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ORIGINAL ARTICLE

Association between serum and synovial fluid Dickkopf-1 levels with radiographic severity in primary knee osteoarthritis patients

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Abstract Primary knee osteoarthritis (OA) contributes to disability among middle-aged and elderly people. Dickkopf-1 (Dkk-1) and sclerostin are inhibitors of Wnt/β-catenin signaling pathway implicated in regulation of cartilage homeostasis and bone formation, respectively. We aim to investigate the association between the serum(s) and synovial fluid (SF) Dkk-1 and sclerostin levels and disease severity in patients with primary knee OA. Forty patients aged 56–87 years with primary knee OA and 20 healthy individuals were recruited. Weight-bearing anteroposterior radiographs of the affected knee were used to determine the disease severity according to Kellgren and Lawrence criteria. Dkk-1 and sclerostin levels in serum and SF were measured by ELISA. SF Dkk-1 levels were significantly higher in the OA, compared to control group (180 ± 182 vs 128 ± 330 pg/ml, $p < 0.001$). However, OA patients did not differ significantly regarding the sDkk-1 concentrations compared to healthy controls (1289.8 pg/ml vs 1214.1, respectively, $p = 0.630$). SF Dkk-1 levels in Kellgren and Lawrence (KL) grade 4 were significantly elevated compared to those of KL grades 2 and 3 (1.97 vs 2.23 pg/ml, $p = 0.017$, log transformed because data were not normally distributed), whereas sDkk-1 levels between those groups demonstrated marginally statistically significant difference (1111.8 vs 1415.9 pg/ml, $p = 0.057$). SFSclerostin and sSclerostin levels did not have any significant difference between the OA and control groups. SF Dkk-1 levels are positively related to the severity of joint damage in knee OA. Sclerostin levels failed to substantiate an association to knee OA progression. Dkk-1 could play a potential role in the degenerative process of OA. Thus, DKK-1 may emerge as a promising future therapeutic manipulation of OA.

Keywords Dickkopf-1 · Kellgren and Lawrence grading system · Knee · Osteoarthritis · Sclerostin · Wnt/β-catenin signaling

This article was originally published with all author names incorrectly listed. All author names have now been transposed and appear correctly above.

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Introduction

Osteoarthritis (OA) is a common chronic degenerative joint disease that features a multifactorial etiology and is characterized by gradual articular cartilage destruction, osteophyte formation, and subchondral bone remodeling. Patients usually complain of pain, swelling, and stiffness in the affected joint(s), which is exacerbated with activity and relieved by rest. OA incidence substantially increases with each decade after the age of 45 years indicating a worldwide distribution. The most common region for OA to manifest is the medial compartment of the knee. Epidemiological studies have demonstrated the importance of both endogenous and exogenous risk factors for OA [1].

Secreted glycoproteins of the Wingless (Wnt) signaling pathway are major regulators of cell growth and survival in a variety of human cell types. Wnt ligands initiate signaling by interacting with a receptor complex encompassing a member of the Frizzled family of seven transmembrane proteins and the co-receptor, low-density lipoprotein (LDL) receptor-related proteins (LRP5/6). In the canonical Wnt/ b-catenin signaling pathway, receptor activation leads to a stabilization of b-catenin, which accumulates and relocates into the nucleus resulting in target gene expression. Dickkopf-1 (Dkk-1) is considered a direct inhibitory ligand of Wnt/ b-catenin signaling pathway's LRP5/6 co-receptors, acting as a critical mediator of osteoblastogenesis and regulating the formation of the skeleton during the development of the embryo [2]. Sclerostin, a SOST gene product, is a monomeric glycoprotein with a cysteine knot-like domain that is expressed by osteocytes and articular chondrocytes. The role of sclerostin that was initially perceived as a BMP antagonist, due to its homology to the deadenylating nuclease (DAN) family, was later established to that of an endogenous inhibitor of Wnt signaling by binding to the LRP5/6 and Frizzled co-receptors on the cell surface of osteoblasts, thus resulting in reduced osteoblastic bone formation [3–6].

