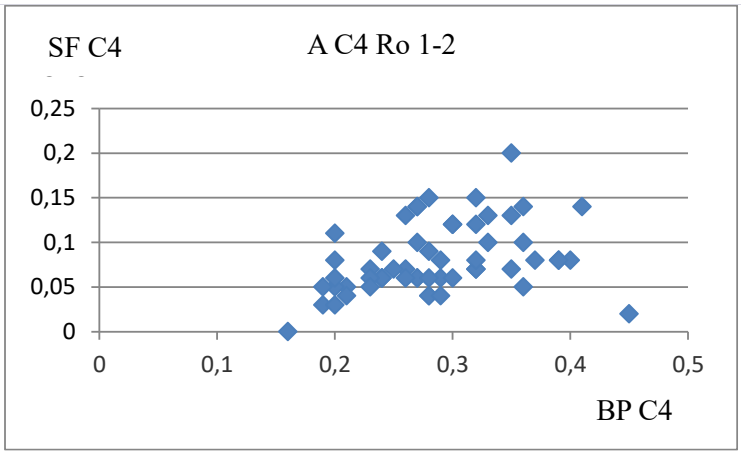
A correlation of r = 0.629 is observed, indicating a significant correlation.



Histogram No. 4: C3 at Radiological Stages III and IV

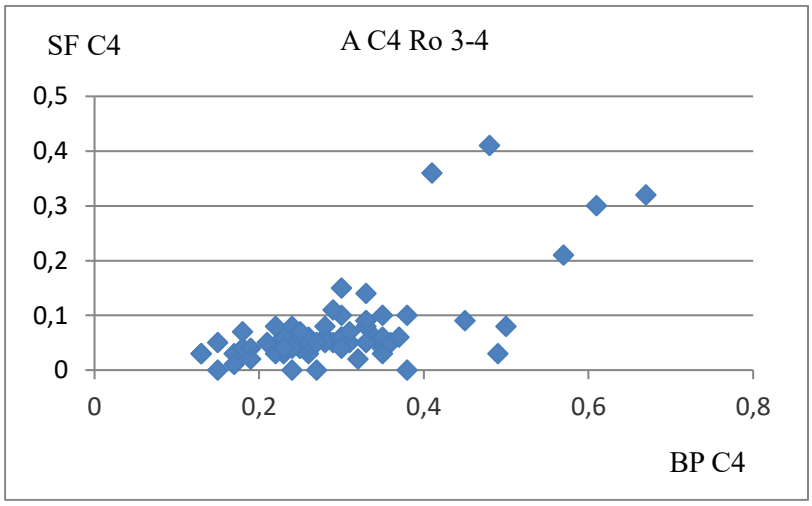
A histogram illustrating C3 levels at radiological stages III and IV.

Correlation: r = 0.388 – (moderate correlation)



Histogram No. 5: C4 at Radiological Stages I and II
A histogram depicting C4 levels at radiological stages I and II.

Correlation: r = 0.371 – (moderate correlation)



Histogram No. 6: C4 at Radiological Stages III and IV

A histogram illustrating C4 levels at radiological stages III and IV.

Correlation: r = 0.665 – (significant correlation)

6. DISCUSSION

6.1. Pre-analytical Challenges

Pre-analytical challenges pose significant obstacles, hindering the comprehensive study of the complement system in the last century. Complement proteins attach to cell membranes and are biologically designed to interact with immunoglobulins. They are known to be temperature and time-labile. The instability of samples is the primary challenge for their study. Coagulation can also activate the complement, as many coagulation enzymes can cleave complement components into activating fragments. In both cases – heat exposure or coagulation – after complement activation, measuring the fractions will yield falsely elevated results. Therefore, freezing the samples immediately after collection is recommended. In vitro coagulation triggers a rapid activation of the complement cascade. This is why complement fractions are studied in plasma rather than serum.

