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PhD THESIS

THE VALUE OF MOLECULAR MARKERS IN BODY FLUIDS IN THE DIAGNOSIS AND PROGNOSIS OF KNEE OSTEOARTHRITIS

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TABLE OF CONTENTS

LIST OF PUBLISHED SCIENTIFIC PAPERS**Error! Bookmark not defined.**
LIST OF ABBREVIATIONS AND SYMBOLS**Error! Bookmark not defined.**
LIST OF FIGURES.....**Error! Bookmark not defined.**
DEDICATION (optional) .....**Error! Bookmark not defined.**
INTRODUCTION.....**Error! Bookmark not defined.**
GENERAL PART.....**Error! Bookmark not defined.**

1. KNEE OSTEOARTHRITIS- GENERALITIES AND ETIOPATHOLOGY**Error! Bookmark not defined.**
   1.1. Definition and classification..... **Error! Bookmark not defined.**
   1.2. Global incidence ..... **Error! Bookmark not defined.**
   1.3. Risk factors and pathologies associated with knee osteoarthritis**Error! Bookmark not defined.**
      1.3.1. Pathophysiology of knee osteoarthritis**Error! Bookmark not defined.**
2. ASSESSMENT OF KNEE OSTEOARTHRITIS ACTIVITY**Error! Bookmark not defined.**
   2.1. Diagnostic methods ..... **Error! Bookmark not defined.**
   2.2. Biomarkers specific to the prognosis and diagnosis of knee osteoarthritis **Error! Bookmark not defined.**
3. MANAGEMENT OF THE KNEE OSTEOARTHRITIS**Error! Bookmark not defined.**
   3.1. Non-invasive therapeutic solutions ..... **Error! Bookmark not defined.**
      3.1.1. Physical therapy in the case of knee osteoarthritis**Error! Bookmark not defined.**
      3.1.2. Drug therapy of knee osteoarthritis..... **Error! Bookmark not defined.**
   3.2. Invasive interventions ..... **Error! Bookmark not defined.**

SPECIAL PART.....**Error! Bookmark not defined.**

4. ADIPONECTIN IN OSTEOARTHRITIS: PATHOPHYSIOLOGY, RELATIONSHIP WITH OBESITY AND PRESUMPTIVE DIAGNOSTIC BIOMARKER POTENTIAL **Error! Bookmark not defined.**

4.1. Introduction..... **Error! Bookmark not defined.**

4.2. Materials and methods..... **Error! Bookmark not defined.**

4.3. Results and discussions ..... **Error! Bookmark not defined.**

4.4. Conclusions..... **Error! Bookmark not defined.**

5. ANALYSIS OF THE LEVEL OF ADIPONECTIN AND SELECTED CYTOKINES IN PATIENTS WITH KNEE OSTEOARTHRITIS**Error! Bookmark not defined.**

5.1. Introduction..... **Error! Bookmark not defined.**

5.2. Materials and methods..... **Error! Bookmark not defined.**

5.3. Results and discussions ..... **Error! Bookmark not defined.**

5.4. Conclusions..... **Error! Bookmark not defined.**

6. IN VITRO ASSESSMENT OF TWO TYPES OF HYALURONIC ACID'S ANTITUMOR POTENTIAL IN OSTEOSARCOMA CELLS**Error! Bookmark not defined.**

6.1. Introduction..... **Error! Bookmark not defined.**

6.2. Materials and methods..... **Error! Bookmark not defined.**

6.3. Results and discussions ..... **Error! Bookmark not defined.**

6.4. Conclusions..... **Error! Bookmark not defined.**

CONCLUSIONS..... **Error! Bookmark not defined.**

PUBLISHED ARTICLES *IN EXTENSO* ..... **Error! Bookmark not defined.**

Introduction

Osteoarthritis of the knee (KOA) is a degenerative joint disease characterized by the gradual breakdown of cartilage in the knee joint. It is the most common type of arthritis, affecting millions of people worldwide, particularly those over the age of fifty [1]. The exact cause may vary from person to person, but it ultimately leads to the deterioration of the joint's structural integrity.