Serum and/or synovial fluid levels of several cytokines have been widely investigated in patients with knee OA, in search for biochemical markers that could determine disease severity and progression [7–9]. However, there have been several conflicting studies on the possible association between serum and synovial fluid levels of Dkk-1 with disease activity in primary knee OA. Voorzanger-Rousselot et al. reported that the relationship between DKK-1 and cartilage breakdown differs between RA and OA patients, suggesting that this finding reflects the difference in the underlying pathophysiologic mechanism of joint degeneration [10]. Weng et al., on the other hand, implicated an increased DKK-1 expression in the cartilage deterioration that takes place in primary OA via promoting chondrocyte apoptosis [11]. Honsawek et al. rebut the previous results, by noticing a significant decrease in plasma levels of DKK-1 of primary knee OA patients compared to healthy controls, which was inversely correlated with the radiographic severity of OA [12]. Moreover, only few studies have investigated the relation of sclerostin levels with the OA. Bone biopsies from patients with OA or ankylosing spondylitis (AS) revealed decreased sclerostin expression, in contrast to no measured increase in RA patients and interestingly greater sclerostin expression in synovial tissue from RA patients as compared with OA patients [13].

In an effort to shed light upon it, we have investigated the serum and synovial fluid levels of Dkk-1 and sclerostin in both knee OA patients and healthy controls and examined the possible association between Dkk-1 and sclerostin in serum and synovial fluid with the radiographic grading of knee OA.

Materials and methods

Study subjects

This study was approved by the ethics committees of Konstantopouleio–"Agia Olga" Hospital and Thriassio Hospital and was conducted in agreement with the Declaration of Helsinki.

All participants (patients and healthy volunteers) gave their informed consent. Forty patients aged 56 to 81 years (27 females and 13 males; mean age 70.1 ± 7.2 years) diagnosed with primary knee OA undergoing total knee arthroplasty in either Konstantopouleio or Thriassio Hospital from May 2013 to May 2014 were enrolled in the study. All primary knee OA patients met the clinical, symptomatic, and radiographic criteria for OA of the American College of Rheumatology [14]. Participants were excluded if they presented with secondary posttraumatic OA, systemic inflammatory or autoimmune disorders, previous knee injury or joint infection, or history of corticosteroid medication. We also recruited a control group composed of 20 gender- and age-matched subjects (11 females and 9 males; mean age 69.5 ± 8.4 years) who underwent arthroscopy to treat traumatic intraarticular knee joint injury such as meniscal or cruciate ligament tears during the same period. The control group subjects were submitted to conventional x-ray examination preoperatively, which were evaluated as radiologically normal (Kellgren and Lawrence grade 0), and showed no abnormalities of articular cartilage during arthroscopic examination.

Radiographic assessment

All participants underwent weight-bearing anteroposterior radiographs to evaluate the structural changes of the affected knee joint. Radiographic severity was determined according to the Kellgren and Lawrence grading system: grade 0 (normal), grade 1 (questionable narrowing of joint space and possible osteophytic lipping), grade 2 (definite osteophytes and possible narrowing of joint space). grade 3 (moderate multiple osteophytes, definite narrowing of joint space, some sclerosis, and possible deformity of bone contour), and grade 4 (large osteophytes, marked narrowing of joint space, severe sclerosis, and definite deformity of bone contour) [15].

Laboratory methods

Synovial fluid was aspirated from the affected knee using sterile knee puncture just prior to surgery, centrifuged to remove cells and joint debris, and stored immediately at −80 °C until the day of measurement. Fasting venous blood samples drawn from the same participants 1 h before surgery were centrifuged and stored at −80 °C until utilized. Double-blind quantitative detection of Dkk-1 and sclerostin in serum and

synovial fluid was performed by sandwich enzyme-linked immunosorbent assays (ELISA) using commercially available test kits in accordance to the manufacturer's protocol (DKK100 Quantikine; DSST00 Quantikine; both R&D Systems, Minneapolis, MN). The manufacturer-reported intraassay precision was 3.3–4.2% for DKK-1 and 1.8–2.1% for sclerostin, whereas inter-assay precision was 4.6–7.6% for DKK-1 and 8.2–10.8 for sclerostin, respectively. The sensitivity of these assays was 4.2 pg/ml for DKK-1 and 1.74 pg/ml for sclerostin, respectively.

