PROJECT DELIVERABLE REPORT

OActive

Project Title:

Advanced personalised, multi-scale computer models preventing osteoarthritis

SC1-PM-17-2017 - Personalised computer models and in-silico systems for well-being

Deliverable number	**D4.1**
Deliverable title	Documentation on the qualification of biomarkers found in serum of OA patients
Submission month of deliverable	M28
Issuing partner	UNIC
Contributing partners	UNIC,
Dissemination Level (PU/PP/RE/CO):	PU
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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 777159

Revision History

Version	Date	Responsible	Description/Remarks/Reason for changes
1.0	15/01/2020	UNIC	First Draft
1.1	26/01/2020	UNIC	Review of the first draft
1.2	03/02/2020	UNIC	Modify second draft
1.3	28/02/2020	UNIC	Final

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Abbreviations

COMP	Cartilage Oligomeric Matrix Protein
ELISA	Enzyme-Linked Immuno-Sorbent Assay
HA	Hyaluronic acid
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
LLOQ	Low Limit of Detection
OA	Osteoarthritis
PIICP	Procollagen II C-Terminal Propeptide
TNF-α	Tumor Necrosis Factor alpha

1. Summary

This report refers to Deliverable 4.1, which relates to the OActive WP4 "Biochemical modeling and inflammation biomarkers" and specifically to Task-4. This report describes in detail the processes and steps that were followed for the quantification of three biomarkers of bone and cartilage degradation and synthesis as well as of three inflammatory biomarkers in plasma samples from osteoarthritis (OA) patient.

OA is a heterogeneous disease characterized by the failure of the synovial joint organ. The anatomical severity of OA is usually assessed by joint imaging using standard radiographic techniques including X-ray and MRI, however, by the time OA is detected on the radiograph, significant cartilage degradation has already occurred. Previous studies suggested that OA progress can be detected by the measurement of specific molecular markers (biomarkers) in serum or plasma samples. Specifically, molecules that are released into the blood, during matrix metabolism of articular cartilage, subchondral bone, and synovial tissue are potential biochemical markers for the detection and monitoring of the process of OA. Even though OA was considered as non-inflammatory joint disease, specific pro-inflammatory mediators, including cytokines and chemokines, that are produced by articular tissues probably implicated in the pathogenesis and progression of the disease. To this end, we measured the levels of 3 three biomarkers of bone and cartilage degradation and synthesis, namely cartilage oligomeric matrix protein (COMP), hyaluronic acid (HA), and C-propeptide of type II procollagen (PIICP), and three pro-inflammatory biomarkers, i.e. tumor necrosis factor-alpha (TNF-α), interleukin 1-beta (IL-1β), and interleukin 6 (IL-6) in plasma samples from 130 patients with established OA, using commercially available ELISA kits. Our results revealed that the levels of the biomarkers of bone degradation and synthesis were elevated in the plasma samples of OA patients and correlated with the severity of the disease. On the contrary, the levels of the three inflammatory biomarkers lied below the lower detection limits of the selected methods (ELISA kits).

2. Introduction

2.1. Selection of Osteoarthritis biomarkers and ELISA kits

It has been previously demonstrated[1] that 12 molecular biomarkers of bone and cartilage synthesis and degradation can be detected and quantified by commercially available ELISA kits. Importantly, these biomarkers have an association with some aspects of OA based on the BIPEDS[2] (Burden of disease, Investigational, Prognostic, Efficacy of Intervention, and Diagnostic biomarkers) classification scheme. Amongst the 12 available Prognostic Biomarkers of Bone and Cartilage Degradation and Synthesis were measure the levels of COMP, PIICP, HA, in plasma samples of patients with established OA (Table1). Previous studies showed that the levels of these biomarkers in plasma/serum are elevated in patients with OA. In addition, specific inflammatory mediators are produced by articular tissues in OA and probably implicated in the pathogenesis and progression of the disease. Thus, we measured the levels of three inflammatory biomarkers, namely TNF-α, IL-6, and IL-1b in plasma samples from OA patients.