KOA accounts for almost 4/5 of the burden of osteoarthritis worldwide and increases with age and obesity [2]. Timely identification of KOA is essential to commence therapy, including weight loss and exercise, which have proven to be successful in preventing the advancement of KOA and surgery [3].

The diagnosis of KOA is primarily based on a combination of clinical evaluation with imaging studies. The extent of osteoarthritis (OA) can differ greatly from person to person, and a thorough evaluation aids in developing a customized treatment strategy that may include medicine, exercise therapy, changes in lifestyle, or, in extreme situations, surgical procedures such as joint replacement. There are situations in which some individuals may have radiographic evidence of KOA on X-rays but do not experience significant symptoms. This is referred to as asymptomatic osteoarthritis. Symptomatic cases involve noticeable pain, stiffness, and reduced function.

Furthermore, the severity of KOA can be determined by assessing the extent of joint space narrowing, using the commonly employed Kellgren-Lawrence K-L grade system, which is widely recognized by healthcare professionals globally [4-6]. K-L classification groups the condition into 5 categories (from 0 to 4), depending on the grade of severity, the fourth being considered the most severe [7].

Biomarkers play a crucial role in aiding the diagnosis, prognosis, and management of KOA. They are measurable substances or indicators present in the body that can reflect normal or abnormal biological processes, disease states, or responses to therapy. Specific biomarkers associated with KOA have been identified for diagnostic and prognostic purposes.

Adiponectin (ACRP-30) is a 244-amino-acid-long collagen-like hormone protein involved in the modulation of energy metabolism, such as glucose and fatty acid oxidation, insulin sensitivity, inflammation, and atherosclerosis, being the most abundant circulating adipokine secreted by the adipose tissue [8-10]. In addition, ACRP-30 has recently drawn a great deal of attention, due to its multifactorial—genetic, biochemical, and functional—associations with obesity-related OA onset.

Cytokines are other signaling molecules that regulate inflammation, immune responses, and tissue repair. In KOA, dysregulated cytokine expression contributes to joint inflammation, cartilage degradation, and pain. Several cytokines are implicated in OA pathogenesis and serve as potential biomarkers for diagnosis and disease monitoring. Measurement of cytokine levels in synovial fluid may aid in knee OA diagnosis and assessment of disease severity.

Emerging from the above, the aim of the present work was to shed light on biomarkers' role in the diagnosis and prognosis of KOA. To achieve this goal, the following objectives were pursued:

- investigation of the current findings regarding the pathophysiology of OA and its relationship with adiponectin;
- analysis of specific biomarkers alike adiponectin, interleukin (IL) 10, Tumour Necrosis Factor alpha (TNF-α), and IL-6 from KOA patients and correlation with clinical data, radiographic changes, and patient-reported outcome measures;
- in vitro testing of two types of hyaluronic acids, widely used in KOA management, in the cancerology field, on osteosarcoma cells.

Results

The first study aimed to investigate the current findings regarding the pathophysiology of osteoarthritis and its relationship with ACRP-30. Results highlighted the dual pro-inflammatory and catabolic functions of ACRP-30 in the development of osteoarthritis. ACRP-30 has been implicated in modulating inflammatory responses within the joint environment. It can stimulate the production of pro-inflammatory cytokines such as interleukin-IL-6 and TNF-α in chondrocytes and synovial cells. This pro-inflammatory milieu contributes to cartilage degradation and perpetuates the inflammatory cascade characteristic of OA. On the contrary, other reports show that ACRP-30 might possess protective and anti-inflammatory roles, preventing cartilage damage and degradation. Treatment with ACRP-30 in chondrocyte cell lines elevated the expression of the

tissue inhibitor for MMP-2 (TIMP-2) and decreased gene expression for IL-1-induced MMP-13. Moreover, Hu et al. demonstrated that ACRP-30 underwent autophagy that was possibly associated with the AMPK/mTOR signaling pathway in H_2O_2-induced apoptosis, thus having a protective role in chondrocytes [11]. Furthermore, patients displaying higher ACRP-30 levels in their serum seem to have a longer resilience of joint replacement, and decreased serum ACRP-30 is correlated with loosening at the 10-year follow-up for joint arthroplasty.