Statistical analysis

The quantitative and qualitative variables are presented by the mean, median, standard deviation, interquartile range and the frequencies and percentages, respectively. The Kolmogorov-Smirnov test was utilized for normality analysis of the quantitative variables.

Comparisons between groups (OA vs control and grades 2–3 vs grade 4) concerning demographic characteristics were performed using independent samples *t* test and Mann-Whitney in case of violation of normality. Using the analysis of covariance (ANCOVA) model, we compared the difference between groups of all biochemical markers controlling for age, gender, and BMI (covariates). Results are presented as adjusted mean (95% CI). Logarithmic transformation was applied when data were not normally distributed.

A receiver operating curve (ROC) analysis was conducted to examine the prognostic ability of biochemical markers to discriminate cases with OA from normal cases calculating the respective areas under the curve (AUC) with 95% CI. Furthermore, the sensitivity and specificity of different cutoff points for these biochemical markers were estimated.

All tests are two-sided, and statistical significance was set at $p < 0.05$. All analyses were carried out using the statistical package SPSS version 17.00 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, IL, USA).

Results

Population characteristics

Forty serum and synovial fluid samples from knee OA patients and 20 serum and synovial fluid samples from healthy controls were acquired for measurement of Dkk-1 and sclerostin concentrations. Demographic and body characteristics of the study population are shown in Table 1. The patient and the control group did not differ significantly regarding age (70.1 ± 7.2 vs 69.5 ± 8.4, $p = 0.550$) and male/female ratio (13/27 vs 9/11, $p = 0.401$). Patients with OA had increased higher body mass index compared to healthy controls (27.7 ± 3.5 vs 24.6 ± 3.1, $p = 0.002$).

Homogeneity and ANCOVA analysis

As demonstrated in Fig. 1a, OA patients did not differ significantly regarding the serum Dkk-1 concentrations compared to healthy controls [1289.8 (1119.7–1459.8) vs 1214.1 (965.5–1462.5 pg/ml), $p = 0.630$]. However, synovial fluid Dkk-1 levels were significantly higher in the OA group, compared to controls [2.14 (2.02–2.26) vs 1.70 pg/ml (1.52–1.87); $p < 0.0005$, log transformed because data were not normally distributed] (Fig. 2a and Supplementary Fig. 2A). The characteristics of the OA subgroups based on the Kellgren and Lawrence (KL) classification are featured in Table 1. In total, 9 patients were KL grade 2, 9 patients were KL grade 3, and 22 patients were KL grade 4 OA. The groups did not differ significantly regarding age, gender, and BMI. The circulating and synovial fluid levels of Dkk-1 were assessed and compared in association with radiological KL grading of OA adjusted for demographics and body characteristics. As shown on Fig. 2a (and Supplementary Fig. 2A), synovial fluid (SF) Dkk-1 levels in KL grade 4 were significantly higher compared to those of KL grades 2 and 3 ($p = 0.017$), whereas sDkk-1 levels between those groups demonstrated marginally statistically significant difference ($p = 0.057$) (Fig. 1a and Supplementary Fig. 1A).

sSclerostin and SFSclerostin levels did not have any significant difference between the OA and control groups ($p = 0.186$ and $p = 0.763$, respectively) (Figs. 1b and 2b, respectively, and Supplementary Figs. 1B and 2B, respectively) or the subgroup grades 2 and 3 vs grade 4 based on the Kellgren and Lawrence (KL) classification ($p = 0.737$ and $p = 0.706$, respectively) (Figs. 1b and 2b, respectively, and Supplementary 1B and 2B, respectively).

ROC analysis

It seems from the ROC analysis, as depicted in Table 2, that synovial fluid Dkk-1 itself is a good indicator to predict the OA AUC 95% CI [0.801 (0.68–0.93)] (Fig. 3). The best cutoff that maximizes (sensitivity + specificity) is 88.2 pg/ml with the sensitivity 0.68 and specificity is 0.92.

