Assessment of Metalloproteinase-3, Tissue Inhibitor of Metalloproteinase-1, Bone Sialoprotein and Chondroitin Sulphate as Markers of Extracellular Matrix Turnover in Patients with Rheumatoid Arthritis : Correlation with Degree of Bone Erosion.

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Abstract: This study aimed to assess extracellular matrix turnover in patients with rheumatoid arthritis (RA) and to find the relationship between serum and synovial fluid levels of metalloproteinase-3 (MMP_3), tissue inhibitor of metallo-proteinase (TIMP-1), chondroitin sulphate (monitior of cartilage degradation) and bone sialoprotein (monitor of bone degradation) and the degree of bone erosion of the knee joints. Fifty six human subjects subdivided into three groups have participated in this study. Group I included ten apparently healthy individuals to represent the control group. Thirty RA patients were chosen to represent group II while sixteen patients with osteoarthritis were chosen to represent group III as a reference arthritis disease group. All patients of group II and III were presented with effusion of knee joints. Metalloproteinase-3 (MMP-3), tissune inhibitor of metalloproteinase-1 (TIMP-1) and bone sialoprotein were assessed by ELISA technique while chondroitin sulphate was measured by a colorimetric method. All parameters were assessed in serum and synovial fluid. All indices were significantly higher in both serum and synovial fluid when compared to controls. Also serum levels of all parameters were correlated with synovial fluid levels. MMP-3 levels of both serum and synovial fluid were significantly higher in RA patients than OA patients. Such difference was not observed in other parameters indicating higher MMP3/ TIMP-1 in RA patients than OA patients. Synovial fluid levels of MMP3, TIMP-1, chondroitin sulphate and bone sialoprotein were all positively correlated with the degree of bone erosion indicating a strong role of MMP-3 in joint degradation in patients with RA. [Journal of American Science 2010;6(10):1081-1089]. (ISSN: 1545-1003).

Keywords: extracellular matrix turnover; patient; rheumatoid arthritis (RA); metalloproteinase-3 (MMP_3); metallo-proteinase (TIMP-1)

Abbreviations

ECM: Extracellular matrix
TIMPs: tissue inhibitors of metalloproteinase
OA: osteoarthritis
RF: Rheumotoid factor
MMPS: Matrix metalloproteinases
RA: rheumatoid arthritis
ND: non detectable
ANA: Antinuclear antibodies

1. Introduction:

Articular cartilage is composed of an abundant extracellular matrix (ECM) made up largely of aggrecans which consist of sulphated proteoglycans that are trapped within a fibrillar network of collagen. Proteoglycans are responsible for retaining water in the tissues that allows cartilage to resist compression (Eyre et al, 2006, Hardinpham,et al,1992). Other matrix non collagenous proteins are believed to mediate additional steps in matrix assembly. These include members of matrilin family and bone sialoprotein which is a bone specific matrix protein, especially enriched at the bone cartilage interface (Henning, et al (2004). Matrilins play a role in mediating interactions between major components of the extracellular matrix such as collagens and proteoglycans (Fauzen, et al (1985).

The primary event in cartilage destruction in arthritis diseases involves active proteases degrading its ECM components. Matrix metalloproteinases (MMPs) are a family of structurally and functionally related zinc-dependent endopeptidases that are believed to play a pivotal role in tissue remodeling (Woessner, (1991)). MMP-3 (stromolysin-1) has been proved to have broad substrate specificity that includes proteoglycans and other matrix

macromolecules [Nagase, and Woessner (1999)]. The expression of MMP-3 has been shown to be enhanced by in vitro treating fibroblasts with proinflammatory cytokines [Distler, et al (2005)]. Meanwhile the activity of MMPs is regulated by several naturally occurring inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) and α2–macroglobulin [Brew, et al(2000)]. TIMP-1 has been identified to be active against a wide variety of MMPs including MMP-3[Martel – Pelletieret al (2001)] with stochiometric ratio of 1: 1. An imbalance between tissue levels of MMPs and TIMPs towards higher levels of the former may account in part, for development of cartilage damage in arthritis diseases [Ishiguro, et al (1999)].

