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Power Doppler sonography, a non-invasive method of assessment of the synovial inflammation in patients with early rheumatoid arthritis

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Abstract

Objective: To detect synovitis is important in both the diagnosis and outcome assessment of early rheumatoid arthritis. This study was meant to assess the validity and reproducibility of ultrasonography (US) as a mean of detection for the knee synovitis, by comparing US findings with clinical examination and histopathological findings in synovial membrane. *Methods*: The study group included 65 patients with early rheumatoid arthritis – below 12 months from the debut, naive for DMARDs, in whom demographic data – gender, age, disease duration, the number of tender and swollen joints, HAQ score (Health Assessment Questionnaire) and serum samples for CRP, RF, anti-CCP2 antibodies (ELISA, QUANTA LiteTM, CCP IgG, INOVA Diagnostics Inc, USA), VEGF (ELISA, VEGF2, DRG International, IRC, USA) determination were recorded. Disease Activity Score for 28 joints (DAS28) was calculated. PDS signal was scored from 0 to 3 according to the overall expression of PDS findings at the knees. A sample of synovial tissue was obtained in 35 patients during the arthroscopy, and the vascularisation of the synovial tissue was evaluated by immunohistochemistry and was analyzed qualitatively by a pathologist who was unaware of the PDS findings. Written, informed consent was obtained from each patient before entering the study. They all had active synovitis of the knee, ultrasonographically confirmed, with the identification of the target biopsy sites. The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova. *Results*: Angiogenesis was evaluated and quantified by immunohistochemical evaluation of synovial VEGF, one of the most specific endothelial growing factors, that proved to correlate significantly with the serum levels of VEGF, DAS28 as well as with the power Doppler sonography (PDS) score. *Conclusions*: The statistical analysis of the data showed that PDS could be used as non-invasive marker with predictive value regarding synovial inflammation and disease progression in early forms of the disease as well as a useful method in the assessment of the therapeutic response.

Keywords: power Doppler sonography, early rheumatoid arthritis, angiogenesis, knee arthritis, VEGF, VEGF-R1.

Introduction

Rheumatoid arthritis is a chronic inflammatory systemic disease, characterized by inflammation of synovial joints, with progressive, destructive evolution, which involves multiple extra-articular manifestations [1]. Most of the knowledge of inflammation in rheumatoid arthritis results from the study of the synovium in an advanced stage of disease, given the fact that the synovial membrane is the main stage for the pathogenic processes. Histological changes in the rheumatoid arthritis are gradually developing, so the current research aims to initiate the treatment in the early stages, before irreversible damage. Angiogenesis represents a major event in promoting the rheumatoid synovitis, offering the necessary blood supply as well as the access of the cellular populations with proinflammatory functions [2, 3]. This way, neoangiogenesis represents a sine qua non condition for the progression of pannus [4]. Thus, the angiogenesis in synovial tissue became one domain of interest in early rheumatoid arthritis. The study of the angiogenesis in the rheumatoid synovium led to identification of several endothelial growing factors, VEGF being the most specific. Induced by hypoxia, VEGF has been detected in serum, synovial fluid and rheumatoid synovium [5, 6].

The aim of this study was to identify another, non-invasive, marker of inflammation in early rheumatoid arthritis, by finding correlations of the power Doppler sonography (PDS) score with the serum and synovial VEGF and the clinical and biological markers of disease activity.

Materials and Methods

The study group included 65 patients with early rheumatoid arthritis – with less than 12 months from the onset, naive for DMARDs, in whom we recorded gender, age, disease duration, HAQ score (Health Assessment Questionnaire) and serum samples for CRP, RF, anti-CCP2 antibodies (ELISA, QUANTA LiteTM, CCP IgG, INOVA Diagnostics Inc, USA) and serum VEGF. The following variables were considered in addition: number of tender joints, number of swollen joints (of a total of 28 joints) and intensity of pain, assessed using a 100 mm visual analogue scale (VAS)

(0, no pain; 100, worst possible pain). Disease Activity Score for 28 joints (DAS28) was calculated based on four variables (NTJ, NSJ, VAS, ESR).