Furthermore, sDkk-1 (AUC 0.684; 0.51–0.86) and SF Dkk-1 (AUC 0.745; 0.59–0.91) are also good predictors of grade 4 OA (Table 3). The best cutoff points that maximize (sensitivity + specificity) are 1100 pg/ml (sensitivity 0.91, specificity 0.56) and 104 pg/ml (sensitivity 0.82, specificity 0.72), respectively (Fig. 4).

Discussion

The Wnt signaling pathway regulates complex cellular processes, such as proliferation, fate determination, differentiation, and polarity, and therefore assumes a crucial role in

Table 1 Homogeneity between compared groups

	OA (n = 40)	Controls (n = 20)		Grade 2 or 3 (n = 18)	Grade 4 (n = 22)	
Age (years)	70.1 ±7.2	69.0 ± 5.5	p = 0.55	68.3 ± 6.5	71.5 ± 7.5	p = 0.161
Male/female n (%)	13 (32.5%)/27 (67.5%)	9 (45%)/11 (55%)	p = 0.401	4 (22.2%)/14 (77.8%)	9 (41%)/13 (59%)	p = 0.312
Weight (kg)	81.0 ± 9.9	77.4 ± 14.8	p = 0.303	80.9 ± 9.3	81.0 ± 10.6	p = 0.961
Height (cm)	171.1 ± 7.1	176.5 ± 8.8	p = 0.012	170.0 ± 6.4	172.0 ± 7.6	p = 0.390
BMI (kg/m^2)	27.7 ± 3.5	24.6 ± 3.1	p = 0.002	28.1 ± 3.4	27.4 ± 3.6	p = 0.560

All variables are presented as mean ± SD

embryonic development, postnatal development, and adult tissue homeostasis [2]. Consequently, Wnt modulators, including Dkks, are also involved in this highly interlaced functional network [16]. The canonical Wnt pathway has been implicated in the pathogenesis of OA [17]. Products of the Wnt, frizzled, secreted frizzled-related protein (sFRP), Dickkopf, and LDL receptor-related protein gene families have crucial roles in the development and maintenance of the bone, cartilage, and joints [18–20].

Dickkopf (Dkk) is a family of cysteine-rich proteins comprising Dkk-1, Dkk-2, Dkk-3, Dkk-4, and a unique Dkk-3-related protein "soggy" [21]. Dkk-1 functions as a natural inhibitor of Wnt signaling pathway that plays substantial roles in vertebrate embryogenesis including head induction,