Table 1. Characteristics of the 6 biomarkers that were analyzed in plasma samples of patients with established OA

I. Prognostic Biomarkers of Bone and Cartilage Degradation and Synthesis			
Biomarker	**Process**	**BIPEDS classification**	**Description**
COMP	Cartilage degradation	Knee: BPD	Elevated levels in Knee OA
HA	Osteophyte burden, synovitis	Knee: BPED	
PIICP	Type II collagen degradation	Knee: D	
II. Inflammatory Prognostic Biomarkers			
Biomarker	**Presumed source**	**Biomarker subgroup**	**Description**
IL-1β	Cartilage, Synovium, Bone	Cytokine/chemokines	Associated with knee OA pathogenesis
TNF-α	Cartilage, Synovium, Bone	Cytokine/chemokines	
IL-6	Peripheral blood leukocytes	Transcriptomic biomarkers	

The levels of the six biomarkers in the plasma sample from 130 patients with established OA were determined using commercially available ELISA kits (Table 2).

Table 2. Description of the ELISA kits that were used for the quantification of the 6 biomarkers in plasma samples of patients ~~of OA patients~~

Biomarker	**Supplier**	**Cat #**	**Sensitivity**	**Range**
I. Prognostic Biomarkers of Bone and Cartilage Degradation and Synthesis				
COMP	R&D	DCMP0	0.036 ng/mL	0.2 - 10 ng/mL
HA	R&D	DHYAL0	0.2 ng/mL	0.6 – 40 ng/mL
PIICP	Abbexa	abx197534	9.375 pg/mL	15.63 – 1000 pg/mL
II. Inflammatory Prognostic Biomarkers				
IL-1β	Invitrogen	KHC0011	1 ng/mL	3.9-250 pg/mL
TNF-α	Invitrogen	BMS2034	5 pg/mL	23-1500 pg/mL
IL-6	Invitrogen	EH2IL6	1 ng/mL	10.2 -400 pg/mL

[1] Hunter et al (2014) Best Pract. Res.: Clin. Rheumatol. 28: 61
[2] Bauer et al (2006) Osteoarth. Cartilage. 14:723

2.2. Recruitment of patients with established Osteoarthritis

A total of 130 patients with knee OA (98 women, 32 men) undergoing knee replacement surgery at Apollonion Hospital, Nicosia, Cyprus were enrolled in this study. Recruitment of patients was carried out by clinicians and OA was defined according to the American College of Rheumatology criteria (https://www.rheumatology.org/) for the classification and reporting of osteoarthritis of the knee. In addition, we used the inclusion/exclusion criteria that are summarized in Table 3. In general, patients below the age of 50 were excluded from the study. Furthermore, patients with post-traumatic osteoarthritis, arthritis due to any autoimmune, infective or inflammatory rheumatological conditions were also excluded for the study. The Kellgren and Lawrence (K&L) scoring system (0-4) was used to assess the radiographic severity of OA. The majority of OA patients (64%) had a K&L score >3. Patients were asked to participate in this research project with a voluntary decision and they should be competent to understand what is involved. To this end, a concept form was prepared. A questionnaire was also prepared to collect specific information from each patient during their recruitment while we got approval from the Cyprus National Bioethics Committee to perform the study. It should be pointed out that the anonymity of the patients was maintained.

Table 3. The exclusion and inclusion criteria used for the recruitment of OA patients

Exclusion criteria	Inclusion criteria
1. Post-traumatic OA	1. Knee pain
2. Autoimmune OA	2. Radiological evidence of OA on plain film
3. Infective/inflammatory OA	3. Crepitus audible/ palpable
4. Rheumatologic conditions	4. Stiffness lasting under 30mins
5. Patient age <50 years	5. Patient age >50 years

3. Determination of biomarkers levels in serum samples of OA patients

3.1. Blood collection, handling, and storage

For the collection of blood, separation of plasma, and long storage of the samples we followed the rules proposed by the Standard Operating Procedures Internal Working Group (SOPIWG)/ Early Detection Research Network (EDRN) for specimen collection[3] (including blood samples and management for biomarker discovery and validation. All samples were stored at 4^{o}C to prevent hemolysis and proceeded within 4h after collection. It should be pointed out that plasma was selected over serum for the determination of the 6 biomarkers in samples from OA patients because during the coagulation process lysis of cells in the clot may occur, releasing cellular components not usually found in serum samples. Moreover, we selected EDTA as an anticoagulant because it does not interfere with the ELISA assays. In addition, we developed our Standard Operating Procedure (SOP) for the blood collection, as well as for storage and handle of plasma samples in order to ensure that all samples were handled in the exact same manner throughout the entire process of biomarkers determination, as described in the following paragraphs.

3.1.1 Blood collection

Ten (10) mL of whole blood were collected for OA patients using needles of diameter >23 gauge to prevent hemolysis and were immediately transferred into commercially available EDTA-treated tubes (lavender tops). Tubes were inverted carefully 10 times to mix blood and anticoagulant and were stored at 4^{o}C until centrifugation.