To the question ,,Can Adiponectin Represent a Future Diagnostic Biomarker for OA?" it was found that in general, ACRP-30 levels are elevated in samples from patients with OA when compared to those from healthy controls. However, it should be noted that statistical significance was not achieved in every instance of comparison. In a recent study it was observed that serum ACRP-30 levels were significantly higher in the group of patients relative to the non-OA subjects, and that OA grade was associated with MMP-3 and leptin levels. In addition, the authors have linked OA severity with the expression of adipokines and proved that the concentration of adiponectin was positively correlated with the augmentation index of arterial stiffness [12]. On the other hand, one study did not find any statistical significance in the differential expression of adiponectin in OA patients vs. controls but discovered, instead, that ACRP-30 levels were remarkably higher in the plasma relative to the paired synovial fluid ($p < 0.001$), and negatively correlated with the OA severity measured by the K-L grading system, suggesting a protective role for adiponectin in OA [13].

The next study aimed to analyze specific biomarkers alike ACRP-30, IL10, TNF-α, and IL-6 in SF and correlate them with KOA patients' clinical data, radiographic changes, and patient-reported outcome measures (PROM's). In addition, the irritating potential of the fluid on the egg-chorioallantoic membrane was analyzed to identify interferences with hemorrhagic processes.

The studied group consisted of 24 patients respecting the inclusion criteria, with a close distribution of both sexes, 10 males (41.7%) and 14 females (58.3%). The mean age was 67 years, and the BMI indicates that most of the subjects involved were overweight (Table 1). The calculated K–L score shows that the majority of patients present grade 2/3 KOA, with possible joint space narrowing, moderate multiple osteophytes, sclerosis, and eventual deformity of bone ends. In addition, most subjects manifest moderate pain, but significant rigidity. The physical function score highlights that the activities of daily living (ADLs) are moderately affected. The

WOMAC pain subscale and the visual analogue scale (VAS) suggest similar moderate pain and discomfort in most cases.

Table 1. Demographic and clinical characteristics of the studied group.

Variable	Mean ± SD or N(%)	Minimum	Maximum
Number of patients	24		
Gender (males/females)	10(41.7%)/14(58.3%)		
Age (years)	67 ± 10.08	51	87
BMI	28.21 ± 3.47	24	35
Kellgren–Lawrence score	2.292 ± 1.16	0	4
WOMAC pain subscale	5.625 ± 2.7	2	12
WOMAC stiffness subscale	1.917 ± 1.06	0	3
WOMAC physical function subscale	20.21 ± 6.77	9	33
Total WOMAC score	28.04 ± 10.2	12	47
VAS score	5.83 ± 1.71	3	9

The mean values of ACRP-30 (ng/mL), IL-6 (pg/µL), IL-10 (ng/µL), and TNF-α (pg/mL) levels found in SF are presented in Table 2.

Table 2. Concentrations of the studied biomarkers in synovial fluid.

Variable	Mean±SD or N (%)	Minimum	Maximum
ACRP-30	3420±1219	1046	6943
IL-6	1518±762.4	11.68	18364
IL-10	6.327±10.83	0.1	50.18
TNF-α	7.15±3.24	3.52	17.48

When comparing demographic and clinical characteristics of the studied group with biomarkers found in patients' SF (Table 3), negative correlations were found between age and ACRP-30

(p=0.0451, r=-0.412), advancing in age being associated with a reduction in adiponectin levels. Also, the IL-10 values are lower in cases where the in-tensity of the pain is more pronounced (p=0.041, r=-0.421). BMI was as well associated with the reduction of both tested interleukins (p=0.168 for IL-6 and p=0.104 for IL-10). Stiffness was related to IL-10 reduction (p=0.15, r=-0.302).

Table 3. Correlation of demographic and clinical characteristics with biomarkers.