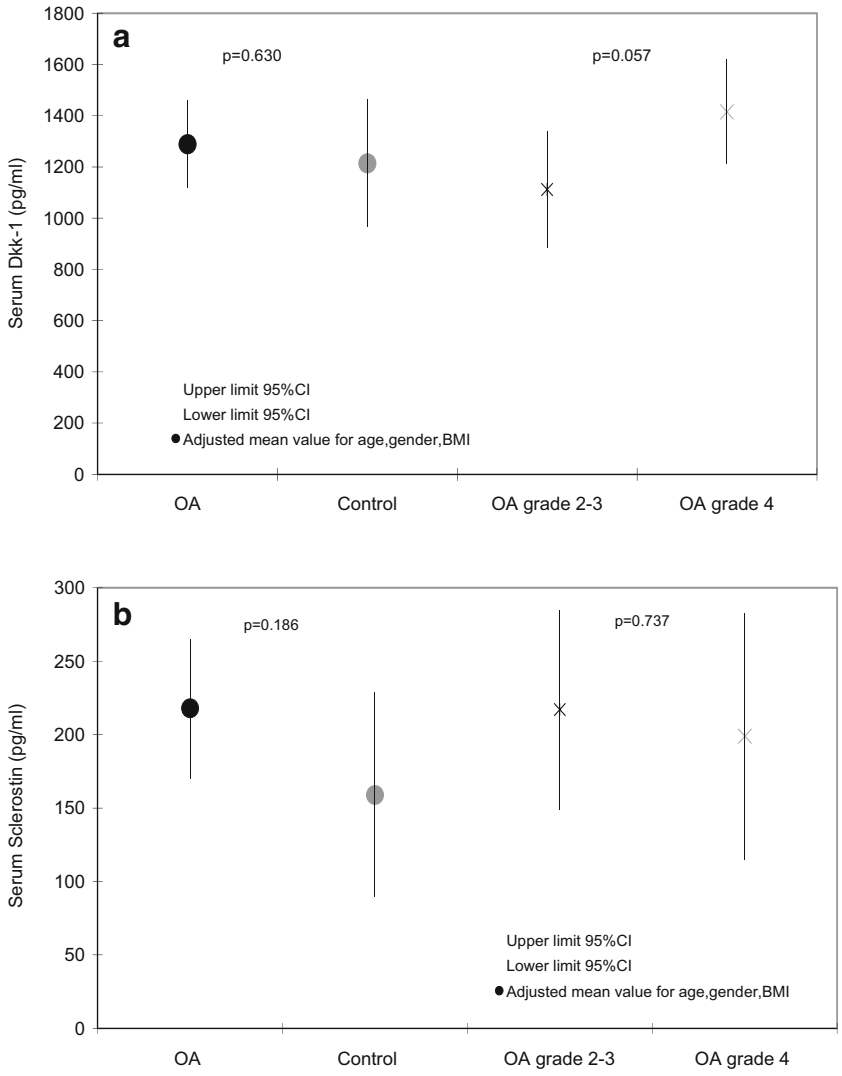

Fig. 1 **a** Scatter plots of serum Dkk-1 in OA (osteoarthritis) vs control group (p = 0.630) and OA grades 2 and 3 vs grade 4 (p = 0.057). **b** Scatter plots of serum sclerostin in OA (osteoarthritis) vs control group (p = 0.186) and OA grades 2 and 3 vs grade 4 (p = 0.737)