Rheumatoid arthritis (RA) is a chronic auto-immune inflammatory disease characterized by inflammation and destruction of articular structures in association with extraarticular manifestations (Woo, (1990)]. Joint destruction in RA patients results from the invasion of cartilage and subcondral bone by the hyper-plastic synovium, with synovial fibroblasts and inflammatory cells. The primary event is activation of synovial macrophages, monocytes and lymphocytes with concomitant release of proinflammatory cytokines that may initiate a cascade of events that ultimately ends with bone erosion (Smith, (1990)].

This study has been conducted to evaluate serum and synovial fluid levels of MMP-3, TIMP-1, chondroitin sulphate and bone sialoprotein to assess ECM turnover in cartilage and bone of the knee joints of patients with RA when compared to the non inflammatory arthritis disease (osteoarthritis) (OA). The study is also a trial to clarify the correlation between these parameters and degree of radiographic alteration of the knee joint.

2. Subjects and Methods

This study has been conducted on three groups of human subjects. Group I included 10 healthy individuals who represented the control group. Group II comprised 30 patients with RA. The diagnosis of RA was based on the revised criteria of the American Rheumatism Association[Arenett, et al (1988)]. All selected patients were presented with knee effusion. Twenty-three patients were receiving methotrexate alone while seven patients were being treated with methotrexate and hydroxychloroquine. Patients receiving oral or intraarticular steroids were not included. Group III included 16 patients with osteoarthritis (OA) who represented a reference group. These patients were diagnosed according to the criteria of clinical osteoarthritis[Altman, (1991)] and were also presented with knee effusion. All RA and OA patients were attending the outpatient clinics of Department of Physical Medicine and Rehabilitation, Tanta University Hospital.

All patients and controls were subjected to the following:-

1-Full history taking and thorough clinical examination.

2-Routine laboratory investigations including complete blood picture, liver function tests and kidney function tests. Patients with altered kidney or liver functions were excluded.

3-Specific laboratory investigations for arthritis diseases including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF) and antinuclear antibody (ANA).

4-Aliquots of serum samples were also frozen at – 70^{o} C until assayed for the required parameters in this study.

5-Synovial fluid was collected from the knee joints of RA and OA patients under strict sterile conditions. Patients with purulent effusion were excluded from this study. Samples of synovial fluid were centrifuged at 3000 rpm for 10 minutes at 4^{o}C and aliquots of the supernatants were kept frozen at – 70^{o} C until assayed.

6-Aliquots of sera and synovial fluids were assayed for the following.

a-MMP – 3 by ELISA technique[Fujimoto, et al (1993)] using a commercial kit.

b-TIMP-1by ELISA technique[Pay, et al (2002)] using a commercial kit.

c-Chondroitin sulphate following the colorimetric method of Mier and Wood [Mier and Wood (1969)] as modified by Shinmei [Shinmei, et al (1992)].

d- Bone sialoprotein by ELISA technique[3] using a commercial kit. All commercial ELISA kits were obtained from Biotrak ELISA system, Amersham Pharmacia Biotech, Little Chalfont, UK.

7-Radiographic imaging of the knee joints of RA and OA patients was carried out. Joint destruction on plain anteroposterior radiographs of affected joints was graded according to the method of Larsen et al [Larsen, (1995)]. In this method grade O was given to normal joints, grade 1 was given to erosions of less than 1 mm or if joint space narrowing was found, grade 2 was given if one or more erosions greater than 1mm were present, grade 3 was given if erosions of significant size were present, grade 4 was given for severe erosions and grade 5 was given when the original articular surfaces have disappeared. In this study bone changes in RA and OA patients were

classified into mild (grade 0 and 1) moderate (grade 2 and 3) and severe (greade 4 and 5).

8-Statistical analysis of the collected data was carried out using student-t test, Chi- square testing and Spearman bivariate correlation r (Gianneli, et al (2004)).

3. Results

Table (1) shows the demographic and clinical data of the three studied groups.

