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Adipocytokines, Receptor Activator of Nuclear Factor-κB Ligand, and Midkine: Intricate Biomarkers Network Involved in Pathogenesis and Activity of Rheumatoid Arthritis in Egyptians

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NAS and WAK reached literature and conceived the study, contributed to biochemical / molecular assays, performed data analysis and wrote the first draft of the paper. Author RME was involved in protocol development, gaining ethical approval, patient recruitment and data analysis. All authors reviewed, edited the manuscript and approved the final manuscript.

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ABSTRACT

Background: Rheumatoid arthritis (RA) is a common autoimmune disease in which a heterogeneous course and different pathogenic mechanisms are implicated in the development of chronic inflammation and subsequent joint damage. Early diagnosis and timely detection of RA progression are of global challenges. However, the lack of sensitivity of the currently available biomarkers has impaired the ability to implement potentially effective therapy in a timely manner. Adipocytokines such as adiponectin, and visfatin have recently emerged as pro-inflammatory mediators involved in the pathophysiology of RA, however they still a matter of debate.
Aim: This current study went further to investigate the roles of Adipocytokines, receptor activator of

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nuclear factor kappa-B ligand (RANKL) levels and midkine (MDK) gene expression axis in patients with early untreated RA and their implication in disease activity monitoring.
Methodology: Forty newly diagnosed untreated patients with RA (20 patients with active RA and 20 patients with inactive RA) in addition to twenty apparently healthy age, sex and race matched controls were enrolled in this study. Serum tumor necrosis factor-alpha (TNF-α), adiponectin, visfatin and RANKL levels by immunoassay and MDK mRNA level in peripheral blood by real-time PCR were assessed.
Results: TNF-α, adiponectin, visfatin and RANKL levels as well as peripheral blood MDK mRNA levels were significantly increased in patients RA with higher values were for active RA with significant positive correlations to disease activity score 28 (DAS-28).
Conclusion: Adipocytokines, RANKL and MDK axis has a role in RA pathogenesis, early diagnosis and disease activity. These results may open new avenues for developing preventive and therapeutic strategies for RA.

Keywords: Rheumatoid arthritis; tumor necrosis factor-α; adiponectin; visfatin; receptor activator of NF-κB ligand; midkine.

1. INTRODUCTION

Rheumatoid arthritis (RA), a chronic autoimmune disease of the joints, is characterized by inflammation, abnormal cellular / immune response and synovial proliferation with subsequent progressive joint damage [1]. Although the definite etiology of RA remains unknown, however, inflammatory and immune cells with their derived cytokine and chemokine may play central role in the pathogenesis of RA [2]. Early diagnosis and early therapeutic intervention improve clinical outcomes. Adipose tissue is a structural component of many organs as heart and joints. Adipocytokines as tumor necrosis factor-alpha (TNF-α), adiponectin, and visfatin are soluble mediators which participate in several metabolic, immune, and inflammatory processes as RA. Adipocytokines are produced by adipose tissue, synovium, cartilage, and mononuclear blood cells [3].

RA causes significant joint pain, deformity and disability with increased risk of bone fracture. In RA osteoclast is responsible for bone resorption, plays a crucial role in bone remodeling and it was clarified as one of the pivotal effector cells in the pathogenesis of erosive bone disease and joint damage [4-5]. T lymphocytes and synovial fibroblasts have been found to produce receptor activator of nuclear factor-kappa B (NF-κB) ligand (RANKL) which promotes differentiation of osteoclast precursors and osteoclast development [6].

Midkine (MDK) is a novel heparin-binding growth factor originally identified by the screening of retinoic acid-responsive genes. MDK plays an important role in inflammation and cancer through modulation of cell transformation, growth, survival and migration in addition to its role in angiogenesis [7].

To the best of our knowledge, no enough studies have identified the combined role of adipocytokines, RANKL and MDK axis in RA, thus this study aimed to unravel their roles in RA pathogenesis, early diagnosis and activity, which may open new avenues for developing preventive and therapeutic strategies for RA.

2. MATERIALS AND METHODS

2.1 Participants and Study Design

A total of forty newly diagnosed untreated patients with RA and 20 age and sex-matched healthy controls were enrolled in this study. All patients have given their informed written consent and the study was institutionally approved by the Research Ethical Committee of Faculty of Medicine, Tanta University, Egypt. This current study included forty newly diagnosed patients with RA, diagnosed according to the American College of Rheumatology (ACR) classification revised criteria [8]. They were enrolled from patients with arthritis referred to the outpatient clinic of Rheumatology & Rehabilitation department, Tanta University Hospital during a period of six months from March to August 2014. Patients were subdivided into two groups: group I (n=20) with active RA; group II (n=20) age and sex matched individuals with inactive RA. In addition, age and sex matched healthy volunteers within the same geographical distribution with no clinical findings suggesting immunological or bone diseases and served as control group III (n=20).