Ultrasound evaluation

We carried out a combined two planes grey scale and power Doppler study in three distinct joint recesses, to assess power Doppler signals in the synovitis areas. All examinations were done out using a high frequency 32 mm linear transducer (ALOKA UST-5412) by an observer, who was not aware of the histopathological findings in the patients. We used standardized anatomical guidelines for the scans of the knee, in the three recesses–suprapatellar recess and lateral and medial parapatellar recesses [7].

The suprapatellar recess is the preferred site for synovial thickening in the knee of the rheumatoid arthritis patients. The synovial thickness of the supra-patellar recess was determined by scanning the zone deep to the quadriceps tendon and the suprapatellar fat pad and superficial to the prefemoral fat pad (supine position; knee joint extended). At the level of the lateral and medial parapatellar recesses, the vertical edge along the medial and lateral border of the kneecap was identified by scanning. Each knee was evaluated as a whole, and the thickest area detected between the three recesses was measured, the resulting value being assumed a measure of synovial thickness [8]. The thickness was graded from 0 to 3 using this scale: 0, if the thickness was <2 mm; grade 1, for a thickness between 2–5 mm; grade 2, for 6–8 mm and grade3 for a thickness >8 mm.

PDS evaluation

Power Doppler sonography was set for high sensitivity, with a low wall filter to allow detection of vessels with low blood flow. Pulse repetition frequency was 750–1000 Hz and medium persistence was used. The color gain was increased until background noise appeared and then reduced until noise was suppressed, thus ensuring maximum sensitivity.

PDS signal was scored from 0 to 3 according to the overall expression of PDS findings at the knees (Table 1), on the most representative images [9].

Table 1 – ***PDS scoring system***

PDS score	
0	Absence of PD signal
1	Single vessel dots – mild hyperemia
2	Confluent vessel dots over less than half the area of synovium – moderate hyperemia
3	Confluent vessel dots over greater than half the area of synovium – marked hyperemia

Synovial biopsy handling

The synovial biopsy was performed in 35 patients using the arthroscopic triangulation technique, in the Department of Orthopedics and Trauma, University of Medicine and Pharmacy of Craiova and the paraffin embedded tissue sections were examined using conventional and immunohistochemical staining.

Histopathological analysis

The histopathological analysis was based on many parameters, but *neovascularization* was evaluated through computerized morphometry (Lucia M), after vessel staining.

The tissues were fixed in formalin and embedded in paraffin blocks. Each block was cut in sections, with a thickness of 4 μm, which were deparaffinized in xylene and rehydrated through graded concentrations of alcohol. Formalin fixation forms protein cross-links that mask the antigenic sites in tissue specimens, giving weak or false negative staining for immunohisto-chemical detection of certain proteins. The Tris-EDTA and the Citrate Buffer break the protein cross-links, unmasking the antigens and epitopes in formalin-fixed and paraffin embedded tissue sections, thus enhancing staining intensity of antibody. Thereby, we performed antigens unmasking by microwave heating in Tris-EDTA (10 mM Tris Base, 1 mM EDTA solution, 0.05% Tween 20, pH 9) for 20 minutes for VEGF and in citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6) for 20 minutes for VEGF-R1. The immunostaining was performed using the CSA II, Biotin-Free Catalyzed Amplification System (Dako-K1497), Monoclonal Mouse Anti-Human Vascular Endothelial Growth Factor (Dako, M7273, 1:500 dilution) and Monoclonal Anti-Mouse VEGF-R1 (Flt-1) Antibody (R&D System, MAB471, dilution 1:300. Background or unspecific staining often occurs during antibody staining. We used as blocking solutions 3% hydrogen peroxide in water (to block the endogenous peroxidase activity) and Normal Goat Serum Blocking Solution in BSA for 30 minutes. The chromogen used was 3,3'-diaminobezidine tetra-hydrochloride (DAB). All sections were then counter-stained with Hematoxylin.

We used the following scoring methods to quantify the VEGF and VEGF-R1 expressions (Table 2) [10].

