Osteopontin in Patients with Primary Knee Osteoarthritis: Relation to Disease Severity

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Abstract:Background: Osteopontin; a small integrin-binding ligand; has been proved to be an important factor in bone mineralization, remodeling and metabolism. Upregulation of osteopontin was noticed in knee osteoarthritis. It may be involved in the molecular pathogenesis of the disease, contributing to progressive degeneration of articular cartilage.
Aim: To measure plasma and synovial fluid osteopontin in patients with primary knee osteoarthritis in order to assess its relation to disease severity. **Patients and Methods:** This study included thirty patients (aged 44-66 years) diagnosed as having primary knee osteoarthritis according to the checklist of American College of Rheumatology criteria. Ten age and sex matched apparently healthy controls were also enrolled in this study. Full history taking, thorough clinical examination, routine laboratory investigations,and plasma osteopontin (OPN) level measurement were done for all patients and controls. While Synovial fluid OPN levels were measured in cases with knee effusion. Disease severity was assessed using Kellgren/ Lawrence (K/L) radiological score. **Results:** Statistically significantly elevated levels of both plasma and synovial fluid OPN were found in patients compared to controls ($P<0.001$&$P<0.05$ respectively). Synovial fluid OPN levels were statistically significantly higher than paired plasma samples ($P<0.001$). A significant positive correlation was found between plasma OPN and synovial fluid OPN levels and both of them showed positive correlation with disease severity grades as assessed by K/L radiological score. **In Conclusion:** Both plasma and synovial fluid OPN levels were increased in primary knee O.A patients and both of them correlated with more severe OA. Measurements of plasma and/or synovial fluid levels of osteopontin could possibly serve as a biochemical parameter for determining disease severity and predicting the progression of osteoarthritic disease process.

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1. Introduction

Osteoarthritis (OA) is a disorder of the hyaline joints characterized by wear, softening and thinning of the articular cartilage and diminished compliance of the subchondral bone (Bijlsma et al., 2011).

There is a great potential in the use of biochemical markers of arthritis to diagnose the disease at an earlier stage, assess its severity and monitor the effect of any treatment ***(Garnero et al., 2002).*** Markers of cartilage degradation have been assessed extensively and show a moderate to good relation with clinical and radiographic variables of osteoarthritis but not enough is known about markers of bone metabolism, like bone morphogenetic proteins and osteopontin which might have an important role in pathophysiology of the disease ***(Honsawek et al., 2009).*** However, no maker has gained complete acceptance for clinical monitoring of osteoarthritis.

Osteopontin, (OPN) a member of the SIBLING (small integrin-binding ligand N-linked glycosylated protein) family, is present in extracellular fluids. It is abundant in the extracellular matrix of mineralized tissues such as bone, where it mediates important cell–matrix and cell–cell interactions ***(O'Regan et al., 2000).***Its expression during chondrocyte maturation is one of the important events involved in cartilage-to-bone transitions in fracture repair ***(Gravallese, 2003).***

Scatena et al., 2007 stated that OPN was a multifunctional molecule highly expressed in chronic inflammatory and autoimmune diseases, Being a secreted adhesive molecule, OPN was found to aid in the recruitment of monocytes-macrophages and to regulate cytokine production in macrophages, dendritic cells, and T-cells. OPN has been classified as a T-helper 1 cytokine. Furthermore, OPN is cleaved by at least 2 classes of proteases: thrombin and matrix-metalloproteases (MMPs). Most importantly, fragments generated by cleavage not only maintain OPN adhesive functions but also expose new active domains that may impart new activities.

Several studies proved that OPN was involved in different physiologic and pathologic events in liver (Ramaiah and Rittling 2008), skeletal muscle myoblasts (Uaesoontrachoon *et al.* (2008), the vascular system (Waller *et al.*, 2010), in cellular transformation and cancer (Weber 2011) and in mineralized tissues (McKee *et al.*, 2011).

Patients with ankylosing spondylitis had high levels of OPN. However, the plasma OPN level in such patients showed correlation with bone remodeling markers rather than with inflammation (Choi *et al.*,

2008). In addition, OPN was expressed by rheumatoid arthritis fibroblast –like synoviocytes (FLS) . It affected FLS and B lymphocyte interactions by supporting the adhesion of B lymphocytes to FLS and enhancing the production of IL-6 (Take *et al.*, 2009).