It has been proven that EDTA is more effective in inhibiting in vitro activation compared to citrate and heparin. Analyzing the effect of temperature and storage time on the samples to avoid falsely elevated values of C3 and C4, recommendations from this clinical study are as follows: samples should be collected in EDTA monovettes and immediately cooled to 4°C. After centrifugation, EDTA-plasma should be frozen at -70°C (if not possible immediately, this should be done within 8 hours, during which the samples should be kept at 4°C). This storage temperature ensures the absence of significant complement activation within 3 years.

According to other publications and laboratory recommendations, blood in EDTA is centrifuged for 15 minutes at a minimum of 2000-3000 rpm to separate plasma from cells. According to the same sources, the biological half-life of CRP is 2-4 hours; stability at a temperature of 20-25°C is 11 days; at 4-8°C is 2 months; at -20°C is 3 years. The biological half-life of C3 is minutes; stability at a temperature of 20-25°C is 4 days; at 4-8°C is 8 days; at -20°C is 8 days. C4 has a biological half-life of 12-24 hours; stability at a temperature of 20-25°C is 2 days; at 4-8°C is 8 days; at -20°C is 3 months.

I f synovial fluid is evaluated without prior processing, viscosity can cause uneven cell distribution in the hemocytometer, and analysis can be challenging for samples with high viscosity. If untreated joint fluid is diluted, the dilution must be done very precisely with a micropipette, and the resulting result should be corrected for the dilution factor of each sample. An alternative is the use of the enzyme hyaluronidase, which prevents the formation of mucinous clots.

Given the aforementioned conditions and recommendations for obtaining and storing the samples, we adopted the working protocol described above.

6.2. Analysis of Results

After analyzing the results obtained from the studied group of 156 patients with activated knee osteoarthritis, it is found that the values of the three studied proteins in the

synovial fluid are on average 42.14% for CRP, 34.90% for C3, and 30.97% for C4 of their values in the blood plasma, with the cited normal ranges of 10% for complement levels and up to 20% for CRP levels in a healthy joint. The relatively lower percentage for C4 compared to C3 can be explained by the fact that the innate immune system, especially the activation of the complement through the alternative pathway, plays a leading role in the inflammation during osteoarthritis. Clinical studies cited in the literature review also find elevated levels of CRP, C3, C5, C6, C7 in osteoarthritic joints, but the question arises as to why this is the case and what is the biological significance of this phenomenon. In the search for an answer to this question, we divided the synovial fluid into samples A and B. The results of the analysis reveal higher levels of C3 and C4 in sample B compared to those in sample A (p-value = 0.0000 for C3 and p-value = 0.0045 for C4). As seen in the introduction, complement fractions are fixed on the cell membrane of degenerating cells, apoptotic chondrocytes, and fragments of the extracellular matrix. With the applied methodology, we quantitatively ascertain that the higher levels of complement fractions in sample B are due to the release during processing (cryogenic cell destruction) of the complement fractions fixed on cell membranes. Departing from the evolutionarily determined role of the complement system as part of the innate immunity and the first line of defense, the study quantitatively assesses this role.

6.3. Observation on the Correlation Strength

The observation regarding the strength of the correlation between the aforementioned results and the radiological stage confirms a stronger correlation of the obtained results in the earlier stages of the disease when the activity of the repair processes is more pronounced.