				WOMAC scale				
Variable	Age (years)	BMI	Kellgren–Lawrence Score	Pain	Stiffness	ADL score	WOMAC total	VAS score
ACRP-30	-0.412	-0.056	-0.122	-0.216	-0.13	-0.025	-0.093	-0.09
	0.045*	0.792	0.567	0.309	0.542	0.904	0.663	0.675
IL-6	0.023	-0.29	0.127	-0.24	-0.116	0.001	-0.095	-0.067
	0.912	0.168	0.553	0.258	0.588	0.993	0.658	0.752
IL-10	-0.457	-0.339	-0.105	-0.421	-0.302	-0.198	-0.247	-0.213
	0.832	0.104	0.622	**0.041***	0.15	0.374	0.242	0.317
TNF-α	0.04	-0.038	0.193	-0.06	-0.023	0.11	0.054	0.148
	0.85	0.857	0.365	0.779	0.913	0.605	0.8	0.488

Statistically significant values are marked with *, as follows: * p < 0.1.

When analyzing the interdependence of the monitored biomarkers, positive correlations were obtained between ACRP-30 and IL-6, adiponectin levels being associated with IL-6 augmentation levels (p=0.045, r=0.413). The increased values of IL-6 were also associated with the significant increase of IL-10 values (p=3.42e-07, r=0.837) (Table 4).

Table 4. Correlation of biomarkers in synovial fluid.

Variable	ACRP-30	IL-6	IL-10	TNF-α
ACRP-30				
IL-6	0.413 0.045 *			
IL-10	0.297 0.158	0.837 <0.001 ****		
TNF-α	0.181 0.398	0.229 0.281	0.249 0.24	

Statistically significant values are marked with *, as follows: * $p < 0.1$; **** $p < 0.0001$.

The next step of the study was to stratify the patients by gender, in order to identify some characteristics specific for men/women. Data correlations are depicted in Figures 1 and 2. In the case of both sexes, age is positively correlated to the WOMAC pain subscale, WOMAC stiffness subscale, WOMAC ADL and total WOMAC scores. Pain is also associated with age in both cases (p = 0.0439 in the case of men and 0.0417 in the case of women). Though in the case of males, stiffness is more related to age (p = 0.0079, r = 0.7993), compared to women (p = 0.0203, r = 0.6223).

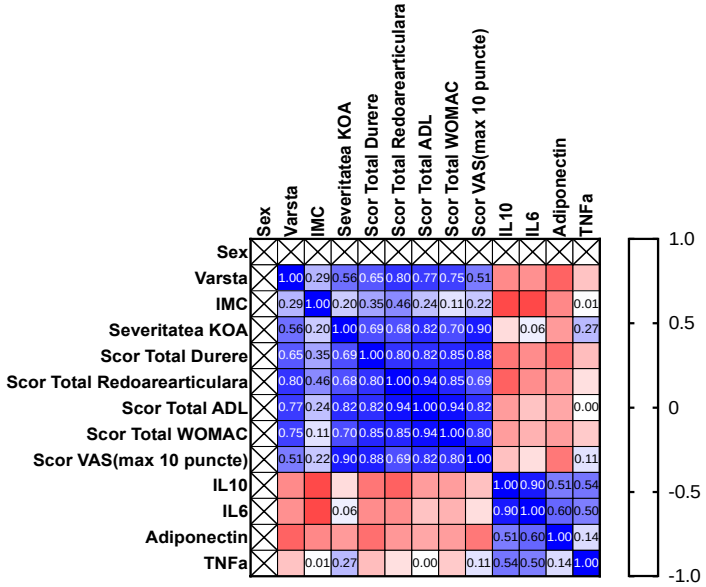

Figure 1. Spearman correlation matrix of demographic, clinical characteristics, and biomarkers found in men.

The KOA severity is positively correlated to the WOMAC pain subscale score, the stiffness, ADL, VAS scores, and the total WOMAC score; however, according to the K–L score, the progression of the disease tends to correlate more intensively with the total WOMAC score's values in the case of women (p = 0.0003, r = 0.8342) (Figure 2), compared with men (p = 0.0289, r = 7013) (Figure 1).