Up regulation of OPN in O.A. was noticed by enhanced expression of osteopontin mRNA in human OA cartilage. Osteopontin may be involved in the molecular pathogenesis of osteoarthritis, contributing to progressive degeneration of articular cartilage (Standal et al., 2004).

The synthesis and degradation of cartilage matrix and the cartilage homeostasis is regulated by chondrocytes via mechanisms that depend, in part, upon the interaction of chondrocytes with the extracellular matrix (ECM) proteins. These include collagens, proteoglycans, and noncollagen-proteins such as fibronectin (FN) and osteopontin (OPN), (Hunter et al., 2009). In addition, OPN was thought to be involved in destruction of cartilage matrix by inducing the production of collagenases in articular chondrocytes (Gao et al., 2010).

Aim of the study:

To measure plasma and synovial fluid osteopontin in patients with primary knee osteoarthritis in order to assess its relation to disease severity.

2. Patients and Methods

This study included thirty symptomatic patients who attended the Physical medicine, Rheumatology and Rehabilitation Outpatient Clinic of Ain Shams University Hospital and were diagnosed as having primary knee O.A according to the checklist of American College of Rheumatology criteria for the classification of knee osteoarthritis ***(Altman et al., 1986)*** as shown in table (1).

Table (1): Checklist of American College of Rheumatology criteria for the classification of idiopathic knee osteoarthritis (Altman et al., 1986):

Clinical and laboratory	Clinical and radiographic	Clinical †
Knee pain	Knee pain	Knee pain
+ at least 5 of 9:	+ at least 1 of 3:	+ at least 3 of 6:
- Age > 50 years	-Age > 50 years	- Age > 50 years
- Stiffness < 30 mins	-Stiffness < 30 mins	- Stiffness < 30 mins
- Crepitus	- Crepitus	-Crepitus
- Bony Tenderness	+ Osteophytes	-Bony Tenderness
- Bony enlargement		- Bony enlargement
- No palpable warmth		- No palpable warmth
- ESR <40 mm/hour		
- RF <1:40		
- SF OA		
92% sensitive	91% sensitive	95% sensitive
75% specific	86% specific	69% specific

ESR = erythrocyte sedimentation rate (Westergren); SF OA = synovial fluid signs of OA (clear, viscous, or white blood cell count <2,000 /mm3).
RF=rheumatoid factor

Ten healthy individuals matched for age and sex with patients were also enrolled in this study as a control group.

Patients with secondary OA, other types of arthritis such as Rheumatoid arthritis, Systemic lupus erythrematosis, Psoriatic arthritis, gout, pseudo gout, and infectious arthritis. Also, patients suffering from osteoporosis, systemic inflammatory or autoimmune disease or malignancy were excluded.

All patients were **subjected to:**

Full medical history: Sex, age, Occupation, Menstrual history, with special emphasis on history of knee pain**,** morning stiffness, jelling phenomenon, joint swelling, other joint affection, constitutional symptoms and extra-articular manifestations. Past history suggestive of secondary of knee OA.

Thorough clinical examination: Weight and height (BMI was calculated). Local examination of knee joint for tenderness, warmth, swelling, effusion, crepitus, Palpable osteophytes, deformities, Synovial hypertrophy, range of motion, muscle wasting, ligament laxity and patella-femoral compression test. Also examination of small joints of hands and feet, shoulders, hip joints, and ankle joints as a screening for primary generalized O.A and gait examination.

3. Laboratory investigations:

A. Routine laboratory investigations:

- -Complete blood count using coulter counter.
- -Erythrocyte sedimentation rate (ESR) by the Westergren method.
- Serum CRP using enzyme linked immunosorbant assay (ELISA) and considering concentrations lower than 10 mg/L within normal levels.

B. Measuring of full length OPN levels in plasma and synovial fluid samples:

Blood samples were collected from all patients, centrifuged, and stored at − 80 °C until assayed.