Table 2 – ***Scoring system for VEGF and VEGF-R1*** **expression**

VEGF score		VEGF-R1 score	
1	Less than 10% positive lining synoviocytes	*1*	Less than 10% of sublining synovial blood vessels
2	10–25% positive cells	*2*	10–25% of SBV
3	25–50% positive cells	*3*	25–50% of SBV
4	More than 50% positive cells	*4*	More than 50% of SBV

Serum evaluation of VEGF

We collected 100 μL of serum from each patient at the time of arthroscopy and serum VEGF levels were determined, with a sensitivity of 40–600 pg/mL, using a standard sandwich enzyme-linked immunoabsorbent assay (ELISA) (VEGF2, DRG International, IRC, USA) according to the manufacturer's protocols.

Statistical analysis

Data were statistically analyzed using the Microsoft Excel software. Correlation between changes of PDS scores and clinical data was studied by Spearman's correlation coefficient and regression analyses. The level

of significance was $p<0.05$. Intraobserver reproducibility was assessed by calculating the intra-class correlation coefficients.

Results

At the time of examination, the mean age of the patients was 50.12 ± 11.35SD years, and the disease duration (mean ± SD) was 7.85 ±2.41SD months. Fifty-four (83%) patients were positive for RF, and 28 (43%) for anti-CCP2, with the mean value for anti-CCP2 being 89.64U±95.28SD. The disease activity score measured on 28-joints (DAS28) was 4.77±1.75 SD, and. the mean value for the CRP was 42.15±11.46. Table 3 shows the characteristics of the study group.

In ultrasound evaluation, we identified synovitis as a hypoechoic area in the joint space, compared with the fat in the neighborhood. Synovial effusion was defined as a hypoechoic or anechoic area, easily compressed by the pressure of the transducer. We found knee synovial proliferation in 62 patients, with the mean synovial thickness being 3.8±2.6 (SD). Suprapatellar joint effusions was seen in 48 (74%) of 65 patients, medial parapatellar in 25 (38%) and lateral parapatellar in 57 patients (87%). 23 patients had knee articular cartilage contour irregularity or thinning and 10 patients had knee bone erosions. Erosions were defined as breaks in the hyperechoic line of the bon, found in two perpendicular planes, according to OMERACT criteria.

Table 3 – ***Demographical and clinico-biological characteristics of the study group***

Variables	Mean (SD)
Age [years]	50.12 (11.35)
Sex F:M	51:14
Duration of disease [months]	7.85 (2.41)
Patient pain [100 mm VAS]	69.6 (15.74)
DAS28	4.77 (1.75)
CRP	42.15 (11.46)
ESR [mm/1 hr.]	48.53 (18.59)
Rheumatoid Factor [U]	60.03 (27.66)
antiCCP2 [U]	106.67 (92.37)
serum VEGF [pg/mL]	685.92 (332.49)

VAS – visual analogue scale, DAS – disease activity score, ESR – erythrocyte sedimentation rate, VEGF – vascular endothelial growth factor.

The intraobserver value for agreement for scoring PDS signals at the knee level in all 65 patients included in the study was 0.87 (SE=0.31).

PDS detected abnormal synovial perfusion in 59 of 65 patients (90.7%) with at least one knee affected. Most of the patients showed a moderate hyperemia: 31 patients (47.7%) had a PDS score of 2, and 16 patients (24.6%) had a score of 3. In 12 (18.40%) patients we found a mild hyperemia (PDS score of 1) and in six patients (9.2%), no PDS signal was detected (Figure 1).