3.1.2 Plasma isolation, aliquoting, and storage

To separate plasma from blood-cells samples were centrifuged at 1500g for 20 min at 4^{o}C using a refrigerated centrifuge. The resulting supernatant was designated as plasma. Following centrifugation, the liquid fraction (plasma) was immediately transferred into clean cryovials using a sterile serological pipette. The samples were maintained at 2–8°C while handling. Based on EDRN guidelines for specimen collection, plasma/serum samples are of better quality for analysis if smaller volume aliquots are initially prepared rather than larger ones that have to be thawed, handled, and refrozen, perhaps multiple times. Thus, we stored plasma samples in 0.5-1.0 mL aliquots, at -80^{o}C in two different locations. In addition, relating freeze-thaw cycles of the plasma samples were avoided. All samples used were clear and transparent.

[3] Tuck et al (2009) J. Proteome Res. 8:113

3.2. Quantification of the 6 biomarkers in the plasma samples of OA patients

3.2.1 ELISA assays

All ELISA experiments were performed according to the instructions provided by the manufacturer of each kit without any modification. All assays employed the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific to the target human antigen of interest (i.e. COMP, HA, PIICP, TNF-α, IL-6, or IL-1β) has been pre-coated onto a microplate. Standards and samples were pipetted into the wells and any antigen present was bound by the immobilized antibody. Unbound substances were removed by extensive wash and subsequently, an enzyme-linked polyclonal antibody specific for the antigen of interest in each case was added to the wells. In all cases, except for the detection of PIICP, following extensive wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color was developed in proportion to the amount of antigen bound in the initial step. The color development was stopped and the intensity of the color was measured. The optical density of each well was determined within 30 minutes, using a microplate reader (PerkinElmer, Waltham, MA) set to 450 nm. Wavelength correction was set to 570 nm. The reading for each standard, control, and sample were averaged and the average zero standard optical density was subtracted. For the detection of PIICP, the standards and samples were added to the wells and incubated. Subsequently, biotin-conjugated anti-PIICP and avidin conjugated to HRP were added to each microplate well and incubated. After the addition of the development solution, only wells that contained PIICP produced chemiluminescence. The intensity of the emitted light was proportional to the amount of PIICP in the sample or standard.

The concentration of the antigens of interest (biomarkers) in plasma samples was determined using a relative standard curve (i.e. a plot of known concentrations of each antigen against the readout obtained for each concentration) for each biomarker (examples are provided in Figure 1).

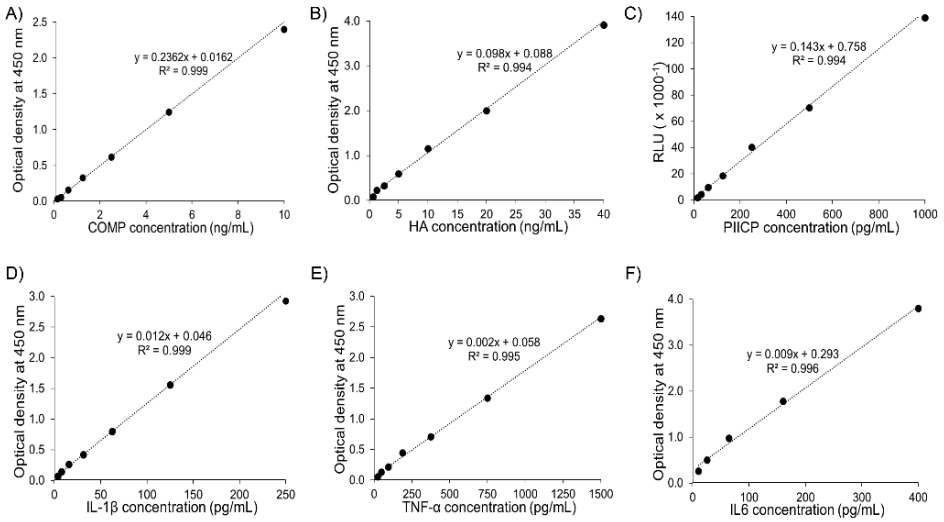

Figure 1. Examples of standard curves that were used for the determination of the concentration of COMP (A), HA (B), PIICP (C), IL-1β (D), TNF-α (E), and IL6 (F) in plasma samples (different dilutions as indicated in the text) of patients with established OA. In C), RLU= Relative Light Units.

