Serum Visfatin is Specific Significant Predictor of Rheumatoid Arthritis Severity: A Comparative Study versus Interleukin-6 and Clinical Severity Scores

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Abstract: Objectives: To determine serum levels of visfatin in patients with rheumatoid arthritis (RA) of varying duration of disease and to correlate it with serum interleukin (IL)-6 and clinical and radiological severity scores. **Patients & Methods:** The study included 70 patients fulfilled either four of seven ACR criteria or having morning stiffness ≥60 minutes, symmetrical arthritis and small joint arthritis for at least 6 months and 20 cross matched age and gender volunteers (Control group). Patients' data including age, gender, weight, height and calculation of body mass index (BMI) were determined. All patients underwent clinical evaluation for disease activity assessed using a 28 joint disease activity score, (DAS-28), pain using visual analogue scale (VAS) and functional disability using the Swedish version of the Stanford health assessment questionnaire (HAQ) to calculate the Disability Index (DI). Postero-anterior radiographs of hands, wrists, and forefeet were taken and joint destruction was classified according to Larsen–Dale index. Blood samples were obtained from patients and controls for ELISA estimation of Rheumatoid factor (RF) and serum IL-6 and visfatin. **Results:** Mean DAS-28 score was 3.9±0.8; range: 1.4-6.8, mean VAS joint pain score was 60.2±5.2; range: 51-71 and mean DI was 12.3±5.1. Erosive lesions were identified in 43 patients (61.4%), while the remaining 27 patients (38.6%). Fifty-one patients (72.9%) were RF positive; 34 had joint erosions and 17 patients were free of erosion. Estimated serum levels of IL-6 and visfatin were significantly higher in patients compared to controls with significantly higher levels in patients had erosive lesions compared to those free of erosion. There was positive significant correlation between presence of radiological evidence of presence bone erosion and patients' age, clinical data and disease severity scores and serum levels of IL-6 and visfatin. Serum levels of IL-6 and visfatin were found to be specific predictors of radiological evidence of bone erosion. **Conclusion:** There was a positive significant correlation between serum visfatin levels and clinical and radiological severity of RA and could be considered as specific predictor for RA radiological severity.

[Khaled Amer and Waleed M. Fathy. **Serum Visfatin is Specific Significant Predictor of Rheumatoid Arthritis Severity: A Comparative Study versus Interleukin-6 and Clinical Severity Scores.** Life Science Journal 2012; 9(1):809-816]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 117

Keywords: Rheumatoid arthritis, interleukin-6, visfatin, prediction of radiological severity.

1. Introduction:

Rheumatoid arthritis is a chronic systemic autoimmune inflammatory disease in which the peripheral joints are the primary sites of inflammation, often leading to destruction of these joints. RA is characterized by symmetrical synovitis, progressive joint damage, pain, fatigue, and disability. The spectrum of RA ranges from benign remitting manifestations to rapidly progressive forms with increased mortality. About 10% of the patients show an intractable rapidly progressive course associated with severe extra-articular manifestations. Within the first three years, 70% of the patients develop radiological erosions of the joints and 31% deformities of the hands. Life expectancy is shortened by 3-18 years **(Kroot *et al.*, 2001, Maille *et al.*, 2004, Rat *et al.*, 2004)**.

Synovitis may explain most of the early symptoms and is also considered to contribute to the development of joint damage and disability. The correlation between inflammation and joint damage has been studied extensively, especially the relevance of inflammatory variables such as C reactive protein and erythrocyte sedimentation rate. Although there is a link between inflammation and the development of joint damage it is well established that damage may progress in spite of decreased inflammatory activity, and erosions may develop in patients who have few clinical signs of inflammation. Thus it has been suggested that pathological processes other than inflammation are involved in the destructive process **(van den Berg, 2001, Fonseca *et al.*, 2009, Plant *et al.*, 2000)**.

Although the exact cause of RA is still unknown, investigation of its pathogenesis has confirmed a role for various pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6. Accordingly, inhibition of these cytokines has become the new therapeutic strategy for RA **(Fonseca *et al.*, 2009, Lioté, 2005)**.

Pre-B cell colony-enhancing factor (PBEF), also known as visfatin, is a highly conserved, 52-kDa protein found in living species from bacteria to humans. It is a one of the recently discovered adipokine produced and secreted primarily by visceral white adipose tissue. PBEF or visfatin is also produced by endotoxin-stimulated neutrophils and inhibits neutrophil apoptosis through a mechanism mediated by caspase 3 and caspase 8 **(Matsui *et al.*, 2009)**.

