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*Modification of osteopontin and MMP-9 levels in patients with
psoriasis on anti-TNF- α therapy.*

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Osteopontin (OPN), also known as early T-lymphocyte activation 1 (Eta-1) that is encoded by the Spp-1 gene, is a phosphorylated acidic glycoprotein initially identified in 1979 (1,2). Senger et al. described it as a secreted protein associated with neoplastic transformation of epithelial and fibroblastic cells (3). Independently of this, Franzen and Heinegard (4) isolated a 44-kDa phosphorylated glycoprotein from mineralized rat bone matrix, which contains an Arg–Gly–Asp (RGD) cell-binding sequence (5) and was named osteopontin.

It is present in many tissues and body fluids (6,7), being produced by various cells, such as osteoblasts, macrophages, dendritic cells, natural killer cells, T and B cells, epithelial cells and vascular smooth muscle cells (8). OPN is a secreted adhesive molecule, and it is thought to aid in the recruitment of monocytes-macrophages and to regulate cytokine production in macrophages, dendritic cells, and T-cells. OPN modulates the inflammatory response at several levels, from immune cell accumulation to activation of T helper 1 (Th1) cytokines and cell survival, thus exacerbating the chronic inflammatory response (9). Besides proinflammatory functions, physiologically OPN is a potent inhibitor of mineralization, it prevents ectopic calcium deposits and is a potent inducible inhibitor of vascular calcification (10).

The diverse biological functions of OPN can be explained by its different conserved sequence motifs some of which only become accessible if the protein is cleaved by thrombin. OPN contains three possible thrombin cleavage sites: the Arg–Gly in the RGD tripeptide and two Arg–Ser-sites at residues 154–155 and 158–159. OPN functions through binding to several receptors (11). This binding can be RGD-dependent mediated by α_v and β_1 integrin subfamilies as well as RGD-independent by CD44 isoforms containing CD44v6-v10 (12,13). At several sites, the OPN molecule can additionally be cleaved by matrix metalloproteinases (MMP) (14). The fact that OPN interacts with multiple cell surface receptors which are ubiquitously expressed makes it an active player in many physiological and pathological processes (15). OPN is highly expressed in inflammatory cells associated with many diseases including cancer (16), arterial restenosis (17), renal tubulointerstitial fibrosis (18), myocardial infarction, stroke (19), wound healing (20) , and in several chronic inflammatory diseases such as experimental autoimmune encephalitis, rheumatoid arthritis (21), and in a number of granulomatous diseases including sarcoidosis, foreign body granulomas, and tuberculosis (22).

Psoriasis is a chronic disease that affects 1-3% of the population (23). It is an immune-mediated inflammatory disorder, where Th1 and Th17 lymphocytes contribute to the pathogenesis through the release of inflammatory cytokines that promote further recruitment of immune cells, keratinocyte proliferation and sustained inflammation (24). Tumor necrosis factor alpha (TNF- α) is a pleiotropic cytokine with a central role in the

pathogenesis of psoriasis. Biological therapy based on monoclonal antibodies against TNF- α has been proven to be effective in patients with psoriatic arthritis on both the arthropathy and the cutaneous symptoms of the disease (25,26). Today there are three main biological agents targeting TNF- α , which are already in use for treating PsA. These are the chimeric monoclonal IgG1 antibody infliximab with human constant and murine variable regions, the fully human anti-TNF- α monoclonal antibody adalimumab and the recombinant 75-kDa TNF- α receptor IgG1 fusion protein etanercept (27). Infliximab and adalimumab bind to cell membrane bound TNF- α , which may lead to cell lysis. Among the multiple effects produced by TNF- α on keratinocytes, the induction of matrix MMP-9, a collagenase implicated in joint inflammatory arthritis which acts as an angiogenesis promoting factor, might represent a key mechanism in the pathogenesis of the disease (28). It has been shown that OPN modulates the expression and/or activity of proteins that regulate extracellular matrix remodeling. For example, OPN has been reported to increase the migration and expression of MMP-9 in human chondrosarcoma cells (29) as well as in cardiac and skeletal muscle (30).

We have previously showed that OPN is increased in psoriasis at peripheral blood mononuclear cells (PBMC) level and in skin (31). Chen *et al.* reported that high plasma OPN levels were an unfavourable factor for development of cardiovascular disease in patients with psoriasis (32). Furthermore high plasma OPN and low plasma selenium levels have been described as predictable factors for occurrence of psoriasis (33).