The best-fit line was determined by regression analysis while the linearity of each model (standard curve) was assessed using the R^2. As illustrated in the example shown in Figure 1, all standard curves have an $R^2 > 0.99$ indicating a good fitting of the experimental data to the proposed models (equations).

3.2.2 Determination of the best dilution factor of the plasma samples

Samples had to be diluted so that the expected concentration falls within the kit's range. However, before the determination of the levels of the 6 biomarkers in plasma samples, we performed preliminary experiments using different concentrations of 3 random plasma samples in order to i) determine the best dilution factor of samples in each assay, ii) confirm that the results (signals/optical density-OD values) fall within the detection range of each assay, and iii) validate the linearity of dilution of each assay. It should be pointed out that dilution is a vital parameter for ELISA experiments and in general, is used as an indication of the suitability of an ELISA assay. The linearity of dilution is determined by measuring multiple dilutions of known samples containing the antigen of interest by ELISA. The concentration of the target antigen (biomarker) was determined by multiplying the dilution factor by the calculated concentration. For the best results, the concentration of the samples should be similar for all dilutions. All samples were analyzed in duplicate while we used the following acceptance criteria:

i. Inter-assay coefficient of variation (% CV) less than 15 was acceptable
ii. A difference of less than 20% among the different dilution factors was acceptable.

Each manufacturer proposes a dilution factor of plasma samples for the determination of each biomarker. The proposed (by the manufacturer of each ELISA kit) dilution factors for the six biomarkers were for COMP: 100-fold, HA: 4-fold, PIICP: 4-fold, while a recommended dilution for the three inflammatory biomarkers was not provided. The proposed dilution factors were used as a starting point, and we increased and decreased their values in order to determine the best dilution factor of plasma samples for each ELISA assay. Student's-*t*-tests were used to compare means. The results are illustrated in Figure 2 and as shown in all cases analyte detection was not affected by the dilution of the sample.

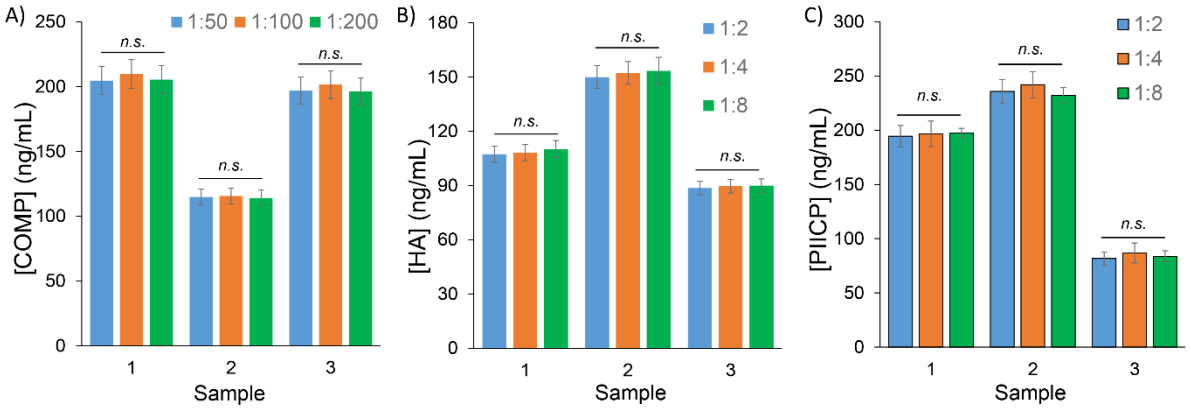

Figure 2. Effect of sample dilution on the determination of the levels of COMP, HA, and PIICP of in plasma samples from OA patients. Comparison of biomarker levels in plasma samples from the same individuals using different dilutions showed slight differences, but these differences were not statistically significant (Student's *t*-test; n.s.= non-significant). Data in (A-C) represent mean ± SEM

The effect of sample dilution on the determination of biomarker levels was further evaluated by calculating the recovery/linearity of each assay of dilution while we took the lower dilution of plasma samples as a basis of our calculations (100% of expected concentration) as illustrated in Table 4.