- Synovial fluid was aspirated using lateral approach technique from the affected knees of 10 patients who had knee effusion, centrifuged and stored immediately at − 80 °C until analyzed.
- Double-blind quantitative detection of osteopontin level in plasma and synovial fluid was performed using enzyme-linked immune sorbent assay (ELISA) (Kit name is Quantikine used for Human Osteopontin (OPN) Immunoassay. Catalog Number DOST00, SOST00P, DOST00) using the following procedure:

Micro-titer plates were coated with capture rabbit polyclonal antibody. Both plasma and synovial fluid samples were diluted at 1:10 with dilution buffer. then added to the plates(100 ul/well) and incubated for 1 h at 37 ^{0}C. The wells were then washed seven times with washing buffer and incubated for 30 min at 4 °C with a horseradish peroxidase-labeled mouse monoclonal detection antibody for human OPN. After extensive washes, 100 ul of tetramethylbenzidine buffer (used as a substrate) was added to each well, and the plate was incubated for 30 min at room temperature in the dark. Finally, the reaction was stopped with the stop solution. A plate reader (Bio-rad, Hercules, CA, USA) was used to quantify the signal at 450 nm. OPN concentrations were calculated by the standard curve. The sensitivity of this assay was 3.3 ng/ml.

4. Knee Radiography:

Standardized plain X-ray weight-bearing antero-posterior radiographs of the knee were performed and evaluated according to the Kellgren and Lawrence (K/L) severity grading scale for knee OA. (Kellgren and Lawrence, 1957).

Statistical Analysis:

Statistical analysis was performed using Statistical package for Social Sciences Software (SPSS) version 15.0.1 . Quantitative variables (clinical and laboratory parameters) were presented as the mean ±SD. Student(t) test was used to compare means of two studied groups. ANOVA test was used to compare more than two groups. Relationships between parameters were analyzed using Pearson Correlation Coefficient. *P* value less than 0.05 was considered significant

3. Results:

This study was conducted on 30 patients with primary knee OA; aged 44 to 66 years with a mean±SD of 52.7±5.3 years, 25 females (83%) and 5 males (17%). Their BMI ranged from 25.6 to 35.0 Kg/m ^{2} with a mean±SD of 32.0±2.34 Kg/m ^{2}. Their Disease duration ranged from 3 to 15 years with a mean ±SD of 6.35±2.98 years. Ten apparently healthy controls were also enrolled in this study; aged 32 to 62 years with a mean±SD of 52.2±9.51 years , 8 females (80%) and 2 males (20%).Their BMI ranged from 27.0 to 33.0 Kg/m ^{2} with a mean± SD of 30.9±1.9 Kg/m ^{2}.

Comparison between patients and controls as regards age and BMI showed no statistical significant difference (P>0.05).

Table (2): The frequency of clinical manifestations of knee OA in the patient group :

Clinical manifestation	NO	%
Morning stiffness	20	66.7%
Pain	30	100%
Crepitus	30	100%
Palpable osteophytes	7	23.4%
Patello-femoral test	20	66.7%
Effusion	10	33.3%
Tenderness	30	100%
Synovial hypertrophy	2	6.7%
Quadriceps atrophy	20	66.7%

No: Number, **%:** percentage

ESR and CRP were normal in all patients and controls.

Evaluation of Plasma OPN levels:

plasma OPN levels in control group ranged from 11ng/ml to 22ng/ml with a mean level of 15.6 ±3.41ng/ml while plasma OPN levels in O.A group ranged from 44 ng/ml to 207ng/ml with a mean level of 171.37±15.96 ng/ml. Comparison between patient and control groups as regards plasma OPN levels revealed a highly statistically significant difference (P>0.001) as shown in fig(1).

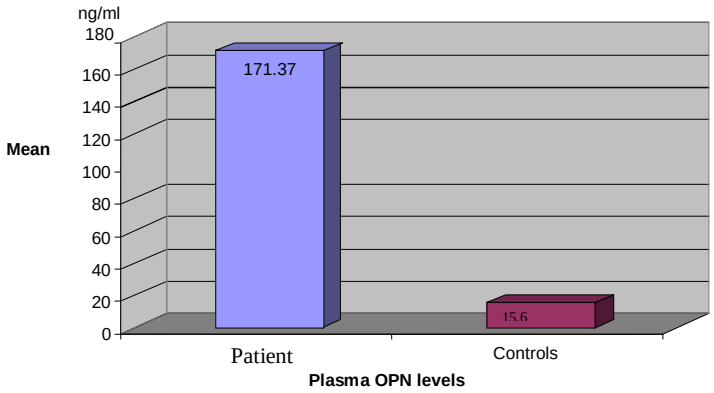

Fig. (1): Comparison between patient and control groups as regards plasma OPN levels.