Table (2) demonstrates serum levels of the different studied parameters. RA and OA patients showed significantly higher levels of MMP-3 when compared to controls Meanwhile MMP-3 serum levels were significantly higher in RA patients when compared to those of OA patients .Although TIMP-1 was significantly higher in RA and OA patients when compared to control, there was no significant statistical difference between levels of TIMP-1 in RA patients when compared to OA patients. The same finding was reported to serum levels of Chondroitin sulphate and bone sialoprotein.

Table (3) shows levels of MMP-3, TIMP-1, Chondroitin sulphate and bone sialoprotein in synovial fluid of knee joints of RA and OA patients. The only significant difference was reported to MMP-3 whose level exerted a significant increment in RA patients than in OA patients. Levels of TIMP-1, Chondroitin sulphate and bone sialoprotein were not statistically different P> 0.05.

Table (4) demonstrates the difference between serum levels and synovial fluid levels of different studied parameters in RA patients and OA patients. A significant increment of synovial fluid levels in comparison to serum levels was revealed for all parameters studied in both arthritis diseases.

Table (5): Shows significant increase in MMP3/TIMP-1 ratio for RA in comparison with OA patients in both serum and synovial fluid

Tables (6,7) demonstrates the difference between synovial fluid levels of different studied parameters in RA and OA patients with degree of bone erosion.

Table (8) shows a significant positive correlation between ESR and CRP in serum of RA patients. Also MMP3,TIMP-1, chondroitin sulphate and bone sialoprotein are significantly positive correlated to each other and to both ESR and CRP.

Table (9) shows a significant positive correlation between MMP3, TIMP-1, chondroitin sulphate and bone sialoprotein and with both ESR and CRP in RA patients.

Table (10) shows a significant correlation between ESR and CRP in serum of OA patients. Also, MMP-3, TIMP-1, chordroitin sulphate and bone sialoprotein are significantly correlated to each other and insignificantly correlated to both ESR and CRP.

Table (11) shows a significant correlation of the increased synovial fluid levels of MMP-3, TIMP-1, chordroitin sulphate and bone sialoprotein to each other in OA patients, however, their correlation to ESR and CRP is insignificant.

Table (1): Demographic and clinical data of different groups under study

	Control (n-10)	RA patients n = 30	OA patients (n=16)
Age (years)	55.6± 5.2	53.1±7.2	57.32± 5.9
Gender (M/F)	7/3	25/5	13/3
ESR (mm/1st h.)	9.5±5.23	68.5±7.33	28.5±7.2
CRP (mg/L)	ND	89.6±17.75	7.1±2.04
RF positive /negative	0/10	23/7	2/14
ANA positive /negative	0/10	18/12	0/16
Larson score			
Mild (scores O and 1)		9	6
Moderote (scores 2 and 3)		11	5
Severe (scores 4 and 5)		10	5

Table (2): Serum levels of MMP-3, TIMP-1, chondroitin sulphate and bone sialoprotein in different groups under study.

	Controls N=10	R A patients N=30	OA patients N=16	Test of Significance (p values)
MMP-3 (ug/ml)	2.46±0.96	64.13±22.33	27.16±12.78	**P1** RA <0.001* **P1**OA <0.001* **P2:** < 0.001*
TIMP-1 (ug/ml)	0.562± 0.095	2.88 ±1.08	3.11±1.45	**P1** RA <0.001* **P1**OA <0.001* **P2:** > 0.05
Chondroitin sulphate mmole/ml	1.39±0.87	30.48±10.83	28.56±10.78	**P1** RA <0.001* **P1** OA <0.001* **P2:** > 0.05
Bone sialoprotein (ng/ml)	10.52±3.39	142.53±52.03	144.63±58.3	**P1** RA <0.001* **P1** OA <0.001* **P2:** >0.05

P1 RA and P1 OA vs control P2: RA vs OA * significant difference

Table (3): Synovial fluid levels of MMP-3, TIMP-1, chordroitin sulphate and bone sialoprotein in RA patients (n=30) in comparison to OA patients (n= 16) .