Table 4. Assessment of linearity of dilution of the assays for the determination of the three biomarkers of bone and cartilage degradation and synthesis

COMP (ng/mL)			**HA (ng/mL)**			**PIICP (pg/mL)**		
1:50	Mean	204.7	1:2	Mean	103.7	1:2	Mean	235.9
	Range	199.0-210.4		Range	100.2-107.3		Range	233.4-238.4
	% of expected[a]	100		% of expected[a]	100		% of expected[a]	100
1:100	Mean	209.7	1:4	Mean	108.2	1:4	Mean	242.0
	Range	204.1-215.4		Range	104.8-111.6		Range	237.3-246.7
	% of expected	102.5		% of expected	104.5		% of expected	102.6
1:200	Mean	205.43	1:8	Mean	110.2	1:8	Mean	232.2
	Range	200.4-201.5		Range	106.9-113.5		Range	228.6-235.8
	% of expected	100.4		% of expected	106.2		% of expected	98.5

[a] The lower dilution was used as a basis for the calculations

As shown in Table 4, the recovery/linearity of all assays were within the acceptance criteria since the same concentration of the three biomarkers were detected in the plasma samples regardless of the dilution of the sample. Based on the results of Figure 2 and Table 4 we used the following dilutions of plasma samples for the quantification of COMP, HA, and PIICP, respectively: 1:100, 1:8, and 1:4.

Our preliminary results revealed that the levels of the three inflammatory biomarkers (IL1-β, TNF-α, and IL6) in the plasma samples were below their respective Lower Limit of Quantification (LLOQ) regardless of the dilution of the sample, even when we used a 1:2 dilution. Thus, for the following experiments, we used a 1:2 dilution for the quantification of IL1-β, TNF-α, and IL6.

3.3 Determination of the levels of six biomarkers in the plasma samples of patients with established osteoarthritis

The levels of the 6 biomarkers are summarized in Table 5. With samples diluted to capture a majority of the cytokines in the optimal reading range, the mean concentrations for COMP, HA, and PIICP were 306.6 (± 170.5) ng/mL, 102.2 (± 72.4) ng/mL, and 205.3 (± 130.1) pg/mL respectively. However, three of the analyzed cytokines (IL-1β, TNF-α, and IL-6) had a large proportion (> 94.6-100%) of the samples with concentrations below their respective LLOQ (Table 5).

Table 5. Expression levels of the 6 biomarkers in the plasma samples from OA patients

I. Prognostic Biomarkers of Bone and Cartilage Degradation and Synthesis								
COMP			**HA**			**PIICP**		
Min-max values (mean [a]), ng/mL		29.5- 995.5 (306.6 ± 170.5)	Min-max values (mean [a]), ng/mL		1.64-385.9 (102.2± 72.4)	Min-max values (mean [a]), pg/mL		8.77- 697.4 (205.3 ± 130.1)
Range, ng/mL	Mean, ng/mL	n (%)	Range, ng/mL	Mean, ng/mL	n (%)	Range [b], pg/mL	Mean, pg/mL	n (%)
<100	74.9	7 (5.4)	<10	4.4	2 (1.5)	<15	8.85	2 (1.5)
100-199	156.0	33 (25.4)	10-99	52.5	71 (54.6)	1-199	128.19	77 (59.2)
200-399	291.4	59 (45.4)	100-199	138.9	42 (32.3)	200-399	277.17	38 (29.2)
400-599	480.1	23 (17.7)	200-300	237.8	14 (10.8)	400-599	464.55	12 (9.2)
600-999	743.7	8 (6.2)	>300	-	1 (0.8)	>600	-	1 (0.8)
II. Inflammatory Prognostic Biomarkers								
IL-1β			**TNF-α**			**IL-6**		
< LLOQ [c], n (%)		130 (100)	< LLOQ, n (%)		123 (94.6)	< LLOQ, n (%)		126 (96.9)
> LLOQ, n (%)		0 (0)	> LLOQ, n (%)		7 (5.4)	> LLOQ, n (%)		4 (3.1)

[a] Data are expressed as mean ± SD
[b] Detection limit: >15 ng/mL
[c] LLOQ: lower limit of detection

Boxplot in Figure 3 illustrates the distribution of COMP, HA, and PIICP in the plasma samples of OA patients and as shown data was not normally distributed.