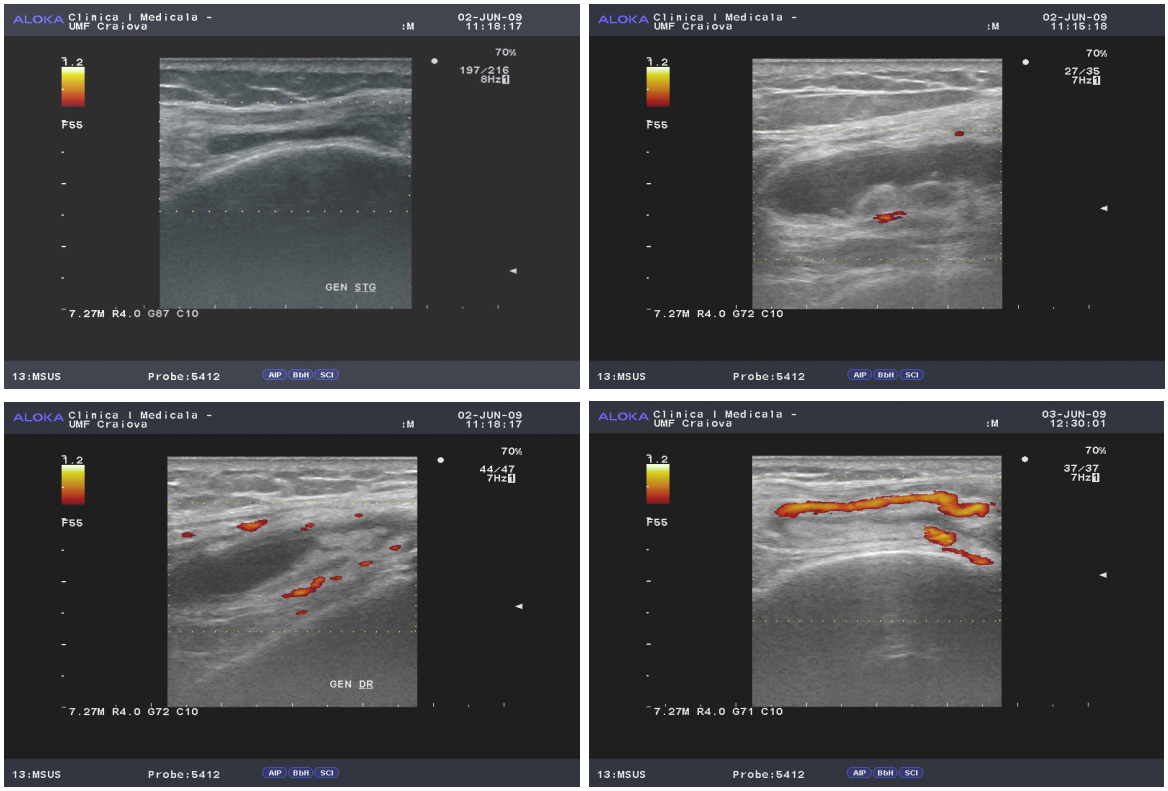

Figure 1 – ***Representative power Doppler sonography (PDS) images obtained on the scan of the dominant knee: (A) Absence of PD signal (PDS score 0); (B) Single vessel dots: mild hyperemia (PDS score 1); (C) Confluent vessel dots over less than half the area of synovium: moderate hyperemia (PDS score 2); (D) Confluent vessel dots over more than half the area of synovium: high hyperemia (PDS score 3).***

The histopathology of the early RA specimens showed a heterogeneous pattern of lesions. The most encountered histopathological aspect was the proliferation of synovial cells, with more than 5–6 rows and the neovascularization. Other common aspect was the lymphoplasmocytic infiltrate, more frequently presented as diffuse infiltration

in the sublining regions and rare as follicular clusters with perivascular distribution. Fibrinoid necrosis and mesenchymoid transformation were encountered in less than 20% of cases.

Synovial expression of VEGF showed positivity predominantly in the lining and sublining synoviocytes, but in the fibroblasts, inflammatory cells and endothelial cells as well (Figure 2).