	RA patients (n=30)	OA patents (n=16)	P
MMP-3 (ug/ml	301±112.62	107.88±15.84	<0.001*
TIMP-1 (ug/ml)	14.62±6.0	15.51±7.22	>0.05
chordroitin sulphate (mml/ml)	166.20±52.8	171.93±64.76	>0.05
Bone sialoprotein (ng/ml)	534.20±204.28	572.88±220.94	>0.05

P: RAvsOA * Significant difference

Table (4): Difference between synovial fluid levels and serum levels of different studied parameters in RA patients and OA patients.

	MMP3 (ug/ml)		TIMP1 (ug/ml)		Chordroition sulphate (mmole/ml)		Bone sialoprotein (ng/ml)	
	Synovial fluid level	Serum level	Synovial fluid level	Serum level	Synovial fluid level	Serum level	Synovial fluid level	Serum level
RA (n=30)	301±112.6	64±12	14.8±5.7	2.88±1.08	166.2±52.8	30.48±10.8	534.2±204.3	142.5±52
p. value	<0.001*		<0.001*		<0.001*		<0.001*	
OA (n=16)	107.8±15.8	27.1±12.9	15.5±7.5	3.1±1.5	171.9±64.8	28.6±10.9	572.9±240.9	144.6±58.3
p. value	<0.001*		<0.001*		<0.001*		<0.001*	

* Significant difference

Table (5): Shows MMP3/TIMP-1 ratio for RA and OA patients in both serum and synovial fluid

	Serum levels		Synovial fluid levels	
	MMP-3/ TIMP-1 in RA	**MMP-3/ TIMP-1 in OA**	**MMP-3/ TIMP-1 in RA**	**MMP-3/ TIMP-1 in OA**
Mean + SD	22.26 + 9.71	8.72 + 4.8	20.33 + 11.2	6.95 + 2.1
t. test	5.42		7.1	
p. value	<0.001*		<0.001*	

* Significant difference

Table (6) Difference in synovial fluid levels of studied parameters between R A patients with mild moderate and severe bone erosion.

	Mild N= 9	Moderate N= 11	Severe N= 10	F Value	P Value
MMP-3 (ug/ml)	40.9±5.7	61.5±5.1	88±19.2	37.52	<0.001*
TIMP-1 (ug/ml)	1.76±0.27	2.76±0.26	4.04±1.08	37.3	<0.001*
chordroitin sulphate (mmole/ml)	18.50±2.85	28.95±2.89	42.93±6.57	72.1	<0.001*
Bone sialopretein (ng/ml)	85.76±14.18	137.7±13.29	199±39.29	47.59	<0.001*

Mild = grades 0 and 1 Moderate = grades 2 and 3 Severe = grades 4 and 5
*Significant = severe>moderate> mild

Table (7): Difference between synovial fluid levels of different studied parameters and O A patients with mild moderate and sever bone erosion in with OA (n= 16) .

	Mild N= 6	Moderate N= 5	Severe N= 5	F Value	P value
MMP-3 (ug/ml)	14.42±5.56	27.42±4.44	42.19±5.28	39.67	<0.001*
TIMMP-1 (ug/ml)	1.8±0.14	2.78±0.29	5±0.74	70.19	<0.001*
chordroitin sulphate (mml/ml)	17.50±2.43	29±3.67	41.40±5.03	54.87	<0.001*
Bone siloprotein (ng/ml)	91.50±21.39	140±13.67	213±43.39	24.36	<0.001*

Mild = grades 0 and 1 Moderate = grades 2 and 3 Severe = grades 4 and 5
*Significant= severe>moderate> mild

Table (8) Correlation between ESR ,CRP and studied serum biochemical parameters in RA patients.

		ESR	CRP	MMP3	TIMP-1	Chondroitin sulphate
CRP	**R-**	0.867	-	-	-	-
	P-	<0.001*				
MMP3	**R-**	0.922	0.904	-	-	-
	P-	<0.001*	<0.001*			
TIMP-1	**R-**	0.915	0.964	0.997	-	-
	P-	<0.001*	<0.001*	<0.001*		
Chondroitin sulphate	**R-**	0.421	0.555	0.499	0.522	-
	P-	<0.05*	<0.05*	<0.05	<0.05*	
Sialoprotien	**R-**	0.903	0.944	0.993	0.992	0.582
	P-	<0.001*	<0.001*	<0.001*	<0.001*	<0.05*

* Significant difference

Table (9) Correlation between ESR ,CRP and studied synovial biochemical parameters in RA patients.