All patients were subjected to full history taking, thorough clinical examination especially of the musculoskeletal system with particular attention to number of the tender and/or swollen joints, calculation of body mass index (BMI) (weight in kilograms divided by the square of the height in meters (kg/m^2) were carried out under proper condition of wearing light clothes and no shoes. Disease activity was assessed clinically using the modified disease activity score 28 (DAS 28). The DAS-28 provides an absolute indication of RA disease activity on a scale of 0.49 to 9.07; DAS 28 value >5.1 corresponds to a high disease activity, DAS-28 value between 3.2 and 5.1 corresponds to a moderate disease activity, and DAS-28 value between 2.6 and 3.2 corresponds to a low disease activity, and DAS 28 value < 2.6 corresponds to remission or inactivity [9].

The following routine laboratory assessments were done for all patients: erythrocyte sedimentation rate (ESR) by Westergren's method [10], C-reactive protein (CRP) and rheumatoid factor (RF) levels by commercially supplied kits (Biosystem, Spain). Patients with any other autoimmune disorder, tobacco smoking, liver / kidney diseases, current or past history of malignancy, cardiovascular disease, infection, hypertension or diabetes mellitus were excluded from the present study.

2.2 Blood Sampling for Biochemical and Molecular Assays

Overnight fasting blood samples were obtained from each participant on sterile EDTA treated and non-treated tubes. The blood in the non-EDTA treated tubes was used for serum separation. Serum and EDTA treated blood were stored at -80℃ till analysis.

2.3 Assessment of Inflammatory and Bone Resorption Status

2.3.1 Biochemical assessments

Serum level of TNF-α was determined using enzyme linked immunosorbent assay (ELISA) technique using commercial kits (Affymetrix eBioscience, USA). Serum RANKL level was determined using ELISA technique using commercial kits (Sunred Biological Technology Co. Shanghai, PRC). Serum adiponectin level was determined using ELISA technique using commercial kits (Ani Biotech Oy, Organium Laboratories Business Unit, Finland). Serum visfatin level was determined using ELISA technique using commercial kits (Ray Biotech, Inc. USA). All ELISA techniques were done according to the manufacturer's protocol and read on microplate reader (Stat Fax®2100, Fisher Bioblock Scientific, France), at 450 nm with correction wavelength set at 570 nm.

2.3.2 Molecular assessment

2.3.2.1 RNA extraction, cDNA synthesis and real-time PCR for MDK gene

Total RNA was extracted from EDTA peripheral blood using Magna pure Compact Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany) according to the protocol provided by the manufacturer. Total RNA was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first strand using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Real-time PCR was carried out with cDNAs. PCR reactions were performed using Roche Light Cycler Fast Start DNA Master ^{PLUS} SYBR Green I kits (Roche Diagnostics) following the manufacturer's instructions. MDK mRNA transcripts were quantified, relative to the housekeeping gene, glyceraldehyde-3-phosphatedehydrogenase (GAPDH) which was used as an internal control. Sequence specific primers were designed by Primer3 software: (http://bioinfo.ut.ee/primer3/) as follows: MDK (No: NM_001012333.2): forward primer (5'-AAGGCGCGCTACAATGCTC-3') and reverse primer (5'-CATCCAGGCTTGGCGTCTA-3'); GAPDH (No: NM_001289746.1): forward primer (5'- AGTGCCAGCCTCGTCTCATAG -3), and reverse primer (5'- CGTTGAACTTG CCGTGGGTAG -3'). The following conditions were used: pre-denaturation at 94℃ for 30 second, 35 cycles of denaturation (94℃ for 30 second), re-annealing (53℃ for 30 second), and extension (72℃ for 40 second). The final results were automatically calculated from the cross-point values of the target and the reference gene by Light Cycler 4 Relative Quantification Software (Roche Diagnostics).

2.4 Statistical Methods

For statistical analysis, Statistical package for social science (SPSS) software version 16 was used. Analysis of the results in the form of mean and standard deviation was done. Student's t-test for comparison between means of two groups and one way analysis of variance

(ANOVA) for comparison between means of three groups were used. The correlation study was calculated using Pearson's correlation. The level of significance was set at $p<0.05$.