Aim of the present study was to investigate the relationship between OPN, MMP-9 and TNF- α in psoriasis, by assessing the presence of OPN and MMP-9 in PBMC and sera of psoriatic patients before and after anti-TNF- α treatment.

Materials and methods

The study was conducted after written informed consent was obtained from all participants. From June 2010 to June 2011, 7 moderate-to-severe plaque psoriatic subjects, candidates to TNF- α antagonists, were enrolled. Before entering the study all subjects underwent a wash-out period of 8 weeks for systemic active drugs for psoriasis. Of the 7 patients enrolled, 3 were treated with etanercept, and four with adalimumab. Etanercept was administered subcutaneously 50 mg twice weekly for 12 weeks and 50 mg once weekly for other 12 weeks, whereas adalimumab 80 mg at week 0, followed by 40 mg every other week for 24 weeks, subcutaneously as well. Clinical parameters including age, sex, body weight, phenotype of psoriasis, psoriasis severity, presence of psoriatic arthritis were collected. Exclusion criteria included known cardiovascular disorders, diabetes and active malignancy. Psoriasis Area and Severity Index (PASI) was used to assess severity of psoriasis and a score >10 was considered as moderate-to-severe psoriasis.

Blood samples were taken at baseline (T0) and after 24 weeks (T1). Control blood samples were obtained from 10 age- and sex-matched healthy subjects. Ficoll-Hypaque (Biochrom Ag, Berlin Deutschland) density gradient centrifugations were carried out to isolate PBMC following manufacturer's instructions.

Total RNA, isolated by High Pure RNA Isolation Kit (Roche Diagnostics, Basel, Switzerland) from 1×10^6 PBMC was transcribed by reverse

transcriptase (Expand Reverse Transcriptase, Roche Diagnostics, Basel, Switzerland) at 42°C for 45 min according to the manufacturer's instructions. Two µl of complementary DNA (cDNA) were amplified in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 200 µM dNTP, and 2.5 units of Taq DNA polymerase (Roche Diagnostics) in a final volume of 50 µl. For the co-amplification of MMP-9, the PCR was carried out in the presence of 0.5 µM sense and antisense MMP-9 primers, and 0.05 µM sense and antisense glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin primers. OPN PCR was carried out in the presence of 0.5 µM sense and antisense OPN primers. RT-PCR was performed in a thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany). GAPDH or β-actin were used as housekeeping genes. PCR products were analyzed by electrophoresis on 1.8% agarose (Eppendorf, Hamburg, Germany) gel in Tris-Borate-EDTA buffer (TBE) (Fluka, BioChemika, Buchs, Switzerland). In Table 1 all sequences are summarized. Densitometric analysis of ethidium-bromide stained agarose gel was carried out by Quantity One 4.6.8 1-D Analyses Software (Bio-Rad Laboratories, Berkeley, CA, USA). The ratio between the yield of each amplified product and that of co-amplified internal control allowed a relative estimate of mRNA levels in the sample analyzed.

OPN concentration in patients' plasma was measured by ELISA assay (Quantikine; R&D System, Abingdon, UK) according to the manufacturer's instructions. The lower limit of detection for both assays was 0.313 ng/ml. Measurements were performed in duplicate. Samples with a coefficient of variation >10% were repeated.

Statistical analysis

Paired *t*-test analysis of variance (ANOVA) was used to statistically analyze OPN plasmatic levels and gene expression levels of OPN and MMP-9 before and after treatment. A *P* value <0.05 was considered as statistically significant.

GENE	FORWARD PRIMER 5'-3'	REVERSE PRIMER 5'-3'
B-Actin	5'-TgAcggggTCACCCACACTgTgCCCATCTA-3'	5- CTAgAAgCATTAgCggTggACgATggAggg-3'
GAPDH	5' CGGAGTCAACGGATTTGGTCGTAT-3'	5' AGCCTTCTCCATGGTGGTGAAGAC-3'
OPN	5' CCAAGTAAGTCCAACGAAAG-3'	5' GGTGATGCTCTCGTCTGTA-3'
MMP9	5' ACGACGTCTTCCAGTACCGA -3'	5' TTGGTCCACCTGGTTCAACT -3'

Table 1. Human primers sense and antisense sequences

Results

A total of 7 psoriatic subjects and 10 healthy volunteers was enrolled. All patients completed the study. There were no significant differences in age and sex between the two groups. None of psoriatic patients was affected by arthropathy too.