Plasma OPN levels in patients with knee effusion showed a mean±SD of 174.5±16. 24 while in patients without knee effusion its mean± SD was 169.8± 16.01. This difference was statistically non significant (P>0.05).

Assessment of radiological findings showed:

Table (3): radiological findings of patients according to K-L score:

		Number	Percentage
K-L grading score	Grade 1	0	00.0%
	Grade 2	6	20.0%
	Grade 3	19	63.3%
	Grade 4	5	16.7%

K-L score: kellgren-lawrence classification

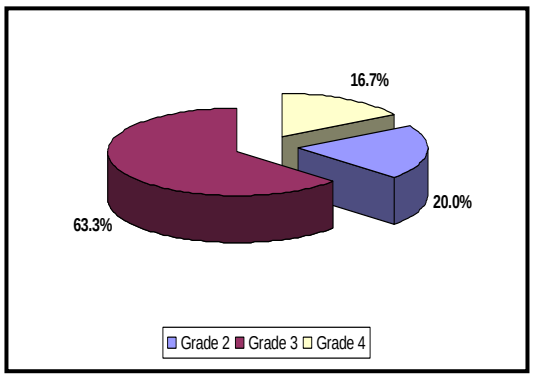

Fig. (2): The frequency of radiological grading among O.A patients

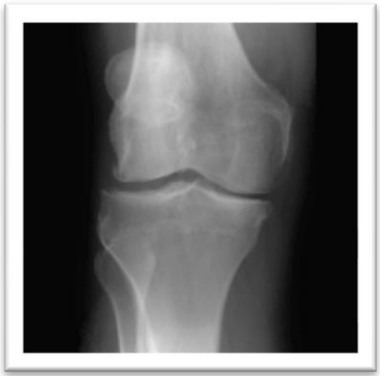

Fig. (3): Plain X-ray AP view of a right knee joint showing K/L grade 2

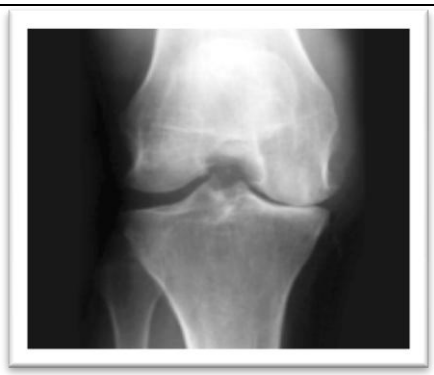

Fig. (4): Plain X-ray AP view of a right knee joint showing K/L grade 3

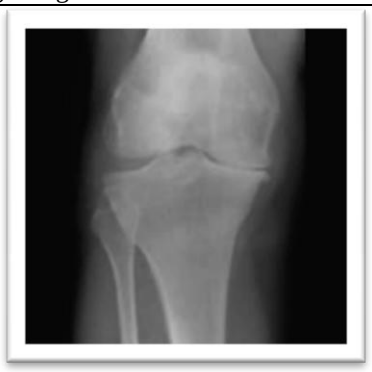

Fig. (5): Plain X-ray AP view of a right knee joint showing K/L grade 4.

Comparison of both plasma and synovial fluid OPN levels with different radiological grading is shown in tables (4 &5) & fig. (6).

Table (4): Comparison of plasma and synovial fluid OPN levels with different radiological gradings:

	Grade 2	Grade 3	Grade 4	F*	P	Sig.
	Mean± SD	Mean± SD	Mean± SD			
Plasma OPN (ng/ml)	150.17± 4.79	170.92 ± 5.92	198.50 ± 8.11	85.42	<0.001	HS
Synovial OPN (ng/ml)	215.00 ± 2.83	228.00 ± 11.73	243.00 ± 1.41	30.94	<0.05	S

S = Significant, **HS = highly significant, ***p***= level of significance, * ANOVA test

Table (5): Post Hoc Test for pair wise comparison between different K/L grades as regards plasma and synovial OPN levels:

Dependent Variable	(I) K-L grading	(J) K-L grading	*P*	Sig.
Plasma OPN	Grade 2	Grade 3	<0.001	HS
		Grade 4	<0.001	HS
	Grade 3	Grade 2	<0.001	HS
		Grade 4	<0.001	HS
	Grade 4	Grade 2	<0.001	HS
		Grade 3	<0.001	HS
Synovial OPN	Grade 2	Grade 3	>0.05	NS
		Grade 4	<0.05	S
	Grade 3	Grade 2	>0.05	NS
		Grade 4	>0.05	NS
	Grade 4	Grade 2	<0.05	S
		Grade 3	>0.05	NS

S = Significant, **HS = highly significant, ***p***= level of significance, NS= non significant.