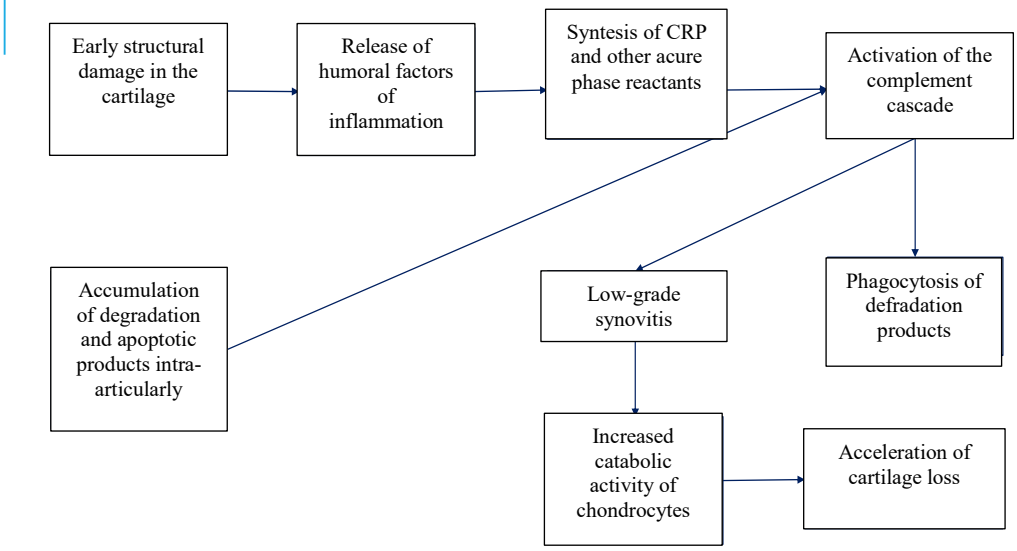
6.4. Gender Differences

No significant differences are observed between the two genders for all investigated indicators.

6.5. Low-Grade Local Inflammation Model in OA

Based on the above results, the following model of low-grade local inflammation in osteoarthritis was developed:

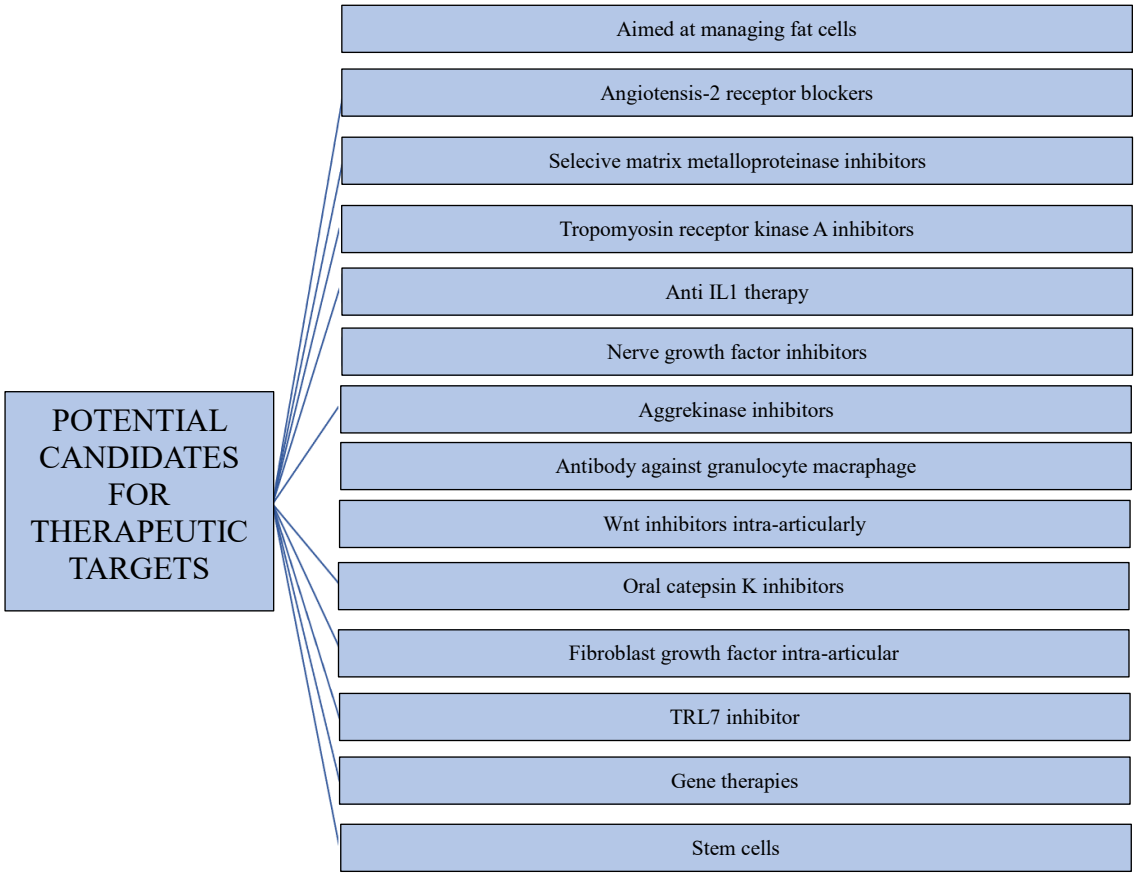
A MODEL OF LOW-GRADE INFLAMMATION IN OSTEOARTRITIS



6.6. Despite the clear role of inflammation in osteoarthritis (OA), previous attempts at anti-inflammatory therapies, including the use of systemic and intra-articular biological agents to inhibit TNFα and IL-1β, have proven disappointing. Studies continue to emphasize the heterogeneous nature of OA, with strong interest in understanding and defining OA phenotypes.

Current recommendations for knee OA treatment primarily consider the disease stage and the degree of symptom manifestation. More work is needed on the molecular pathways initiating and sustaining synovial inflammation, as understanding these pathways will provide new therapeutic possibilities.

6.7. OA is a heterogeneous, multifactorial, multidimensional, and polygenic disease. The main challenges for disease modification in OA include identifying the appropriate patient population for anti-inflammatory therapy, staging the disease, and slowing/stopping structural damage. Synovitis as a predictor of cartilage loss is more common in the early stages (evident from statistical analysis of the representative group). The need to identify and quantitatively determine inflammation in OA before the onset of irreversible structural joint damage holds future therapeutic promise, similar to the paradigm already well accepted for the early aggressive treatment of rheumatoid arthritis (RA).



T reatment of key aspects of synovial inflammation holds promise for analgesia as well as structural modification. The question arises whether controlled and balanced activation of the complement system could be a future therapeutic strategy in the treatment of osteoarthritis (OA) to prevent its progression and enhance...

7. CONCLUSIONS:

7.1. This current scientific study provides evidence for the involvement of CRP and the complement system as humoral factors of innate immunity in the pathogenesis of activated osteoarthritic disease.

7.2. It objectifies the fact that complement activation in OA occurs through the alternative pathway (higher C3 values in synovial fluid compared to C4).

7.3. Complement fractions C3 and C4 directly participate in the process of eliminating degradation products from the joint space in knee OA.

7.4. A stronger correlation between elevated clinical indicators and radiographic changes in the knee joint is observed in the early stages of the disease when the activity of reparative processes is more pronounced.

7.5. By objectifying the pathogenic role of complement in the arthritic process, this study may guide future therapeutic strategies.

7.6. The current evidence regarding the involvement of innate immunity in the pathogenesis of osteoarthritic disease and the potential of the complement system for homeostatic regulation, coping with infections, and apoptotic products support the need for further complement research in osteoarthritis. Complement proteins in synovial fluid could serve as biomarkers for disease activation on one hand and illustrate the progression of structural damage on the other.

8. Contributions:

8.1. Original Contributions:

- Quantitatively and statistically proves the leading role of innate immunity in the pathogenesis of activated osteoarthritis for the first time in Bulgaria.
- Proposes a pathogenetic model of low-grade inflammation in OA.
- Establishes, with statistical significance, the correlation between elevated levels of CRP, C3, and C4 in synovial fluid and the activation of arthritis.
- Results support the concept of pathways initiating and sustaining synovial inflammation in this patient cohort.
- Identifies a stronger correlation between elevated clinical indicators and radiographic changes in the knee joint in the early stages of the disease when reparative processes are more active.