PBEF exert three distinct activities of central importance to cellular energetics and innate immunity. Within the cell, PBEF functions as a nicotinamide phosphoribosyl transferase, the rate-limiting step in a salvage pathway of nicotinamide adenine dinucleotide (NAD) biosynthesis, so through regulation of cellular levels of NAD and so impact not only cellular energetics but also NAD-dependent enzymes such as sirtuins. Although it lacks a signal peptide, PBEF is released by a variety of cells, and elevated levels can be found in the systemic circulation of patients with a variety of inflammatory diseases. As an extracellular cytokine, PBEF can induce the cellular expression of inflammatory cytokines such as TNF-alpha, IL-1beta, and IL-6. Finally, PBEF has been shown to be an adipokine expressed by fat cells that exerts a number of insulin mimetic and antagonistic effects. PBEF expression is up-regulated in a variety of acute and chronic inflammatory diseases including sepsis, acute lung injury, rheumatoid arthritis, inflammatory bowel disease, and myocardial infarction and plays a key role in the persistence of inflammation through its capacity to inhibit neutrophil apoptosis **(Busso *et al.*, 2008, Luk *et al.*, 2008, Neumann *et al.*, 2007, Popa *et al.*, 2005, Sethi *et al.*, 2005)**.

The current prospective comparative study aimed to determine serum levels of visfatin in patients with RA of varying duration of disease and to correlate it with serum IL-6 levels and clinical and radiological severity scores.

2. Patients and Methods

The present study was conducted at Rheumatology and Rehabilitation Department in conjunction with Clinical Pathology Department since Sep 2009 till Sep 2010. After obtaining patients' fully informed written consent, all patients had rheumatoid arthritis (RA) attending the outpatient clinic for first time or for follow-up were enrolled in the study so as to collect 70 patients with varied duration of disease. Only patients who fulfilled either four of seven ACR criteria or having morning stiffness ≥60 minutes, symmetrical arthritis and small joint arthritis (metacarpo-/metatarso-phalangeal joints/wrists) for at least 6 months were included in the study. Acute phase reactions were measured by erythrocyte sedimentation rate (ESR; mm/h) and C-reactive protein (mg/l) using standard laboratory methods and performed at hospital laboratory. The study also included 20 cross matched age and gender volunteers free of any form of joint affection chosen from those attending hospital blood bank for blood donation after passing the preliminary laboratory investigations required for blood donation according to hospital protocol to serve as control group.

Patients' data including age, gender, weight, height and calculation of body mass index (BMI) according to the equation: BMI= weight (kg)/height (m^2) were determined and duration of disease were determined.

All patients underwent clinical evaluation of disease activity as assessed by the disease activity score, using a 28 joint score (DAS-28), as follows: ≤3.2: inactive, >3.2-≤5.1: moderate activity and >5.1: very active disease ***(*Prevoo *et al., 1995)***. Pain was assessed by a 0–100 mm horizontal visual analogue scale (VAS), with 0 indicates no pain and 100 indicates the worst intolerable pain and VAS score of 0-25 indicates mild pain, >25-50 indicates moderate pain, >50-75 indicates severe pain and >75 indicates intolerable pain **(Scott & Huskisson, 1976)**.

Functional disability was evaluated using the Swedish version of the Stanford health assessment questionnaire (HAQ) to calculate the Disability Index (DI). The eight categories assessed by DI are 1) dressing and grooming, 2) arising, 3) eating, 4) walking, 5) hygiene, 6) reach, 7) grip, and 8) common daily activities. The difficulty during each of these acts was assessed as follows: 0: without any difficulty, 1: with some difficulty, 2: with much difficulty and 3: unable to do, then the sum of the categories scores is calculated and divided by the number of categories. This gives a score in the 0 to 24 range **(Ekdahl *et al.*, 1988)**.