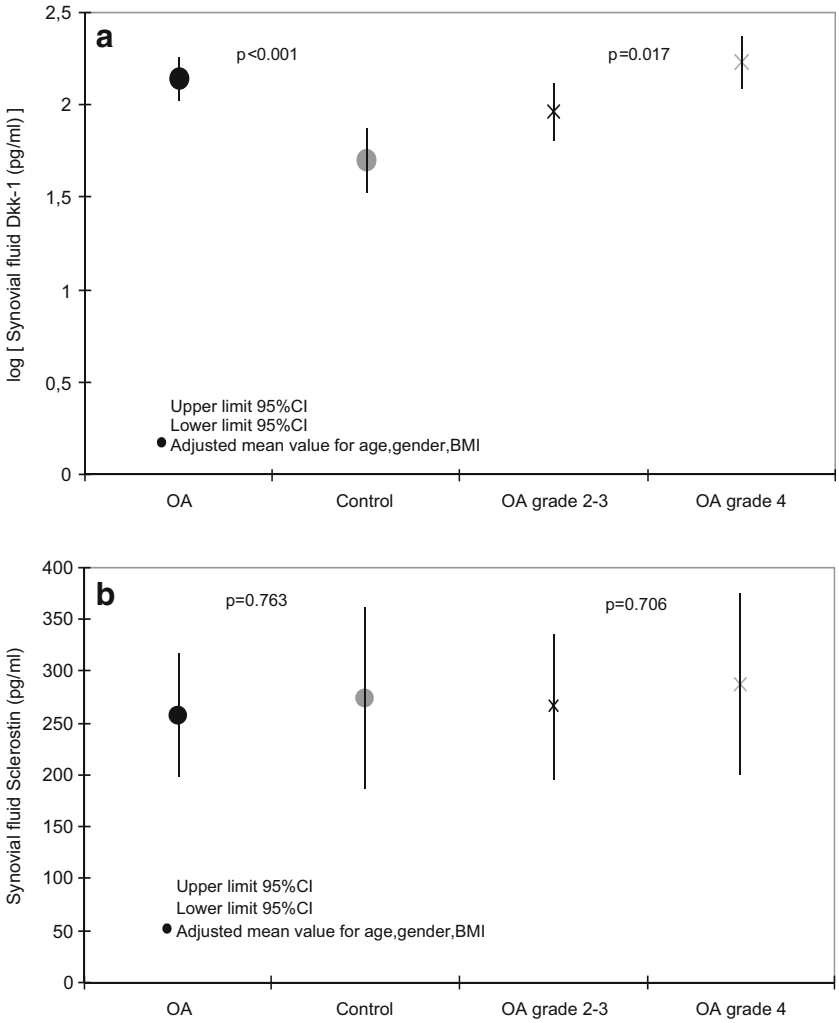

Fig. 2 **a** Scatter plots of log synovial Dkk-1 in OA (osteoarthritis) vs control group ($p < 0.001$) and OA grades 2 and 3 vs grade 4 ($p = 0.017$). **b** Scatter plots of synovial sclerostin in OA (osteoarthritis) vs control group ($p = 0.763$) and OA grades 2 and 3 vs grade 4 ($p = 0.706$)

skeletal development, and limb patterning. DKK-1 binds to the LPR5/6 receptor and a cell surface co-receptor, Kremen-1/2, promoting internalization of the receptor complex and impeding the Wnt signal [16, 22]. It has been previously established that circulating Dkk-1 is present in rheumatoid arthritis, AS, and OA, while studies showed that Dkk-1 was expressed in synovial cells, articular cartilage chondrocytes, and subchondral bone osteoblasts in OA knees [17–20].

In our study, serum and synovial fluid Dkk-1 levels were measured in a well-defined knee OA population at every stage of disease, and according to our results, synovial fluid Dkk-1 concentrations were significantly higher in end-stage knee OA patients compared to early OA patients. This observation suggests a significant increase in the local expression of Dkk-1 in patient with advanced knee OA. According to the ROC analysis, among the studied markers, only the synovial fluid Dkk-1 could predict the OA and moreover could predict the severe (grade 4) OA.

Dkk-1 possibly has multiple roles in OA [20]. Previous reports have demonstrated that increased Dkk-1 levels were correlated with the pathogenesis of joint disorders [23, 24]. Weng et al. advocated that expression of Dkk-1 contributes to cartilage deterioration in primary OA via mediating inflammatory suppression of Akt activation and nuclear β-catenin accumulation and subsequently chondrocyte apoptosis [11].

Table 2 ROC analysis of biological markers as predictors of osteoarthritis

Variable	Area under the curve	95% CI		*P* value
Serum Dkk-1 $n = 60$	0.521	0.362	0.680	0.790
Synovial fluid Dkk-1 $n = 60$	0.801	0.678	0.925	<0.0005
Sclerostin $n = 45$	0.536	0.353	0.718	0.700
Synovial fluid sclerostin $n = 45$	0.614	0.432	0.797	0.215