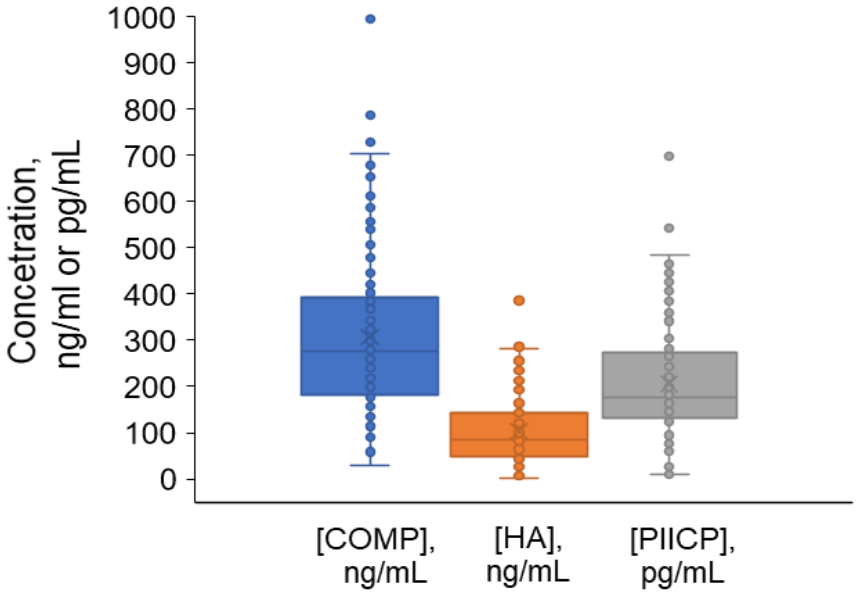

Figure 3. Boxplot showing the distribution of COMP, HA, and PIICP in plasma samples of the 130 patients used in this study.

3.4 Correlation of biomarker levels and severity of osteoarthritis

Spearman's rank correlation coefficient was used to examine correlations between the levels of the biomarkers of bone and cartilage degradation and synthesis and K&L score. All reported *p*-values are two-tailed and a *p*-value < 0.05 was considered statistically significant. Statistical analysis was performed using Prism version 6.0 software (GraphPad Software Inc., La Jolla, CA, USA). The results are summarized in Table 6 and as shown there is a correlation (r > 0) between the levels of COMP, HA, and PIICP and K&L score, however, CIICP levels were not statistically correlated with the severity of the disease (K&L score).

Table 6. Correlation analyses between the biomarkers of bone and cartilage degradation and synthesis and severity of osteoarthritis (K&L score)

Biomarker	Mean ± SD (range)	K&L score			
		Left knee		Right knee	
		r	*p*	r	*p*
COMP (ng/mL)	306.6 ± 170.5 (29.5- 995.5)	0.3446	< 0.0001***	0.2834	0.0011**
HA (ng/mL)	102.2± 72.4 (1.64-385.9)	0.08253	0.3506	0.1940	0.0270*
PIICP (pg/mL)	205.3 ± 130.1 (8.77- 697.4)	0.07242	0.4129	-0.0146	0.8693

Correlation analyses between biomarker levels and severity of knee osteoarthritis (K&L score), was performed using Spearman's rank correlation coefficient (r). *p*-Values < 0.05 were considered statistically significant and are indicated with asterisks: * $p < 0.05$; ** $p < 0.01$, *** $p<0.001$

4. Conclusions

The aim of this work was to determine the levels of 6 biomarkers at the end-stage of osteoarthritis (i.e. last stage before surgery for a total knee replacement). Our preliminary analysis indicated that there is an association between the elevated levels of the biomarkers of bone and cartilage degradation and synthesis (COMP, HA, PIICP) and the severity of the disease (KL scores). On the other hand, the levels of the three inflammatory biomarkers (IL1-β, TNF-α, and IL6) in the plasma samples were below their respective LLOQ. Pro-inflammatory mediators including IL-1β, TNF-α, IL-6, IL-15, IL-17, and IL-18 are known to disrupt metabolic homeostasis by promoting catabolic processes and enzymatic cartilage degradation. Importantly, IL-1β, TNF, and IL-6, in cartilage as well as synovial fluid and membrane, are known to play a key role in the pathogenesis of OA. In general, activation of macrophages and T cells that secrete proinflammatory cytokines is observed during the onset of the disease and that IL-1β and TNFα, as well as IL6, are the first cytokines that are resealed after tissue damage or infection. These proinflammatory cytokines have been shown to play important roles in the destruction of cartilage, synovitis, and pain, however, in our cohort (established OA) their concentrations were expected to be low since it has been previously demonstrated that in end-stage osteoarthritis their levels are relatively low. Further studies using patients at the early stages (onset) of the disease will elucidate the role of the aforementioned pro-inflammatory cytokines in the pathogenesis of OA.