Postero-anterior radiographs of hands, wrists, and forefeet were taken at inclusion in the study and joint destruction was classified by comparison with standard reference films according to the Larsen–Dale index **(Larsen *et al.*, 1977)**. The joints assessed for this index are the wrists, where all metacarpo-phalangeal joints (=10), all proximal interphalangeal joints (=8), both first interphalangeal joints in the hands (=2), metatarso-phalangeal joints II–V (=8), and both first interphalangeal joints in the feet (=2). Thus 32 joints are scored in all. Each joint is graded 0–V, as follows: grade 0: no abnormality; grade I: slight abnormality with one or more of the following criteria: soft tissue swelling, juxta-articular osteoporosis, slight narrowing of the joint space; grade II–V: erosion and narrowing of the joint space of increasing severity as illustrated in the standard

reference radiographs referring to the grade of damage of bone and cartilage, respectively. The degree of erosive damage is the most decisive criterion in grading and the finding of at least one definite erosion on any of the hands or feet radiographs was sufficient to consider the patient as having erosive disease.

Whole blood sample (5 ml) were obtained from patients and controls under complete aseptic conditions and were collected in plain tube and allowed to clot and centrifuged at 5000 rpm for 10 minutes and serum was separated and kept at -80 C° for ELISA estimation of:

1. Rheumatoid factor IgM isotype was analyzed using the ELISA kit for RF IgM quantitation (Orgentec Diagnostika GmbH, Germany) according to the manufacturer's instructions. The titre of 20 IU/ml was regarded as positive, **(Kleveland *et al.*, 1988)**.
2. Serum IL-6 was measured with an ELISA kit from Pelikine™ Inc., Concord, USA. IL-6 values in fresh serum of healthy individuals are <20 pg/ml **(Gaines-Das & Poole, 1993)**.
3. Serum visfatin was measured by Visfatin C-terminal ELISA kit (Phoenix Pharmaceuticals, Inc, Burlingame, CA, USA), **(Fukuhara *et al.*, 2005)**.

Statistical analysis

Obtained data were presented as mean±SD, ranges, numbers and ratios. Results were analyzed using Wilcoxon's Ranked test for unrelated data and Chi-square test. Possible relationships were investigated using Pearson linear regression. Predictors for evaluation of radiological evidence of erosion were evaluated using the receiver operating characteristic (ROC) curve analysis judged by the area under the curve (AUC) and Regression analysis (Stepwise Method). Statistical analysis was conducted using the SPSS (Version 10, 2002) for Windows statistical package. *P* value <0.05 was considered statistically significant.

3. Results

The study included 70 patients; 47 females (67.1%) and 23 males (32.9%) with mean age of 52.9±6.7; range: 40-64 years. All patients had fulfilled the criteria of ACR with a mean duration of disease of 4.7±1.8; range: 1.6-10 years. Mean DAS-28 score for disease activity was 3.9±0.8; range: 1.4-6.8, mean VAS joint pain score was 60.2±5.2; range: 51-71 and mean DI was 12.3±5.1. Mean ESR level was 31.5±13.5; range: 9-60 mm/h and mean CRP level was 16.5±10.8; range: 6-72 mg/l. Details of patients' enrollment data are shown in table 1.

Erosive lesions were identified in 43 patients (61.4%) with a mean Larsen score of 33.2±8.7; range: 13-45 (Erosive group), while the remaining 27 patients (38.6%) with a mean Larson score of 7.3±1.3; range: 4-9 (Non-erosive group) with a mean total Larsen score of 23.1±14.3; range: 4-45, (Fig. 1).

Fifty-one patients (72.9%) were rheumatoid factor positive; 34 had joint erosions and 17 patients were free of erosion. Nineteen patients (27.1%) were rheumatoid factor negative; 9 had joint erosions and 10 patients were free of erosion. There was non-significant difference between erosive and non-erosive groups as regards frequency of rheumatoid factor positivity.

Estimated serum levels of IL-6 (Fig. 1) and visfatin, (Fig. 2) in studied patients were significantly higher, both as total and categorized according to radiological evidence for presence of erosion, compared to control group. Moreover estimated serum levels of IL-6 and visfatin were significantly higher in patients had radiological evidence of presence erosion compared to those free of erosion (Table 2).

There was positive significant correlation between presence of radiological evidence for presence bone erosion and patients' age, clinical data and disease severity scores and serum levels of IL-6 and visfatin, (Table 3). ROC curve analysis of correlated factors versus presence of radiological evidence of presence bone erosion showed that all of them could predict it specifically (Table 4, Fig. 3).