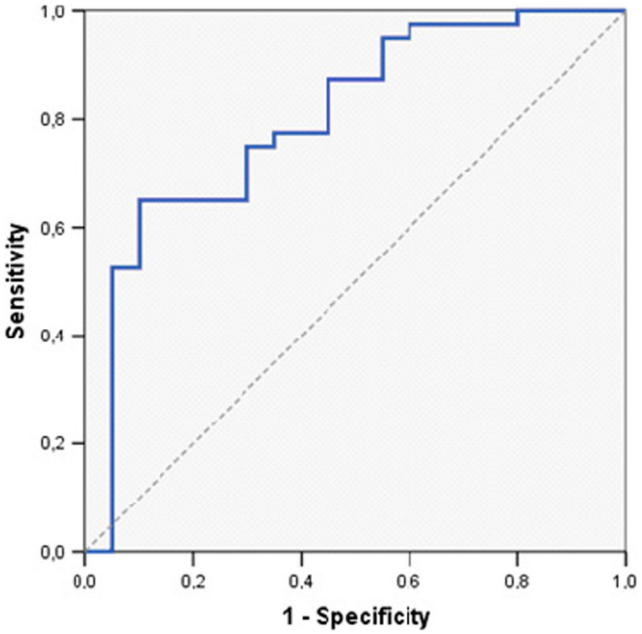

Fig. 3 ROC analysis of synovial fluid Dkk-1 as predictor of osteoarthritis

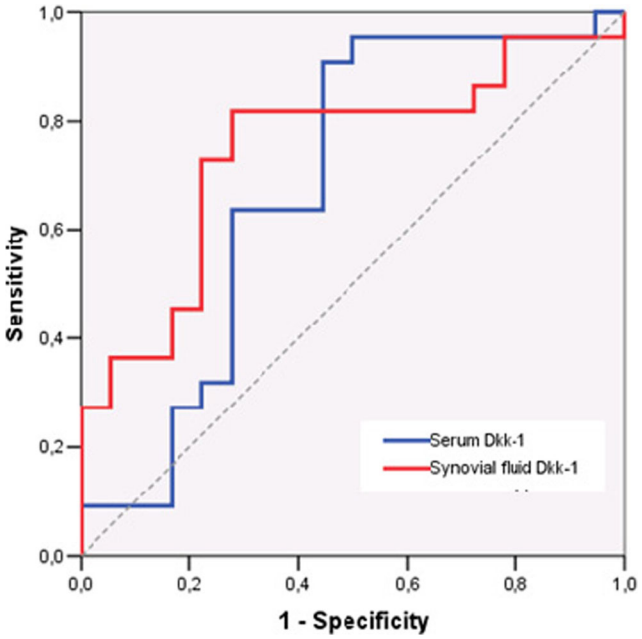

Fig. 4 ROC analysis of serum Dkk-1 and synovial fluid Dkk-1 as predictors of osteoarthritis grade 4

Interestingly, according to their results, Dkk-1 mediated the IL1β-induced apoptosis of human chondrocyte cultures, regulating the transcription of pro-apoptotic and antiapoptotic genes [11]. The same research group, in another study, noted that systemic administration of Dkk-1 antisense oligonucleotides ameliorated osteoarthritic changes in articular cartilage and subchondral bone in rats [23]. Moreover, recent studies have illustrated that aberrant expression of Dkk-1 in myeloma cells was associated with increased bone erosion in human multiple myeloma, while deletion of a single allele of Dkk-1 enhances bone mass in mice [25, 26].

In contrast to our results, Honsawek et al. have reported on an inverse correlation between circulating as well as synovial fluid Dkk-1 levels and disease progression in knee OA; of note, they also found that concentrations of Dkk-1 were decreased in plasma of patients with primary knee OA compared to the controls, implying a reduced systemic production of Dkk-1 in OA; however, a limitation of the study was the lack of synovial fluid samples from the control group [12]. Additionally, Voorzanger-Rousselot et al. have revealed that circulating Dkk-1 levels were lower in patients with knee OA compared to healthy controls [10]. In the same line, Lane and colleagues in a retrospective study have documented that elevated circulating levels of Dkk-1 appeared to be associated with delayed progression of radiographic hip OA in elderly women [24]. In the present study, Dkk-1 levels in serum and SF were increased in direct relation to the severity of knee OA; indeed, circulating and synovial fluid Dkk-1 levels were lower in early knee OA patients (KL grades 2 and 3) compared to end-stage knee OA (KL grade 4). The mechanism(s) implicated in the enhancement of Dkk-1 expression in the synovial fluid of OA patients, in terms of its joint tissue, remains to be investigated further.

Sclerostin is secreted by osteocytes and has been shown to negatively regulate bone mass [27]. Sclerostin binds to the co-receptor, LRP5/6, on the cell membrane to inhibit the Wnt/β-catenin signaling pathway acting on osteocytes in an autocrine manner or osteoblasts through paracrine mechanism [28, 29]. In the present study, we found no differences in serum and synovial fluid sclerostin levels between OA and control groups. Additionally, there was no relation between the progression of the OA and the sclerostin levels.