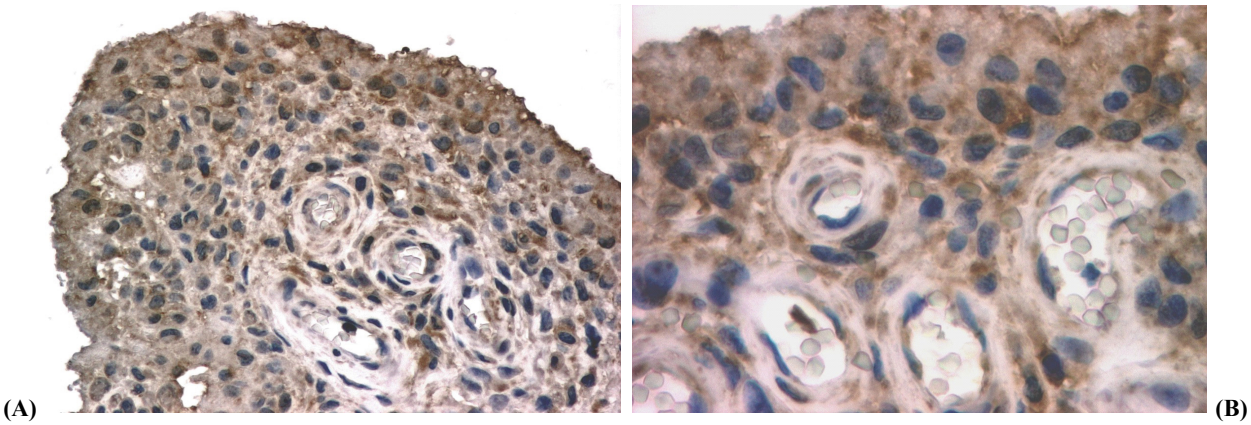

Figure 2 – ***VEGF positive lining synoviocytes, endothelial cells and synovial fibroblasts: A – ×200, B – ×400.***

The pattern of VEGF reaction was cytoplasmic with a more intensity in the lining synoviocytes, endothelial cells, neutrophils and macrophages. VEGF-R1 expression analysis showed positivity mostly in the endothelial cells but in the lining and sublining synoviocytes and the inflammatory cells. The pattern and intensity of VEGF-R1 reaction in the synovium was the same as VEGF.

The semiquantitative evaluation of VEGF immunoexpression in synoviocytes revealed scores of 1 in 22.85%, 2 in 28.57% of cases, 3 in 40% of cases and 4 in 8.57% of cases.

Semiquantitative quantification of VEGF-R1 (Figure 3) showed values considered as a score of 1 (17.14.0%), 2 (34.28%), 3 (37.14%) and 4 (11.42%) (Figure 4).

The PDS score obtained in the dominant knees correlated with the synovial VEGF score (r=0.69, p<0.0001), but it showed a stronger correlation with the VEGF-R1 (r=0.75, p<0.0001).

We found only a tendency to positive correlation between PDS score and serum VEGF (r=0.5, p<0.0001) (Figure 5, a–c).