		ESR	CRP	MMP3	TIMP-1	Chondroitin sulphate
CRP	**R-**	0.867	-	-	-	-
	P-	<0.001*				
MMP3	**R-**	0.923	0.939	-	-	-
	P-	<0.001*	<0.001*			
TIMP-1	**R-**	0.916	0.947	0.989	-	-
	P-	<0.001*	<0.001*	<0.001*		
Chondroitin sulphate	**R-**	0.912	0.939	0.970	0.987	-
	P-	<0.001*	<0.001*	<0.001*	<0.001*	
Bone Sialoprotien	**R-**	0.898	0.937	0.989	0.993	0.979
	P-	<0.001*	<0.001*	<0.001*	<0.001*	<0.001*

* Significant difference

Table (10) correlation matrix of different studied parameters in serum of OA patients to each other and with ESR.

		ESR	CRP	MMP3	TIMP-1	Chondroitin sulphate
CRP	**R-**	0.411	-	-	-	-
	P-	>0.05				
MMP3	**R-**	0.180	0.308	-	-	-
	P-	>0.05	>0.05			
TIMP-1	**R-**	0.080	0.273	0.958	-	-
	P-	>0.05	>0.05	<0.001*		
Chondroitin sulphate	**R-**	0.185	0.302	0.983	0.973	*
	P-	>0.05	>0.05	<0.001*	<0.001*	
Bone Sialoprotien	**R-**	0.229	0.337	0.976	0.969	0.973
	P-	>0.05	>0.05	<0.001*	<0.001*	<0.001*

* Significant difference

Table (11) correlation matrix of different studied parameters in synovial fluid of OA patients to each other and ESR and CRP.

		ESR	CRP	MMP3	TIMP-1	Chondroitin sulphate
CRP	**R-**	0.411	-	-	-	-
	P-	>0.05				
MMP3	**R-**	0.168	0.300	-	-	-
	P-	>0.05	>0.05			
TIMP-1	**R-**	0.085	0.273	0.955	-	-
	P-	>0.05	>0.05	<0.001*		
Chondroitin sulphate	**R-**	0.196	0.298	0.981	0.970	-
	P-	>0.05	>0.05	<0.001*	<0.001*	
Bone Sialoprotien	**R-**	0.203	0.316	0.982	0.971	0.978
	P-	>0.05	>0.05	<0.001*	<0.001*	<0.001*

4. Discussion:

MMP-3 is one of the most important metalloproteinases that can cause bone and cartilage erosions (Distler, et al. (2005). In this current study, MMP-3 levels have been shown to be significantly increased in serum of patients with RA and OA when compared to normal control subjects. Meanwhile serum and synovial fluid levels of MMP-3 showed highly significant elevation in RA patients when compared to OA patients. A positive correlation exists between serum levels and synovial fluid levels of MMP-3 in both groups of arthritis diseases indicating that the articular cartilage is the main site of increased MMP-3 expression. There has been a wide variety of evidences confirming the presence of high serum and synovial fluid levels of different MMPs in different arthritis diseases including OA and RA(Martel – Pelletier, et al (2001) and Gianneli, et al (2004)). Moreover the presence of augmented increase in MMP-3 in RA patients has been explained on the basis that joint destruction in RA results from the invasion of cartilage and subchondral bone by the hyperplastic synovium(Woo, (1990)) with synovial fibroblasts and inflammatory cells mainly macrophages and lymphocytes playing a key role in this process(Smith, (1990) and Kanbe, et al (2002)). Proinflammatory cytokines when released in such situations will promote critical events including induction of MMPs release (Distler, et al (2005). Also cytokine-induced overexpression of MMPs has been confirmed in tissue culture fibroblasts (Distler, et al. (2005) and in expiremental animals presented with autoimmune arthritis (Hegemann, et al (2003). The close association between MMP-3 overexpression and the inflammatory process in RA was also confirmed in this study by the presence of a positive correlation between serum MMP-3 levels and values of ESR and CRP in RA in face of absence of such correlation in OA.