In contrast with our findings, Mabey et al. demonstrated a negative correlation of both plasma and synovial fluid sclerostin levels with knee OA severity leading to the hypothesis of a protective role in OA process [30]. In the same line, Wu et al. revealed a reduced sclerostin expression in the

Table 3 ROC analysis of biological markers as predictors of grade 4 of osteoarthritis

Variable	Area under the curve	95% CI		*p* value
Serum Dkk-1 $n = 40$	0.684	0.505	0.864	0.047
Synovial fluid Dkk-1 $n = 40$	0.745	0.587	0.903	0.008
Sclerostin $n = 30$	0.356	0.140	0.573	0.189
Synovial fluid sclerostin $n = 30$	0.519	0.290	0.747	0.866

subchondral bone and the activation of the Wnt/β-catenin signaling pathway during the progression of OA, although in this study, control group was lacking and the decreased expression of sclerostin was demonstrated at tissue level by western blot analysis and not in synovial fluid [31]. Finally, Appel et al. showed that sclerostin expression in cortical bone was low in patients with diseases associated with the formation of bony spurs such as AS and OA, but not in healthy subjects or patients with RA, where bone proliferation does not exist [13].

Differences in disease progression, studied populations, or assays applied, even incomplete control of confounding variables such as physical activity and eating, could be implicated in the discordance of our results in regard to those of previous studies.

Among the strengths of our study is that serum and SF levels of Dkk-1 were measured both in knee OA patients, presenting with different radiographic severities, and in controls. We consider of special significance the fact that SF has been sampled from control subjects in order to substantiate even further our findings. SF Dkk-1 levels were significantly higher in knee OA patients compared with controls. Serum Dkk-1 levels were demonstrated to be high and not significantly different between OA patients and healthy controls. The major finding of the present study was that SF Dkk-1 levels increased with the advancement of KL grade and were positively correlated with KL scores in OA patients. After adjusting for potential confounders, there was still a significant association between SF Dkk-1 levels and KL scores. These results indicate that SF Dkk-1 levels may be useful for evaluating the severity of OA, while the interaction between Dkk-1 and its receptors may also be a pathogenic factor in the degenerative process of OA [32]. Otherwise, we could hypothesize on a possible feedback mechanism that induces Dkk-1 expression in an attempt to inhibit the OA advancement, which in the case of OA patients obviously does not suffice to compensate the activation of Wnt pathway. Van den Bosch et al. demonstrated the role of alarmins S100A8/A9 in experimental OA development mediated by canonical Wnt signaling pathway activation; interestingly, they showed that enabling Dkk-1 as an inhibitor of Wnt pathway, S100A8 has b-catenin-independent effects on OA pathology [33]. Further investigations in terms of measuring alarmins S100A8/A9 serum and SF levels additionally to DKK-1 levels in OA patients could elucidate a certain interaction pattern.

The potential limitations of the present study require consideration. Firstly, this cross-sectional study included only a small sample size of enrolled Greek patients and further study with a larger cohort as well as synovial fluid aspiration of both involved knees per case would be required to substantiate our results.

Secondly, due to the complex functional nature of the Wnt pathway, other regulators than Dkk-1 concentration in serum and SF should be concurrently measured, in order to assess it thoroughly.

The development of preventive strategies and early-stage interventions for OA requires a better understanding of the molecular mechanisms and the identification of reliable biomarkers that reflect specific biological or pathological processes associated with the development and progression of OA. Therapeutic interventions, such as pharmacological agents targeting Dkk-1 signaling pathways, to hinder the degenerative process of OA, endorse further investigations [3, 34, 35].

In conclusion, the present study has revealed a significant increase in SF Dkk-1 of OA patients and illustrated a pronounced positive correlation with radiographic severity grading in patients with primary knee OA. The data of this study postulate that Dkk-1 may be used as a prognostic parameter to reflect the disease severity of primary knee OA. Further studies will be required to define the mechanisms underlying this association. Additional investigations should be considered to elucidate a possible role of Dkk-1 in the pathogenesis of chronic degenerative joint disorder, aiming to the development of effective therapeutic approaches to restrain the progression to OA.

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Compliance with ethical standards This study was approved by the ethics committees of Konstantopouleio–"Agia Olga" Hospital and Thriassio Hospital and was conducted in agreement with the Declaration of Helsinki. All participants (patients and healthy volunteers) gave their informed consent.

Disclosures None.

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