Age, duration of disease, DAS-28 score, VAS pain score, DI, serum IL-6 and visfatin were verified using Regression analysis (Stepwise method) excluding the non-significant and least significant factors as predictors of the presence of radiological evidence of bone erosion defined serum levels visfatin as specific predictor that was persistently significant in four regression analysis models, followed by serum IL-6 in three models, age in two models and DI in one model (Table 5), thus indicating that visfatin could be used as specific significant predictor for RA severity.

Table (1): Patients' enrollment data

Data			Findings	
			Number	mean±SD
Age (years)	Strata	40-50	22 (31.4%)	45.2±3.6 (40-49.8)
		>50-60	34 (48.6%)	54±2.7 (50-58.8)
		>60	14 (20%)	62.4±1 (60-64)
	Total		70 (100%)	52.9±6.7 (40-64)
Duration of disease (years)	Strata	≤5 years	43 (61.4%)	3.6±0.9 (1.6-5)
		>5 years	27 (38.6%)	6.5±1.1 (5.2-10)
	Total		70 (100%)	4.7±1.8 (1.6-10)
DAS-28 activity score	Inactive disease		14 (20%)	2.3±0.5 (1.4-3.1)
	Moderately active disease		47 (67.1%)	4±0.5 (3.2-5)
	Very active disease		9 (12.9%)	6±0.5 (5.3-6.8)
	Total score		70 (100%)	3.9±0.8 (1.4-6.8)
VAS pain score	Mild pain		11 (15.7%)	18.7±2.6 (15-23)
	Moderate pain		42 (60%)	34.8±6 (26-45)
	Severe pain		17 (24.3%)	60.2±5.2 (51-71)
	Total score		70 (100%)	38.4±15.3 (15-71)
Mean DI	<10		31 (44.3%)	7.5±1.4 (5-9)
	10-20		33 (47.1%)	15.1±2.9 (10-20)
	>20		6 (8.6%)	21.5±0.8 (21-23)
	Total		70 (100%)	12.3±5.1 (5-23)

Data are presented as numbers, ratio & mean±SD; percentages & ranges are in parenthesis

Table (2): Serum levels of IL-6 and visfatin estimated in studied patients categorized according to radiological presence of erosion compared versus control group

	Control (n=20)	Patients		
		Non-erosive (n=27)	Erosive (n=43)	Total (n=70)
IL-6 (ng/ml)	4.49±3.3 (1.5-11.6)	14.1±7.1* (4.8-23.4)	25.7±6.77*† (12.7-41.7)	21.2±9* (4.8-41.7)
Visfatin (ng/ml)	3.24±0.91 (2.1-5.4)	6.61±1.21* (4.7-9.87)	10.65±3.18*† (5.6-18.9)	9.1±3.26* (4.7-18.9)

Data are presented as mean±SD; ranges are in parenthesis *: significant difference versus control group
†: significant difference versus non-erosive group

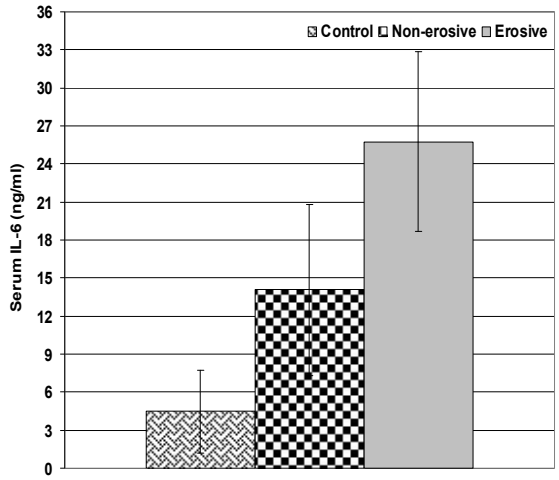

Fig. (1): Serum levels of IL-6 estimated in studied patients categorized according to radiological evidence of presence of erosion

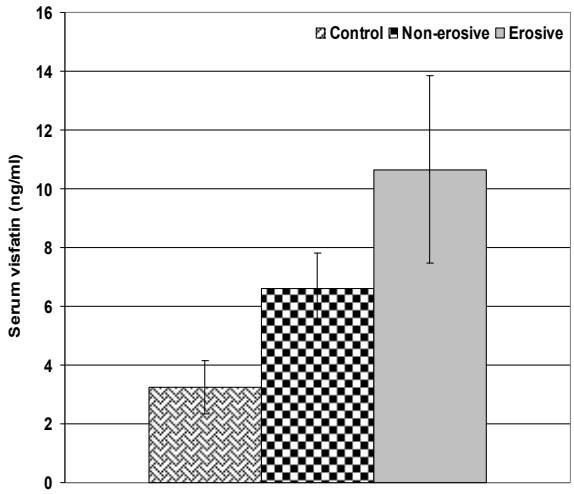

Fig. (2): Serum levels of visfatin estimated in studied patients categorized according to radiological evidence of presence of erosion