To detect any overlap between different K/L grades as regards plasma and synovial fluid OPN levels, Post Hoc curve was performed.

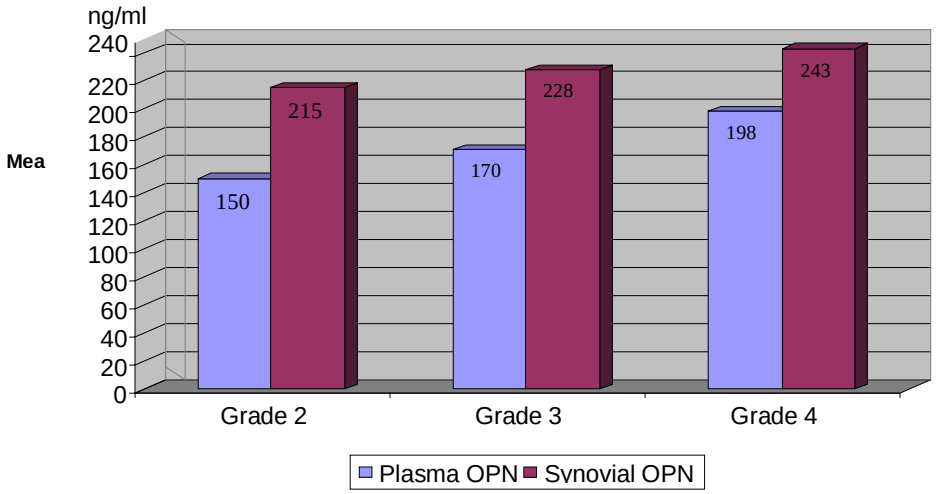

Fig.(6): Comparison between patients with different K-L grades as regards plasma and synovial OPN levels.

Correlating plasma OPN levels to age and BMI of the patients, and disease duration of OA revealed non-significant correlations (P>0.05.)

Correlating both plasma and synovial fluid OPN levels to K/L grading showed statistically significantly positive correlations (P <0.001 and P<0.05 respectively) as shown in table (6) and figs.(7&8).

Table (6): Correlations between both plasma and synovial fluid OPN levels and K/L grading:

		Plasma OPN	Synovial OPN
K/L grading	r	.923	.727
	P	<0.001	<0.05
	Sig	**HS**	**S**

r: Pearson correlation test, **S** = Significant, **HS** = highly significant, ***P*** = level of significance.

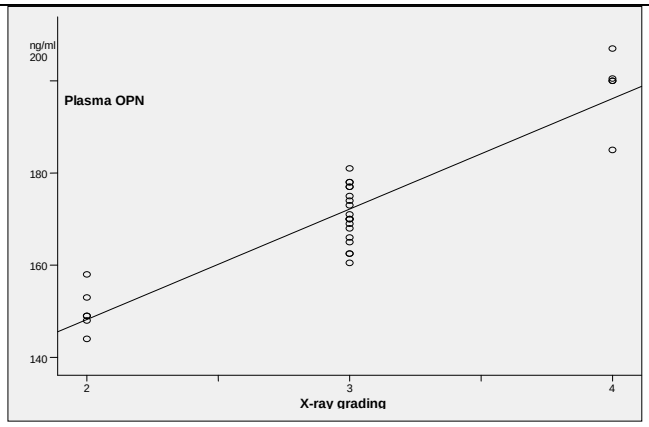

Fig.(7): Correlation between plasma levels of OPN and K/L grading.