8.2. Contributions with Scientific-Applied Nature:

- This analysis can be applied in clinical practice for pathogenetic differentiation of inflammatory processes in knee effusion.
- Controlled complement activation can be a therapeutic target in patients with OA and an inflammatory phenotype.
- Results argue for the use of drugs affecting low-grade inflammation as a disease-modifying treatment model.
- The study results confirm the need for therapeutic control of inflammation in the arthritic joint at an early radiological stage of the disease.

8.3. Confirmatory Contributions:

- The study sample from the Bulgarian population confirms previous publications' data regarding the significance of elevated levels of CRP and complement fractions in the arthritic joint—affirming the role of humoral factors of innate immunity in the pathogenesis of OA.
- The study results confirm the involvement of low-grade inflammation in disease progression.

9. PUBLICATIONS RELATED TO THE DISSERTATION:

1. I. Momcheva, I. Kazmin. "Algorithm for the Diagnosis of Osteoporosis in Premenopausal and Perimenopausal Women." Journal "Medinfo," Issue 1, January 2019, pp. 44-46.
2. I. Momcheva, I. Kazmin, S. Hristova, V. Madzhova. "Role of Inflammation in the Pathogenesis of Osteoarthritic Disease." Journal "Rheumatology," 2021, vol. XXXIX, No 2: 52-55.
3. I. Momcheva, Kazmin I, Hristova S., Madzhova V. "Osteoarthritis and Immunity." Journal "Rheumatology," Vol. XXIX, No 1, 2021, pp. 44-46, 2021.

SCIENTIFIC REPORTS RELATED TO THE DISSERTATION:

1. I. Momcheva, I. Kazmin, D. V'lchev, "Therapeutic Efficacy and Safety of Intra-Articular PRP Injections in Knee Osteoarthritis - Clinical Observation." National Conference on OA and OP, Plovdiv, December 11-13, 2014 (oral presentation).
2. I. Momcheva, "Results from the Partnership between Vitamin K2 and Vitamin D3 in Patients with Osteopenia." National Conference on OA and OP, Borovets, November 2018.
3. I. Momcheva "Increased Level of C3 in Synovial Fluid in Patients with Activated Gonarthrosis as a Diagnostic and Therapeutic Target." Annual Conference on Osteoarthritis and Osteoporosis - Summer Rheumatology Days. Pravets, June 2, 2023 (oral presentation).

10. APPENDICES:

- Table 1: Investigated indicators and reference range.
- Figure 1: Innate immune system - the first line of immune defense. Model of complement activation in OA.
- Figures 2-4: Average percentages of CRP, C3, and C4 in synovial fluid compared to plasma levels.
- Figures 5-8: Distribution of patients according to radiographic stage and ratios of CRP, C3, C4 between synovial fluid and plasma.
- Figure 9: Mean values of CRP, C3, and C4 in blood plasma, synovial fluid A, and synovial fluid B.
- Histograms 1-6: Distribution of patients based on CRP, C3, and C4 values in synovial fluid relative to plasma levels and ratio B/A (51-121).

Acknowledgments: I express my sincere gratitude to:

- Prof. Dr. Valentina Madzhova, MD, for trust, professionalism, patience, and lessons in humanity.
- My academic supervisor Assoc. Prof. Zhenya Ruseva, MD, for constructive advice and unwavering support.

- Dr. Ivan Kazmin, my rheumatology teacher, for motivating me to undertake this work and being my guide throughout.
- Dr. Plamena Haralanova and Dr. Svetlana Hristova, MD, who worked tirelessly and with proverbial precision alongside me.
- Dr. Vasil Kostadinov and Tatyana Kostadinova from the "Lina" laboratory for material processing, result accuracy, and assistance in supplying consumables.
- My family for their patience and understanding.
- I thank everyone who showed interest in my scientific work!

Irina Momcheva