Table (3): Correlation coefficient between radiological presence of erosion versus age, clinical data and serum IL-6 and visfatin levels

	Age	Duration of disease	DAS-28 score	Pain VAS score	DI	Serum IL-6	Serum visfatin
"r"	0.373	0.365	0.486	0.504	0.413	0.390	0.607
p	=0.001	=0.002	<0.001	<0.001	<0.001	=0.001	<0.001

Table (4): ROC curve analysis of age, clinical data and serum IL-6 and visfatin levels as predictors for presence of radiological evidence of erosion as judged by area under curve

	Area under curve
Age	0.693
Duration of disease	0.717
DAS-28 score	0.752
Pain VAS score	0.789
ID	0.746
Serum IL-6	0.742
Serum visfatin	0.876

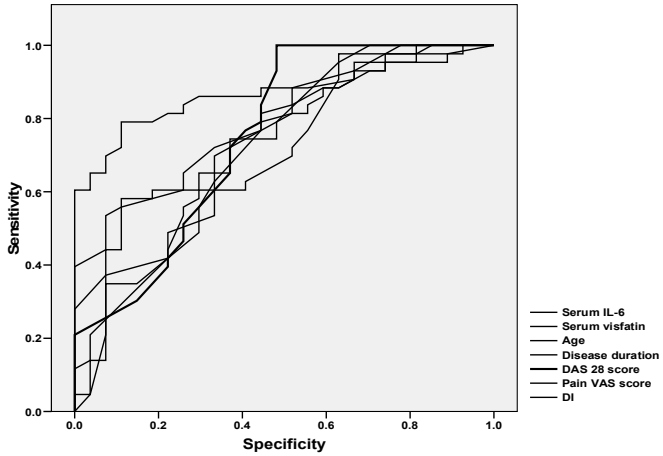

Fig. (3): ROC curve analysis of age, clinical data and laboratory findings as predictors of radiological presence of erosion

Table (5): Regression analysis models "Stepwise method" to identify the significant predictor for presence of radiological evidence of erosion

		ß	SE	t	p
Model 1	Age	0.235	0.006	2.809	=0.007*
	Duration of disease	0.128	0.145	1.475	>0.05
	DAS-28 score	0.104	0.284	1.081	>0.05
	VAS pain score	0.146	0.166	1.401	>0.05
	DI	0.185	0.008	2.096	=0.040*
	Serum IL-6	0.329	0.005	3.795	=0.004*
	Serum visfatin	0.485	0.013	5.814	<0.001*
Model 2	Age	0.231	0.006	2.695	=0.009*
	Serum IL-6	0.402	0.004	4.940	=0.001*
	Serum visfatin	0.531	0.013	6.204	<0.001*
Model 3	Serum IL-6	0.386	0.005	4.552	=0.002*
	Serum visfatin	0.604	0.013	7.117	<0.001*
Model 4	Serum visfatin	0.607	0.015	6.293	<0.001*

ß: standardized coefficient SE: Standard error *: significant parameter

4. Discussion

The current study aimed to enroll all patients with RA manifestation for more than 6 months; all enrolled patients were symptomatizing with a mean DAS-28 score for disease activity of 3.9±0.8, mean VAS joint pain score was 60.2±5.2 and mean DI of 12.3±5.1. These data could be attributed to that previously reported by **Gerber *et al.*(2003)** and **Scott *et al.* (2003)** who found the active joint count predicts subsequent performance and function for patients with recent onset, inflammatory synovitis more effectively than whether patients met ACR criteria for RA.

Disease severity assessment relied on evaluation of pain using VAS pain score, join affection score (DAS 28), disability index and laboratory evaluation of ESR and CRP levels. Such combination helped for patients' selection and goes in hand with **Klarenbeek *et al.* (2011)** who compared nine disease activity indices versus the American College of Rheumatology/European League against Rheumatism remission criteria in RA and tried to relate these indices to physical function and joint damage progression and found clinical DAS and simplified DAI were the most stringent definitions of remission, DAS28 and DAS28-CRP had the highest proportions of remission and concluded that all indices, higher levels of disease activity were associated with decreased physical functioning and more radiological damage progression.