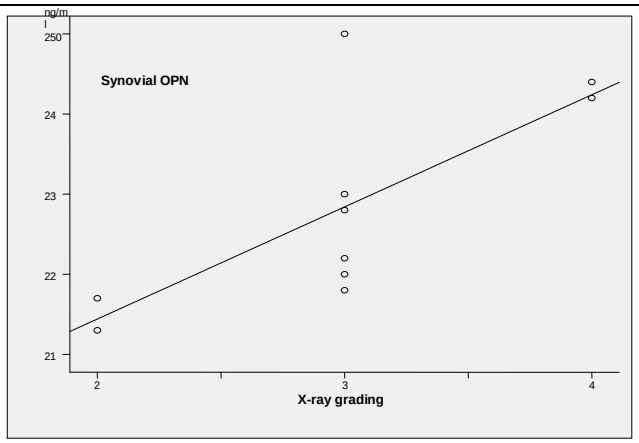

Fig. (8): Correlation between synovial fluid levels of OPN and K/L grading.

Correlation between plasma and synovial fluid OPN levels revealed statistically significant correlation ($p<0.05$) as shown in table (7) and figure (9).

Table (7): Correlation between plasma and synovial fluid OPN levels:

		Synovial OPN
Plasma OPN	r	.585
	P	<0.05
	Sig	S

r: Pearson correlation test, **S** = Significant, ***P*** = level of significance

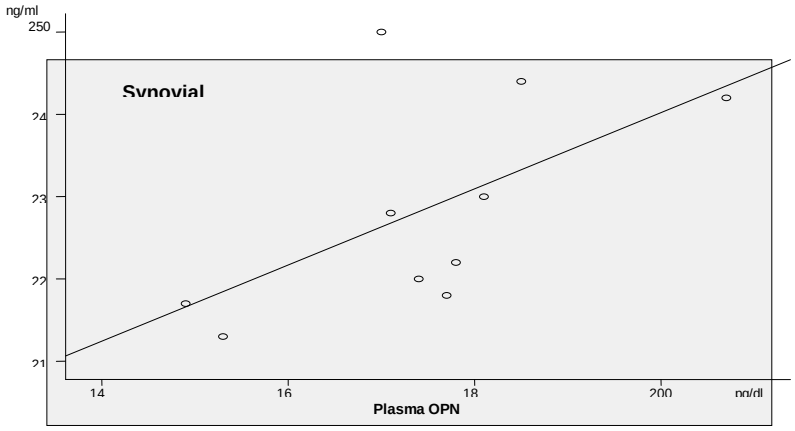

Fig. (9): Correlation between plasma and synovial fluid OPN levels.

4. Discussion

In osteoarthritis, the presence of bone marrow lesions (BMLs) adjacent to the subchondral plate on magnetic resonance images has been strongly associated with disease progression and pain. The defects related to BMLs appear to be sclerotic due to increased bone volume fraction and increased trabecular thickness. The mineral density in these defects, however, is reduced and may render this area to be mechanically compromised, and thus susceptible to attrition ***(Hunter et al., 2009).***

OPN; a non collagenous extracellular matrix (ECM) protein; has been proved to contribute directly to the regulation of bone mineralization and growth (Alford and Hankenson, 2006). Furthermore, it has been implicated as an important factor in bone remodeling and metabolism (Choi *et al.*, 2008).

It has been suggested that osteopontin (OPN), fibronectin (FN), as well as other ECM proteins in the pericellular environment of the chondrocytes such as collagen, and proteoglycan play a role in specific induction and repression of chondrocyte gene (***Saito et al., 1999).*** ***Attur et al.,*** **2000** added that FN and soluble ECM proteins such as vitronectin and OPN are ligands for integrins . They noticed that addition of fragments or recombinant

extra domain regions of these ECM proteins to cartilage up-regulated inflammatory mediators and induced chondrolysis via the ligation of the chondrocyte to integrin. Rosenthal *et al.,* 2007 reported that OPN was found in increased quantities in the pericellular matrix of osteoarthritic cartilage.

On the other hand, Matsui *et al.,* 2009 suggested that OPN was required for cartilage homeostasis and reported that OPN deficiency; and not increase; was associated with more severe OA in mice.

Thus the aim of this study was to measure the plasma and synovial fluid osteopontin levels in patients with primary knee osteoarthritis, in order to assess its relation to disease severity.