3. RESULTS

3.1 Descriptive Statistics of All Studied Groups

There was no statistically significant difference of age, sex and BMI (kg/m^2) between patients with active and inactive RA and control group ($P>0.05$) (Table 1).

3.2 Routine Laboratory Investigations and Disease Activity Score of RA Patients (Group I & II)

There was no statistically significant difference of RF and CRP between patients with active and inactive RA. Meanwhile, first hour ESR and DSA 28 were statistically significantly higher in active RA patients when compared to inactive RA group ($P< 0.05$) (Table 2).

3.3 Assessment of Inflammatory Status

Adipocytokines; TNF-α, adiponectin and visfatin levels were statistically significantly increased in RA patient groups when compared to control group ($P< 0.05$). Inter-group comparison showed that the levels of previously mentioned parameters in patients with active RA were statistically significantly increased when compared to inactive RA patient group (Table 3).

3.4 Assessment of Bone Resorption Status

Bone resorption biomarkers, MDK mRNA and RANKL levels showed statistically significant increase in RA patient group when compared to control ($P<0.05$). Inter-group comparison showed that MDK mRNA and RANKL levels in active RA patient group were statistically significantly increased when compared to inactive RA patient group (Table 3).

3.5 Correlation Study between Different Biochemical, Molecular Findings and Disease Activity Score 28 (DAS-28)

Using Pearson correlation, TNF-α, adiponectin, visfatin, RANKL and MDK mRNA levels in RA patient groups were positively correlated with DAS 28 reflecting their link to RA disease activity (Table 4).

3.6 Correlation Matrix

Correlation matrix showed statistically positive correlation between all studied parameters (Table 5).

Table 1. Descriptive statistics of all studied groups (groups I, II & III)

Groups/Parameters		Group I (Active RA) (n=20)	Group II (Inactive RA) (n=20)	Group III (Control group) (n=20)
Age (years)		37.1±4.6	38.2±3.6	37.9±3.1
Sex	Female (n, %)	17 (85%)	16 (80%)	17 (85%)
	Male (n, %)	3 (15%)	4 (20%)	3 (15%)
BMI (kg/m^2)		23.2±2.2	22.7±2.9	21.9±3.5

*Data are mean ± standard deviation of each group. Statistical analysis is carried out using SPSS computer program version 16 to compare significance between groups one-way analysis of variance (ANOVA) with post hoc test was used. *Significant at p value < 0.05. RA: Rheumatoid Arthritis, BMI: Body Mass Index*

Table 2. Routine biochemical characteristics and disease activity score 28 (DAS 28) of RA patient groups (groups I & II)

Groups/Parameters	Group I (Active RA) (n=20)	Group II (Inactive RA) (n=20)
RF (IU/ml)	167.31±9.428	165±12.535
ESR (mm/1sthr)	51.07±10.402*	25.1±9.115
CRP (mg/ml)	40.59±7.341	39.56±5.992
DAS-28	3.91±1.008*	1.69±0.391

*Data are mean ± standard deviation of each group. Statistical analysis is carried out using SPSS computer program version 16 to compare significance between the two groups student t- test was used. *Significant at p value < 0.05. RA: Rheumatoid arthritis; RF: Rheumatoid factor; ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; DAS-28: Disease activity score 28*

Table 3. Comparative statistics for the studied biochemical and molecular biomarkers

Groups/Parameters	Group I (Active RA) (n=20)	Group II (Inactive RA) (n=20)	Group III (Control group) (n=20)
Serum TNF-α level (pg/ml)	51.24±12.061[ab]	35.06±7.471[c]	17.20±3.397
Serum RANKL level (pmol/L)	28.66±2.075[ab]	21.44±3.451[c]	11.75±1.356
Adiponectin level (ng/ml)	36.45±2,34[ab]	27.56±3,24[c]	7.56±2,34
Visfatin level (ng/ml)	34.6±1.2[ab]	28.4±3.2[c]	19.2±2.5
MDK mRNA level	0.69±0.15[ab]	0.5±0.092[c]	0.16±0.129

*Data are mean ± standard deviation of each group. Statistical analysis is carried out using SPSS computer program version 16 to compare significance between groups one-way analysis of variance (ANOVA) with post hoc test was used. *Significant at p value < 0.05. [a] Significant difference between active RA & control groups, [b] Significant difference between active& inactive RA groups; [c] Significant difference between inactive RA & control groups. RA: Rheumatoid arthritis; TNF-α: Tumor necrosis factor alpha; RANKL: Receptor activator of NF-κB ligand, MDK: Midkine*

Table 4. Correlation study between different biochemical, molecular findings and disease activity score 28 (DAS-28) in RA patients (group I & II)