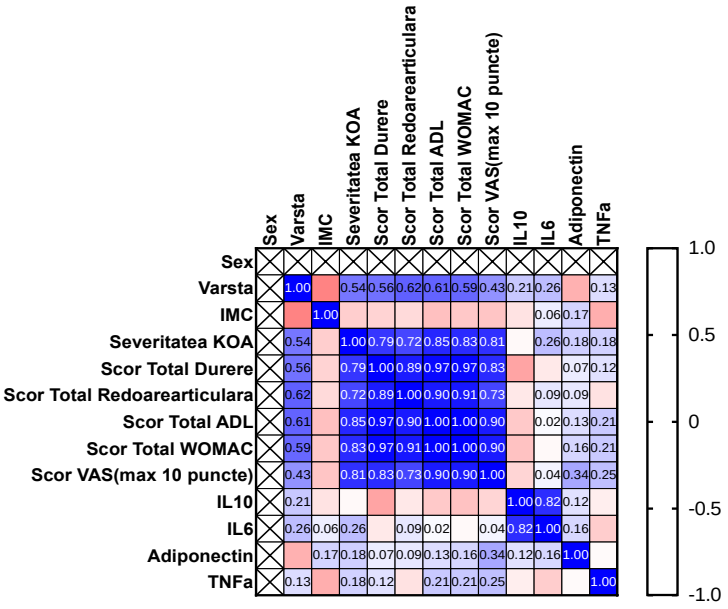

Figure 2. Spearman correlation matrix of demographic, clinical characteristics, and biomarkers found in women.

Using the HET-CAM method, three major effects at the level of the vascular plexus were monitored: hemorrhage, vascular lysis, and vascular coagulation, in order to determine the irritant potential of the two synovial fluid samples - P23 and P24. Both samples were collected with the ESAOTE MyLab Omega ultrasound device, immediately after the clinical evaluation to confirm the joint effusion as well as to guide the fluid aspiration. P23 represents the SF collected from a male with a 2 KOA grade. P24 was collected from a woman with a 4 KOA grade. In addition, a negative control was used - water - and a positive control was used - sodium dodecyl sulfate 1% - to facilitate the interpretation of the results. According to the calculated irritation score, SDS had the highest irritation score (19.57), whereas water had the lowest irritation score (0.07). P23 and P24 had relatively similar irritation scores, 0.72 for P23 and 0.82 for P24 (Table 5). Additionally, SDS induced massive hemorrhage as well as vascular lysis and coagulation at the level of the chorioallantoic membrane. In contrast, the two samples did not induce any significant changes,

with only a slight lysis and vascular stasis observed (Figure 3). However, in the case of P24, it is observed a slight reduction in time of lysis and coagulation, compared to p23.

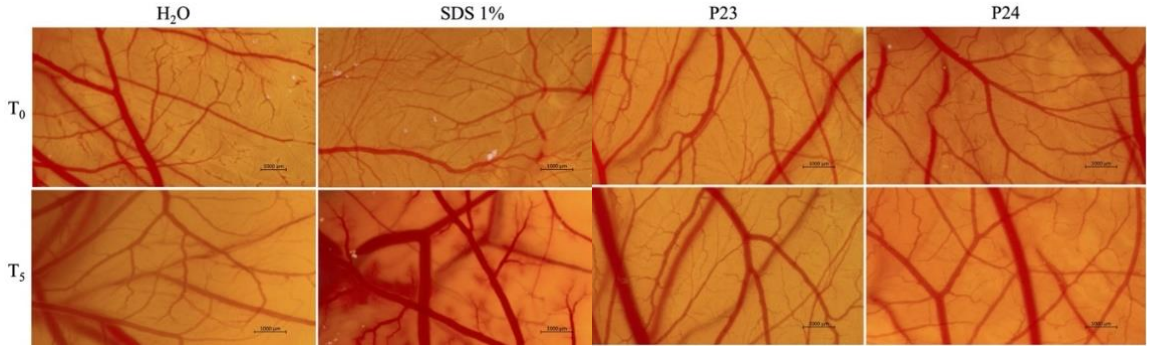

Figure 3. Analysis of P23 and P24's irritant potential using the HET-CAM method. Images of stereomicroscopes of CAMs inoculated with H2O as a negative control, SDS as a positive control, and samples.

Table 5. Irritation score (IS) for P23 and P24 and the occurrence time of hemorrhage (tH), lysis (tL), and coagulation (tC).