This study proved a significant increase in plasma OPN levels in patients with primary knee OA in comparison to control group. These results were in agreement with Honsawek *et al.,* 2009, who suggested enhanced systemic production of OPN in the primary knee osteoarthritis and added that overexpression of OPN may be involved in the molecular athogenesis of osteoarthritis and contribute to progressive degeneration of articular cartilage. Wang and Denhardt, 2008 explained that Upregulation of OPN was found to induce production of proinflammatory chemokines and cytokines including IL1B, TNF ccl2 and cxcl and activation of nuclear factor kappa P pathway. It is already well known that these cytokines are closely associated with functional alterations in synovium, cartilage and subchondral bone. These proinflammatory mediators appear to be first produced by the synovial membrane, and then diffuse into the cartilage through the synovial fluid. They activate the chondrocytes, which in turn produce catabolic factors such as proteases and multiple pro-inflammatory cytokines ***(Standal et al., 2004).***

In our study, there was no statistical significant correlation between plasma OPN levels, age, disease duration and BM of patients..

It has been noticed in this study that OPN levels in synovial fluid were statistically significantly higher with respect to paired plasma samples. These findings were supported by Honsawek *et al.,* 2009. Moreover, Gao et al., 2010 studied OPN expression in both synovial fluid and articular cartilage and reported that synovial fluid OPN levels in O.A patients were higher compared to controls and that expression of OPN was highly up-regulated in human OA cartilage, as compared with normal cartilage. Previous studies have demonstrated; by immuno-histochemical staining; the expression of OPN in fibroblast-like synoviocytes (FLS) and articular chondrocytes (Pullig et al., 2000 , Ohshima et al., 2002). So, the source of elevated OPN in the synovial fluid of OA patients is presumably to be the local tissues such as the synovial membrane and articular cartilage. It was also suggested that cell adhesion, migration or inflammation could be involved in the release of OPN (Standal et al., 2004). However in OA, the degenerative changes of articular cartilage were likely to be the facilitating factors in the release of OPN ; residing in extracellular matrix, into the synovial fluid simply by exposing the subchondral bone to the synovial fluid, so that bone may be a source of synovial fluid OPN ***(Gao et al., 2010).***

Regarding correlation studies, a significant positive correlation was found between plasma OPN and synovial fluid OPN levels. Similar results were reported by ***Honsawek et al., 2009.***

According to our study, both plasma and synovial fluid full length OPN levels were significantly correlated with the severity of primary knee O.A determined by K/L radiological grading. Our findings are in agreement with Honsawek ***et al., 2009. Also, Gao et al., 2010*** proved that synovial fluid OPN levels showed a positive correlation with articular cartilage OPN expression which in turn correlated with disease severity. Therefore, determining the levels of OPN in synovial fluid, may be indirectly predictive of the degree of cartilaginous damage and disease severity and this emphasis our results. In a recent study by ***Hasegawa et al., 2011*** stated that a thrombin cleaved OPN (OPN N-half) was the one significantly higher in synovial fluid of OA knees than in controls and a statistically significant correlation was found between its elevated synovial fluid levels and disease severity by Kellgren-Lawrence grades 1, 2, 3, and 4 ($r = 0.274$, $p < 0.001$). They also added that Immunohistochemistry of the synovium showed stronger reactivity for this thrombin -cleaved OPN in samples from subjects with advanced OA, denoting local expression of thrombin-cleaved OPN which increased with greater OA severity. Interestingly enough, they reported contradictory results about concentrations of full-length OPN in synovial fluid of OA knees which were according to their study; not statistically different from those of controls ($p > 0.05$). Explaining those contradictory results, they stated that the thrombin-cleaved form of OPN was the proinflammatory form which correlated well with various inflammatory diseases. .Another possible reason for differences in those results might be different molecular fragility of OPN types. Our results disagree also with Matsu *et al.,* 2009 who noticed that OPN deficiency; and not increase caused more severe OA changes in OPN-deficient mice. They explained their results by suggesting that OPN might be required for cartilage homeostasis, so its deficiency and not increase was associated with more severe OA. Another explanation may be acceptable differences between their in vitro findings based on experimental animals and others' in vivo findings such like ours.

Conclusion

Both plasma and synovial fluid OPN levels were increased in primary knee OA patients and both of them correlated with more severe OA. Measurements

of plasma and/or synovial fluid levels of OPN could possibly serve as a biochemical parameter for determining disease severity and may be predictive of prognosis with respect to the progression of osteoarthritic disease process.

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