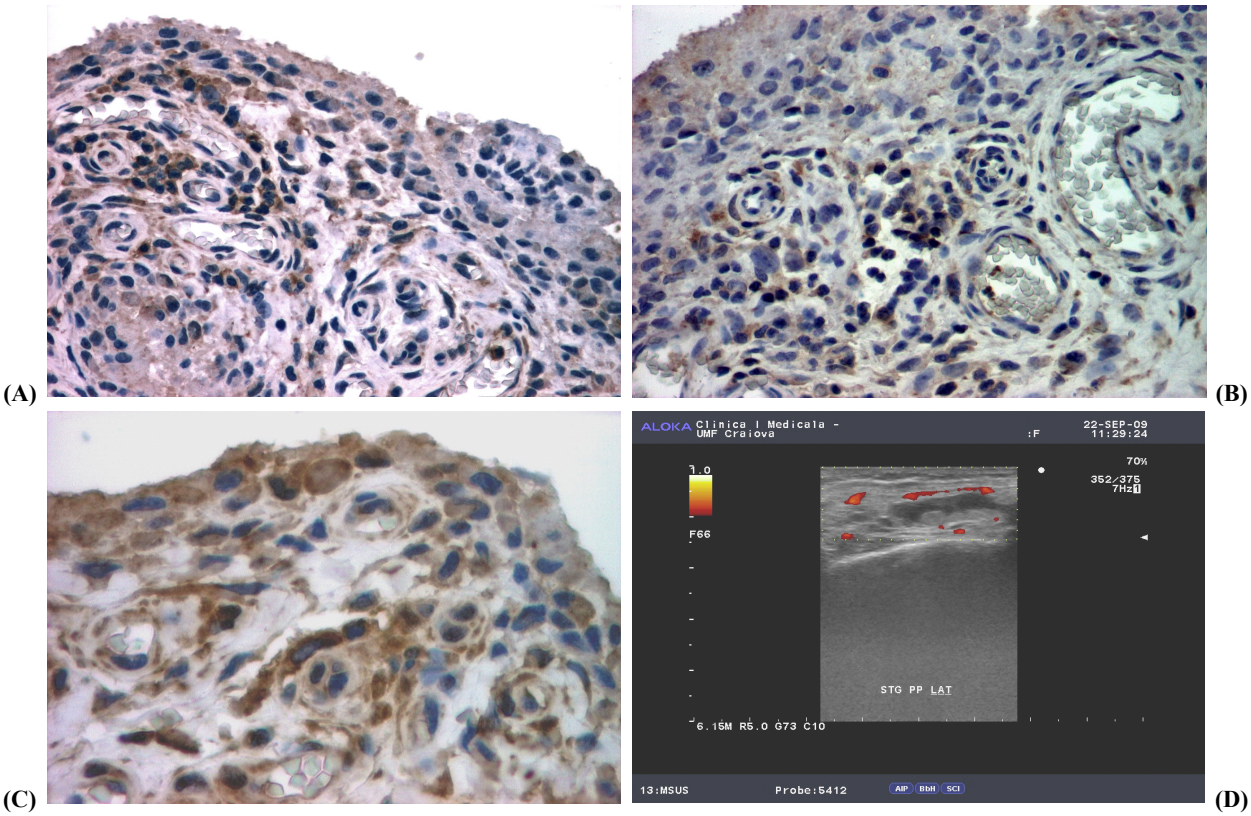

Figure 3 – ***Rheumatoid arthritis: (A, B) VEGF-R1 positive (brown) sublining synoviocytes and vessels beneath the lining synovium, positive VEGF-R1 inflammatory cells, ×200; (C) VEGF positive lining synoviocytes (brown) and endothelial cells (brown), ×200; (D) Vessel dots over less than half the area of synovium: moderate hyperemia (PDS score 2).***

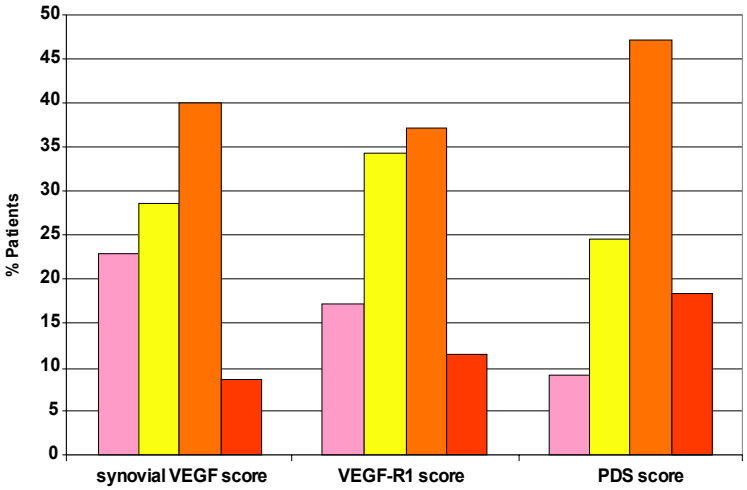

Figure 4 – ***Semiquantitative evaluation of VEGF, VEGF-R1 and PDS.***

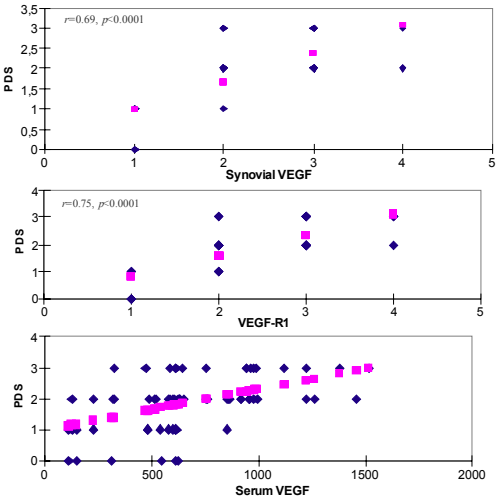

Figure 5 – ***(A) Correlation between the PDS score and the synovial VEGF; (B) Correlation between the PDS score and the synovial VEGF-R1; (C) Correlation between the PDS score and the serum VEGF.***