Tissue inhibitors of MMPs are the major inhibitors of MMPs in joints (Brew, K., et al (2000). These inhibitors exhibit high affinity for binding with MMPs and eventually inhibiting them. In this current study serum levels of TIMP-1 were significantly elevated in patients with RA and OA when compared to normal controls. However serum and synovial

fluid levels of TIMP-1 although were slightly elevated in OA patients than those with RA, this elevation was not of significant difference. MMP-3 / TIMP-1 ratio were significantly higher in RA than those of OA. This may point to an imbalanced level of parameters of extracellular matrix turnover towards more joint degradation in RA joints. This finding was supported by Pay et al.,((2002). Grays, Firestein (2009) who reported elevated synovial fluid levels of MMP-3/ TIMP-1 ratio in patients with RA when compared to other arthritis disease including Behcets disease, Familial Meditrranean fever and OA.

MMP-3 is active against different components of extracellular matrix including proteoglycans and other matrix macromolecules. Proteoglycans are major components of articular cartilage and it is believed that proteoglycan degradation is one of the initial events in the process of cartilage destruction (Mankin and Lippie loc (1971) and Rizkalla, et al (1992)). The release of sulphated glycosaminoglycans such as chondroitin sulphate is largely reflective of proteoglycan degradation (Fawthrop, et al. (1997). In this current study, levels of chondroitin sulphate were significantly elevated in sera of RA and OA patients when compared to normal control subjects. However serum and synovial fluid levels were not significantly different in RA when compared to OA patients. Serum levels and synovial fluid levels of chondroitin sulphate were positively correlated in both arthritis diseases. In RA patients serum and synovial fluid levels were correlated with the degree of bone erosion being highly elevated in patients with sever grades than other grades. (Charni-Ben and Garnero, 2007). The same finding was reported in OA patients confirming the role of chondroitin sulphate in reflecting the degree of joint damage in arthritis diseases. Different studies have reported differences in proteoglycan levels among patients with RA and OA. Menicourt et al provided an evidence of increased rate of proteoglycan degradation in RA patients than those with OA while Yishiqura et al(Ishiquro, (2001)) reported reduced proteoglycan aggrecan degradation in RA when compared to OA. However the lack of significant difference between chondroitin sulphate levels in RA and OA in face of higher MMP-3 / TIMP-1 ratio in the former may highlight a different mode of joint destruction in both arthritis diseases. In RA joints the extracellular matrix components are not affected by the aging process found in OA and higher levels of MMPs may be needed to destroy the joints (Pay, et al (2002)).

Bone sialoprotein is a bone specific matrix macromolecule that is mainly polydispersed in the immediate subchondral layer of bones. The levels of bone sialoprotein in the sera of RA and OA patients were significantly higher than their corresponding control subjects. Sera of OA patients were presented with higher but insignificant level of bone sialoprotein than those of RA patients. Serum and synovial fluid levels of bone sialoprotein were positively correlated with corresponding levels of MMP-3 indicating a possible role of this metalloproteinase in degrading bone sialoprotein. In RA increased levels of bone sialoprotein were also reported to be correlated with different grades of larsen classification with the highest level being expressed in sera and synovial fluid of patients with severe degree of erosion .These data can be supported by Mansson et al(Mansson, (2001)) who were able to report significantly higher levels of bone sialoprotein in synovial fluid of RA patients with destructive than those with non destructive changes or in patients with psoriatic arthritis without bone involvement. However Conrazier et al(Conrozaie, (1998)) were able to provide an evidence of presence of normal serum level of bone sialoprotein in patients with hip osteoarthritis in face of higher levels in RA patients, a finding which was attributed also to the more aggressive and destructive coarse of joint destruction in RA patients.

Thus it can be concluded that MMP-3 is playing a critical role in extracellular matrix turnover in joints of RA patients that may be induced by the inflammatory process. Strategies of inhibiting the inflammatory process or the release of proinflammatory cytokines are considered promising trials to alleviate the aggressive course of RA.

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