After 24 weeks of therapy (T1) 5 out of 7 patients reached PASI 75 (improvement of 75% of PASI score), whereas the remaining 2 achieved PASI 50. Psoriatic subjects showed significant higher levels of OPN expression, respect to healthy controls at baseline. After 24 weeks of TNF- α antagonists all subjects showed a strong reduction of OPN gene expression reaching comparable levels as controls ($P < 0.001$) (Fig. 1). No difference was detected between the 2 anti-TNF- α drugs (data not shown). In order to evaluate OPN release, ELISA assay was performed on plasma blood samples of healthy donors and psoriatic patients before and after treatment. OPN plasma levels were highly increased in psoriatic subjects respect to healthy controls ($P < 0.001$). As showed in Fig. 2 OPN plasma levels were strongly reduced after anti-TNF- α drugs, resulting lower than controls.

Since it has been reported that TNF- α can modulate MMP-9 expression (34), we sought to analyse effects of TNF- α antagonists on MMP-9 expression in PBMC. MMP-9 mRNA was increased in PBMC of psoriatic patients respect to healthy controls and anti-TNF- α treatment reduced these levels returning to normal values ($P < 0.001$) (Fig. 3). As for OPN,

the 2 anti-TNF- α drugs induced a comparable decrease (data not shown). Difference in PBMC gene expression between T0 and T1 (Δ) was calculated in all subjects for OPN ($\Delta=0.31$) and MMP-9 ($\Delta=0.44$); no statistically significant difference was detected, suggesting a similar decreasing trend with anti-TNF- α treatment (Fig. 4).