	H_2O	SDS 1%	P23	P24
IS	0.07	19.57	0.72	0.82
tH	300	27	297	300
tL	300	18	287	283
tC	300	21	290	288

Hyaluronic acid (HA) is commonly used in the treatment of knee pathologies, thus in the last study two types of hyaluronic acid (Fluicondrial 80 and Juvederm) were evaluated at the level of osteosarcoma cells (SAOS-2), another significant bone desease, in terms of viability, cell morphology, structure of the nuclei and the impact at the level of markers with implications in cell apoptosis (Bax, Bad and Bcl-2).

According to the results, both samples (HA-Fu and HA-Ju) have a concentration-dependent effect on cell viability, but with a different intensity. Consequently, HA-Fu concentrations of 50 and 100 µg/mL did not cause a significant decrease in viability, comparable to the control, unstimulated cells. Alternatively, the concentrations of 500 and 1000 µg/mL caused

a more marked decline in the percentage of viable cells, which was 81% and 80%, respectively (Figure 4).

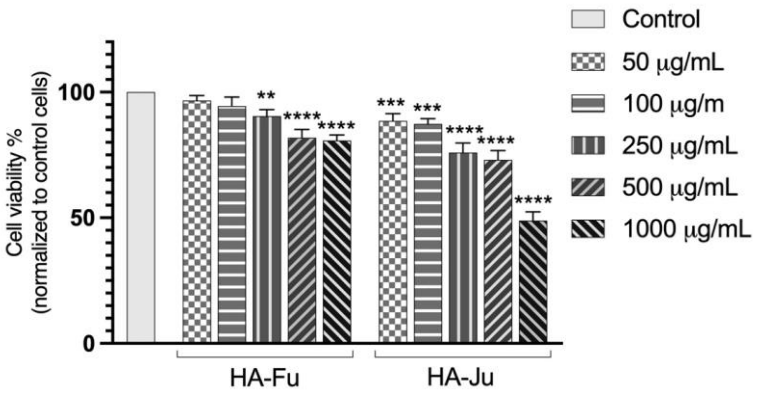

Figure 4. Evaluation of the cytotoxic effect on SAOS-2 cells after 24 hours of stimulation with HA-Fu and HA-Ju (50, 100, 250, 500 and 1000 μg/mL).

The next step in evaluating the effects of HA-Fu and HA-Ju on osteosarcoma cells was to determine the impact on the nuclei's structure. Therefore, the HA-Fu induced structural changes in the nuclei starting at a concentration of 250 μg/mL. Compared to the control cells, there was a decrease in nuclei number, and chromatin was slightly condensed. In addition to chromatin condensation and the decrease in the number of nuclei, the concentration of 1000 g/mL caused massive changes which included the appearance of apoptotic bodies and nuclear fragmentation. Comparatively, HA-Ju caused more substantial alterations than HA-Fu. A massive condensation of chromatin and the appearance of apoptotic bodies could be observed even at the concentration of 250 μg/mL, whereas at the concentration of 1000 μg/mL, the signs of nuclear damage were even more evident, suggesting apoptotic effects (Figure 5).

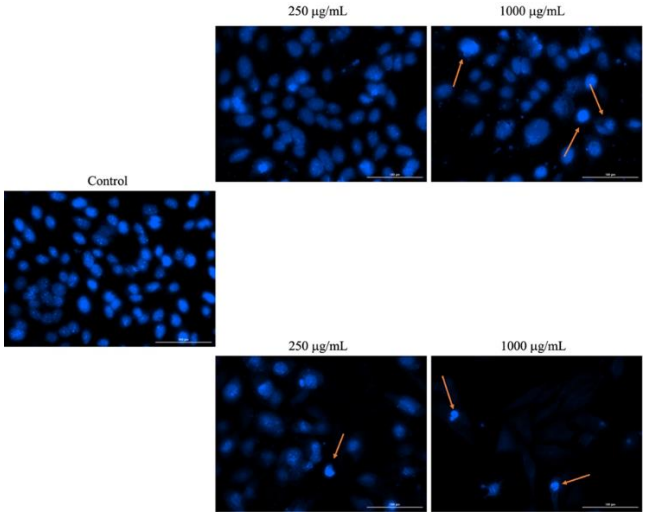

Figure 5. SAOS-2 nuclei stained with DAPI dye after 24 h treatment with HA-Fu and HA-Ju (250 and 1000 µg/mL). The orange arrows indicate signs of apoptosis. The scale bars represent 100 µm [186].