		DAS-28
Serum TNF-α level (pg/ml)	r	0.717
	P	0.001*
Serum adiponectin level (pg/ml)	r	0.324
	P	0.026*
Serum visfatin level (ng/ml)	r	0.213
	P	0.037*
Serum RANKL level (pmol/L)	r	0.777
	P	0.001*
MDK mRNA level	R	0.440
	P	0.002*

Correlation study was carried out using Pearson correlation.
**Significant at p value < 0.05. RA: Rheumatoid arthritis; DAS-28: Disease activity score 28; TNF-α: Tumor necrosis factor alpha; RANKL: Receptor activator of NF-κB ligand, MDK: Midkine*

Table 5. Correlation matrix between all studied biochemical and molecular biomarkers in RA patients (group I & II)

	Serum TNF-α level (pg/ml)		Serum RANKL level (pmol/L)		Adiponectin level (ng/ml)		Visfatin level (ng/ml)	
Serum RANKL level (pmol/L)	R	0.896	-		-		-	
	P	0.001*						
Adiponectin level (ng/ml)	R	0.929	r	0. 924	-		-	
	P	0.002*	P	0.001*				
Visfatin level (ng/ml)	R	0.884	r	0.918	r	0.879	-	
	P	0.001*	P	0.001*	P	0.001*		
MDK mRNA level	R	0.929	r	0. 938	r	0. 951	r	0.925
	P	0.001*	P	0.002*	P	0.001*	P	0.001*

*Correlation study was carried out using Pearson correlation. *Significant at p value < 0.05. RA: Rheumatoid arthritis; TNF-α: Tumor necrosis factor alpha; RANKL: Receptor activator of NF-κB ligand, MDK: Midkine*

4. DISCUSSION

Rheumatoid arthritis is a chronic inflammatory connective tissue disease characterized by joint destruction and disability due to bone loss and erosion which are the chief unsolved problems in RA [11]. Early diagnosis of RA and detection of progression are of global challenges. However, the lack of sensitivity of the currently available biomarkers has impaired the ability to implement potentially effective therapies in a timely manner. Adipocytes have long been considered as none-bioactive cells and only responsible for energy storage. Adipocytokines produced by adipocyte

as adiponectin, leptin, visfatin and resistin play an important role in regulating immune and inflammatory processes [12].

TNF-α, play a central role in the pathophysiological mechanisms of RA through binding to its receptors on target cells which triggers cell survival and transcription factor nuclear factor-κB (NF-κB) activation [13]. NF-κB is a family of transcription factors that contributes to the transcriptional regulation of several genes required for the immune/inflammatory response, apoptosis, cell proliferation and angiogenesis [14]. In this study, TNF-α levels were significantly increased in patients with RA with higher values were for patients with active RA. Also TNF-α was correlated positively with DAS-28. These results can be explained by increased joint infiltration with fibroblast, inflammatory and immune cells like macrophages. Moreover, TNF-α relation to disease activity may be due to their effects on; activation of NF-κB pathway, proliferation of T lymphocytes and increased neutrophil infiltration in addition to the stimulation of osteoclasts proliferation and differentiation with subsequent induction of bone resorption and joints damage [15]. Moreover, TNF-α up-regulates matrix-metalloproteinase (MMP) which is responsible for the increased matrix breakdown, loss of cartilage and bone with further joint destruction [16]. The result of the present study was in agreement with those obtained from previous studies [17-18]. Using monoclonal antibodies or soluble receptors to neutralize cytokines as TNF-α have been developed as treatments for RA but they are not curative with failure of response being common. So that, other cytokines may play a role in the pathophysiology of inflammation in RA patients [19].

Adiponectin is an adipocytokine produced by adipocyte, osteoblasts, RA synovial fibroblast (RASF) with a protective role in vascular diseases, but also act as pro-inflammatory factor promotes matrix degradation in joints [20]. Adiponectin shares strong homologies with the complement factor C1q and TNF-α. Thus, it belongs to the C1q-TNF-superfamily which is involved in synovial pathophysiology and inflammatory joint disease [21]. Ehling et al. [22] reported an increase in interleulin-6 (IL-6) and pro-MMP-1 production by synovial fibroblasts in response to adiponectin. AS fibroblast, adipocytes, and osteogenic cells are mesenchymal cells derived from a common multi-potentent stem cell and it has been demonstrated that adipocytes can be re-differentiated into fibroblast-like cells; synovial fibroblasts can be differentiated into adipocytes so their ability to produce a pro-inflammatory response in response to adiponectin stimulation could be common for mesenchymal cells [23].