Serum levels of IL-6 and visfatin were significantly higher in patients compared to controls and in those had radiological evidence of erosion compared to patients free of erosion. These findings go in hand with **Otero *et al.* (2006)** who investigated plasma levels of adipocytokines (leptin, adiponectin, visfatin and resistin) in patients with RA in comparison to levels estimated in healthy controls, and found patients with RA showed considerably higher plasma levels of leptin, adiponectin and visfatin than healthy controls, but no marked difference was observed in resistin levels between patients and controls. **Senolt *et al.* (2011)** found serum visfatin levels were significantly higher in patients with RA compared with healthy controls and significantly decreased following treatment with anti-B cell therapy.

The increased levels of both IL-6 and visfatin indicated pathogenic relation between both cytokines. In support of this assumption, there was a positive significant correlation between serum levels of both parameters on one side and between both and presence of radiological evidence of erosion.

Multiple studies tried to explore the relationship between IL-6 and visfatin and presence of RA and its severity; **Nowell *et al.* (2006)** experimentally found IL-6 trans-signaling regulated PBEF in a STAT-3-dependent manner, PBEF was regulated by the IL-6-related cytokine oncostatin M and that the involvement of PBEF in arthritis progression was confirmed in vivo, where induction of antigen-induced arthritis resulted in a 4-fold increase in the synovial expression of PBEF. On reverse, **Brentano *et al.* (2007)** found that in RA synovial fibroblasts, PBEF was up-regulated by Toll-like receptor ligands and PBEF itself activated the transcription factors NF-kB and activator protein 1 and induced IL-6, IL-8 and metalloproteinases 1 and 3 in RA synovial fibroblasts as well as IL-6 and TNF-α in monocytes.

Niederer *et al.* (2011) analyzed the expression of sirtuin 1 (SIRT1) which plays an important role in maintaining metabolic homeostasis in synovial tissues and cells of patients with RA and found SIRT1 was constitutively upregulated in synovial tissues and cells from patients with RA compared to osteoarthritis, silencing of SIRT1 promoted apoptosis in RA synovial fibroblasts, whereas SIRT1 over-expression protected cells from apoptosis and knockdown of SIRT1 resulted in a reduction of proinflammatory IL-6 and IL-8 in RA synovial fibroblasts.

Among clinical data including age, duration of disease, DAS-28 score, VAS pain score, DI and serum IL-6 and visfatin, Regression analysis as predictors of the presence of radiological evidence of bone erosion defined serum levels visfatin as specific predictor that was persistently significant in four regression analysis models.

In line with the specificity of visfatin for RA, **Nowell *et al.* (2006)** found that synovial fluid levels of PBEF were significantly higher in RA patients than in osteoarthritis patients. **Rho *et al.* (2009)** who found visfatin concentrations were associated with higher Larsen scores, and this association remained significant after adjustment for age, race, sex, disease duration, BMI, and inflammation. Thereafter, **Rho *et al.* (2010)** examined the relationship between adipocytokines and insulin resistance and coronary atherosclerosis among patients with RA and reported increased serum levels of examined adipocytokines and increased concentrations of leptin were associated with a higher insulin resistance index, even after adjustment for age, race, sex, BMI, traditional cardiovascular risk factors, and inflammation mediators, but concentrations of visfatin, adiponectin and resistin showed no association with insulin resistance.

Klein-Wieringa *et al.* (2011) found levels of IL-6, TNF-α, visfatin, and adiponectin were positively associated with radiographic progression over 4 years and this association was independent of

BMI and concluded that adipokines are predictors of radiographic progression in RA, possibly through distinct underlying biologic mechanisms. **Senolt *et al.* (2011)** reported that lack of change in the serum visfatin levels between baseline and week 16 following treatment with rituximab predicted worsening disease activity between weeks 16 and 24.

It could be concluded that there was a positive significant correlation between serum visfatin levels and rheumatoid arthritis severity as manifested clinically or radiologically and as serum IL-6 levels. Serum levels of visfatin could be considered as specific significant predictor for radiological severity and wider scale studies are advocated for evaluation of its utility as screening test for early cases.

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2/19/2012