Due to previous studies suggesting that both types of hyaluronic acid promote apoptosis, the next step was to examine their impact on the expression of pro-apoptotic (Bax and Bad) and anti-apoptotic genes (Bcl-2). Both types of tested samples revealed an increase in the expression of the pro-apoptotic genes Bax and Bad, with the most intense increase being observed in the HA-Ju 1000 µg/mL sample. Meanwhile, a significant reduction in the antiapoptotic gene was observed only after stimulation with HA-Ju 1000 µg/mL for 24 hours (Figure 6).

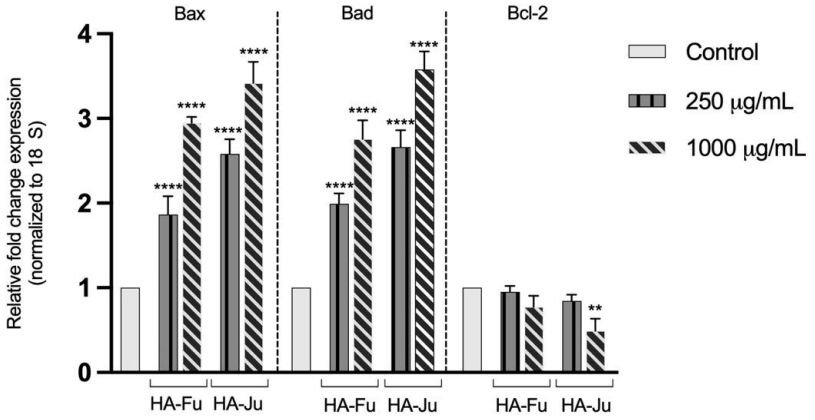

Figure 6. Relative fold expression of mRNA expression of pro- and anti-apoptotic mitochondrial markers in SAOS-2 cells after stimulation with HA-Fu and HA-Ju (250 and 1000 µg/mL) for 24 h.

Conclusions

Following the first study carried out, it was concluded that adiponectin has a dual or more complex role in OA diagnosis. This was highly associated with different forms and stages of OA; however, its exact role in OA pathogenesis remains unclear, since study reports reveal contradictory results, both destructive and protective properties. Despite the accumulation of evidence from in vitro and in vivo studies, the precise and direct mechanism by which adipokines contribute to the onset and development of OA remains largely unexplored and poorly understood.

The results of the second study highlighted a significant correlation between age and ACRP-30, and ACRP-30 and IL-6, suggesting that advanced age may contribute to adiponectin reduction, and this also manifests a synergistic effect with IL-6. At the same time, IL-6 increase

attracts IL-10 augmentation. Comparing men with women, it was observed that men's age is more related to rigidity, and IL-6 and IL-10 are directly correlated to BMI. Rather, women seem to be more sensitive to pain and stiffness.

The last study aimed to highlight the potential of hyaluronic acid, which is widely used in the case of KOA on osteosarcoma cells. According to the obtained results, hyaluronic acid was found cytotoxic, causing a decrease in cell viability and changes in cellular morphology in a dose-dependent manner. The nuclei also showed changes characteristic of cellular apoptosis, including the condensation of chromatin and the appearance of apoptotic bodies. In addition to these findings, RT-PCR analysis indicated that hyaluronic acid increases the expression of pro-apoptotic genes (Bax and Bad) while decreasing the expression of anti-apoptotic genes (Bcl-2).

In conclusion, KOA is a topical problem that affects the daily comfort of millions of people, and the identification of an advanced diagnostic method could significantly improve the prognosis. Biomarkers show promising results in this regard, but additional information is needed to confirm their accuracy and mechanism of action. In addition, the complementary identification of the therapeutic spectrum can significantly improve the quality of life of patients with KOA.

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