In this study, adiponectin level was significantly increased in patients with RA with higher values were for patients with active RA when compared to inactive RA patients. Also, adiponectin level was correlated positively with DAS-28. The results of the present study were in agreement with those obtained previously [24] and can be explained by increased joints infiltration by fibroblast, inflammatory and immune cells. Kusunoki et al. [25] demonstrated that the exposure of rheumatoid arthritis synovial fibroblasts to adiponectin induced the expression of cyclooxygenase (COX-2) and membrane-associated prostaglandin E synthase-1 (mPGES-1), key enzymes involved in PGE2 synthesis, thus adiponectin may play a role in the pathogenesis of synovitis in RA patients.

Visfatin is an adipocytokine involved in early B-cell development and produced by visceral adipose tissue, immune cells and synovial fibroblasts. Visfatin has an important role in immunity and inflammation [26]. In this study, visfatin level was significantly increased in patients with RA with higher values were in patients with active RA. Also visfatin level was correlated positively with DAS-28. These results can be explained by increased joint infiltrations with monocytes/macrophages and immune cells. Also visfatin was shown to be involved in synovial fibroblast activation / migration with the release of high amounts of chemokines, pro-inflammatory cytokines, and MMP which are synthesized by these cells [27]. So visfatin inhibition may reduce the severity of RA. From results of this study it seems that adipocytokines may be a valuable therapeutic target in the future. The results of this study were in harmony with results obtained previously [28,29].

Osteoclast plays an important role in bone remodeling in RA, however, the mechanism of osteoclast activation or differentiation is still unclear. The development, activity, and survival of osteoclasts require an essential osteoclastogenic mediator which is RANKL. RANKL is a membrane protein on osteoblasts produced by both T lymphocytes and synovial fibroblasts [30]. RANKL interacts with RANK and induces NF-κB activation, marrow macrophages differentiation into osteoclasts and activate bone-

resorbing activity / survival of osteoclasts [31]. Osteoprotegerin (OPG), a soluble receptor of RANKL, acts as its natural decoy receptor by blocking the RANK/RANKL interaction. RANKL/OPG balance is important for maintaining osteoclast homeostasis [32]. Results from the present study revealed significant increase in serum RANKL level in patients with active RA versus patients with inactive disease. Also serum RANKL level correlated positively with DAS-28. Adiponectine stimulates RANKL and inhibit osteoprotegerin expression in human osteoblasts via the mitogen activating protein kinase (MAPK) signaling pathway so it can regulating bone metabolism. Moreover, the balance of RANKL/OPG is regulated by numerous factors as TNF-α that support the results obtained from this study [33]. The results of the present study were in harmony with results obtained from previous studies [34-35].

MDK has been shown to play an important role in inflammation, tumor-genesis in addition to its anti- apoptotic effect in many cell types [36]. Previously it was found that MDK aggravates experimental autoimmune encephalomyelitis by decreasing regulatory T cells which regulate the development of autoimmune response through the suppression of tolerogenic dendritic cells development [37]. In 2007, Kuo and his co-workers provided the first direct evidence that MDK was a NF-κB inducible gene [38]. Furthermore, You et al., 2008 reported that MDK gene has a NF-κB-binding site in the 5' non-coding region [39]. The present study revealed a significant increase in MDK mRNA level in patients with RA in relation to control with a statistically significant increase in patients with active RA. Also MDK correlate positively with DAS-28. Therefore, it is possible that increased MDK expression may be due to increased TNF-α which was identified as the strongest inducer of MDK expression through NF-κB pathway [39]. In addition to previous reports, inhibiting MDK may be a useful strategy to treat autoimmune diseases for the following reasons: (1) recombinant human MDK promoted the differentiation of osteoclasts and the osteoclast-inducing activity of MDK together with RANKL was more stronger. Moreover, inhibition of MDK expression by MDK-specific small interfering RNA (siRNA) or inhibition of MDK activity by chondroitin sulfate suppressed the development of antibody-induced arthritis [40]; (2) MDK caused an induction of RANKL expression in osteoblasts with subsequent bone damage and loss [41] that greatly supports the results obtained from this current work in studying this intricate network of biomarkers.

5. CONCLUSION

Adipocytokines, RANKL and MDK axis represents an important mediator of bone resorption in RA so that, they may be considered as potential biomarkers for RA pathogenesis and activity. Therefore, monitoring and inhibition of adipocytokines and RANKL production may be potential targets to control activity and bone damage in RA. Moreover, anti-MDK therapy may be particularly useful strategy for treating RA and improve quality of life in a majority of RA patients.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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