Discussion

Rheumatoid arthritis is a chronic inflammatory disease characterized by an inflammatory erosive synovitis that often progresses to destruction of the articular cartilage. Early changes in the synovium are represented by neovascularization, inflammatory cell infiltration [11], this "tumor-like" pannus destroying the bone, cartilage, tendons, ligaments, and capsule [12].

VEGF is important in the RA pathogenesis by its dual activities as an endothelial-cell mitogen and a modulator of changes in vascular permeability. According to our results data from the literature proved that in RA patients, VEGF is produced by macrophages, fibroblasts surrounding microvessels, vascular smooth muscle cells, synovial lining cells [13], neutrophils of synovial fluid [14], and peripheral blood mononuclear cells [15]. In addition, VEGF levels are significantly higher in the serum and synovial fluids of patients with RA than in either patients with OA or normal controls [16–19]. Also, it was demonstrated that serum VEGF levels of RA patients correlate with scores of disease activity and signs of radiologic progression [6]. Moreover, it seems that high serum VEGF levels at an early RA stage may predict the size of subsequent damage of joints.

The VEGF effects are mediated by its specific receptors, especially by VEGF-R1 and VEGF-R2. Many studies prove that both receptors have high mRNAs levels in endothelial cells from pannus microvessels [5, 20], being also overexpressed in the human RA synovium [21, 22] and osteoclast precursors and osteoclast cells [23]. In addition, it has been suggested that VEGFR1 induces pathologic angiogenesis without affecting physiologic angiogenesis [24, 25]. Moreover, starting from the observation that VEGF-R1 are overexpressed in inflamed synovium [26], Luttun A *et al.* [20] proved that anti-VEGFR1 antibody could suppress joint destruction in an animal model of RA.

In light of these findings becomes evident that inhibition of inflammation and angiogenesis mediated by VEGF and its receptors within the joint is an attractive therapeutic approach in arthritis [27–34].

Although the synovial immunoexpression of VEGF is one of the best markers of angiogenesis, the synovial biopsy still remains an invasive method.

At this moment, PDS has been demonstrated to be a sensitive tool for assessing angiogenesis and, thus the disease activity. The PDS is a safe, inexpensive and non-invasive imaging method, which provides a quick and sensitive method of visualization of synovial hyper-perfusion in joints with inflammatory disease. Several studies showed that PDS has good intra-observer reproducibility and a good inter-observer reproducibility has also been reported by other investigators [35].

The correlation between DAS28 and PDS score is positive, but not strong enough. This can be explained by the fact that PDS is a sensitive tool which can show even minimal increases in synovial perfusion, that could be missed by both clinical and laboratory evaluations [36]. Rheumatoid arthritis may appear to be inactive clinically but it may progress radiologically, so the assessment of the neovascularization of synovial tissue provides important information about disease activity and, most important, may predict erosive changes [37–40].

The role of PDS in the assessment of synovial inflammation has been recently investigated and significant positive correlations have been found by comparing its findings with those obtained using magnetic resonance imaging and arthroscopy [41–43].

Our study supports the correlation between the serum levels of VEGF, synovial VEGF, VEGF-R1 and the PDS score. Thereby, our data suggests that PDS is a feasible and sensitive imaging tool for assessing the synovial inflammation in patients with rheumatoid arthritis.

Conclusions

PDS detected a significant synovial perfusion at the knee joints of patients with rheumatoid arthritis, thus providing a quick, inexpensive, non-invasive, but sensitive method of evaluation in joints with

inflammatory disease. More than this, it may permit better informed management decisions in order suppress synovitis and improve treatment outcomes.

Contribution Note

First two authors contributed equally to this work.

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