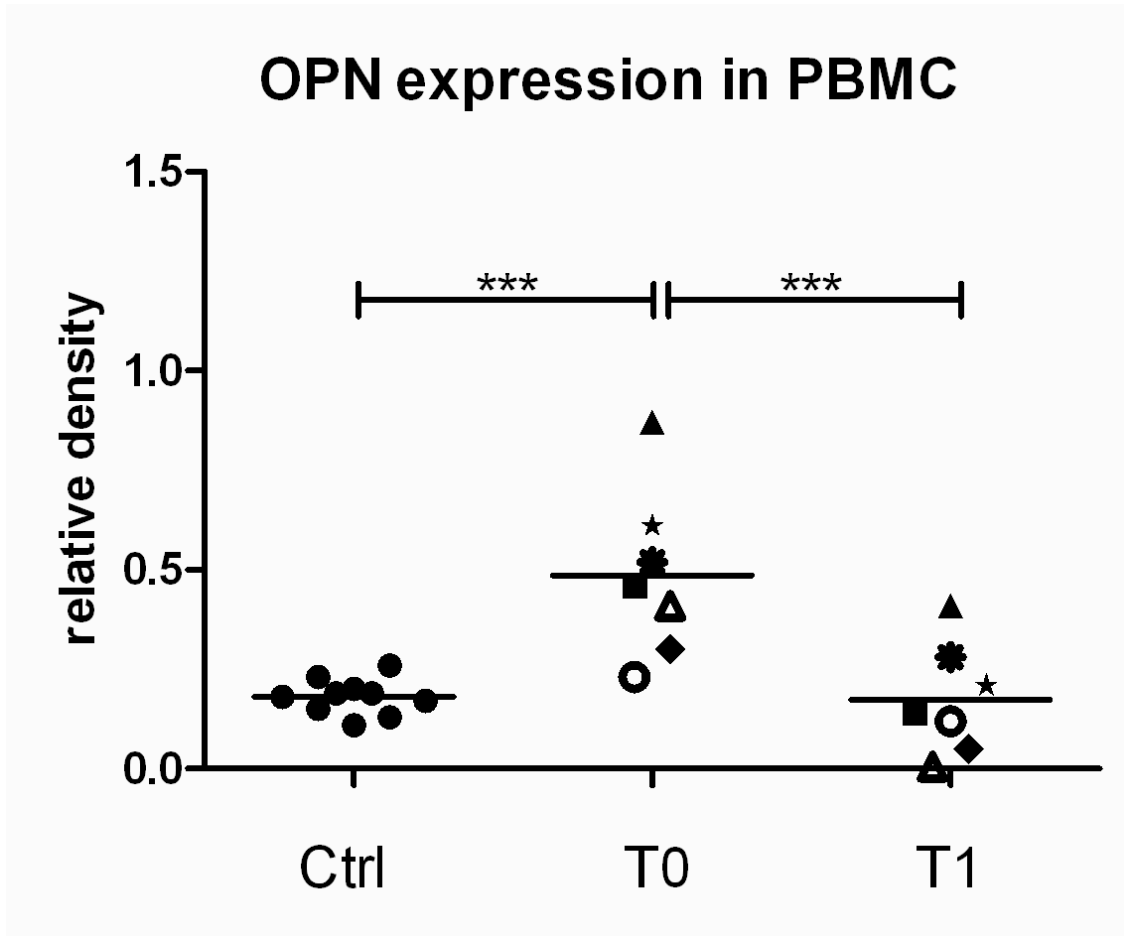


Figure 1. OPN gene expression in PBMC in healthy controls (Ctrl) and psoriatic patients before (T0) and after 24 weeks of therapy with anti-TNF α drugs (T1); $P < 0.001$ (***)

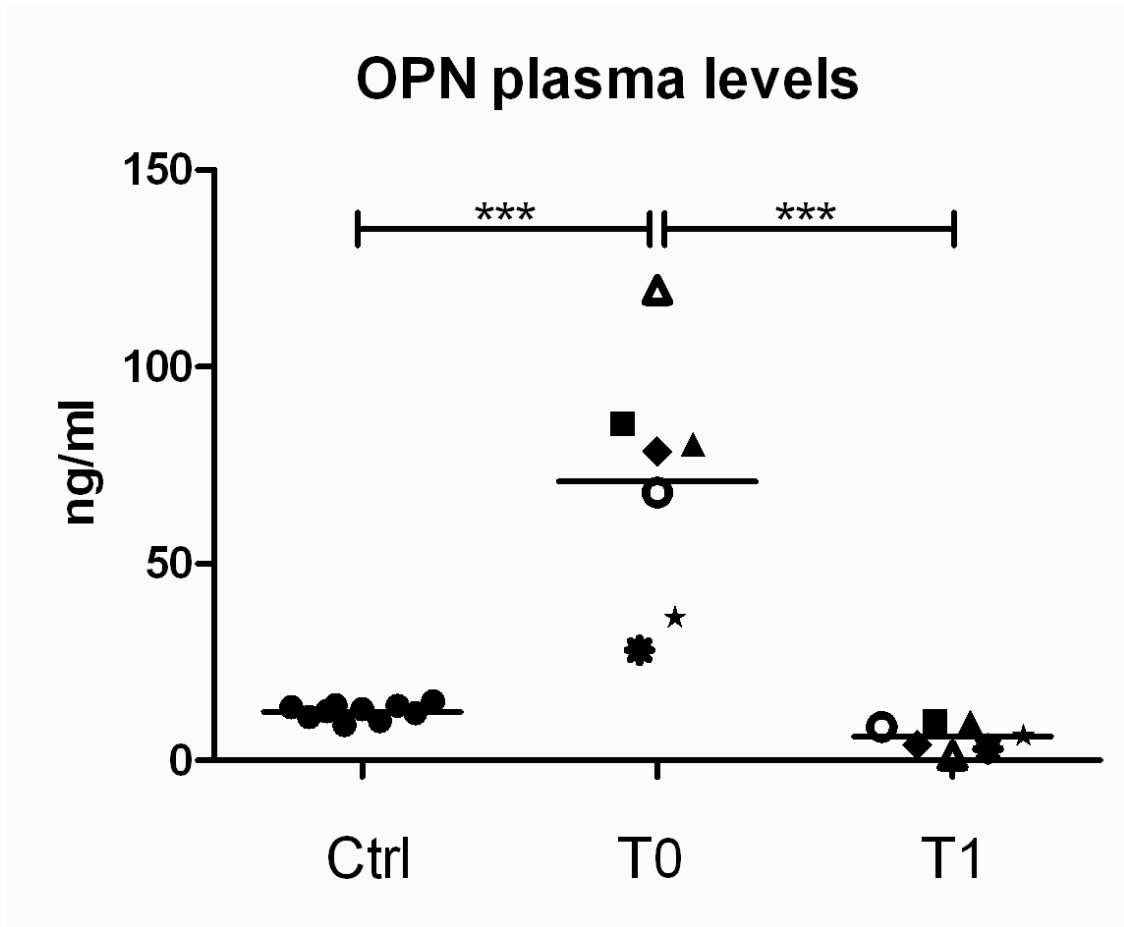


Figure 2. OPN plasmatic levels in healthy controls (Ctrl) and psoriatic patients before (T0) and after 24 weeks of therapy with anti-TNF α drugs (T1); $P < 0.001$ (***)).

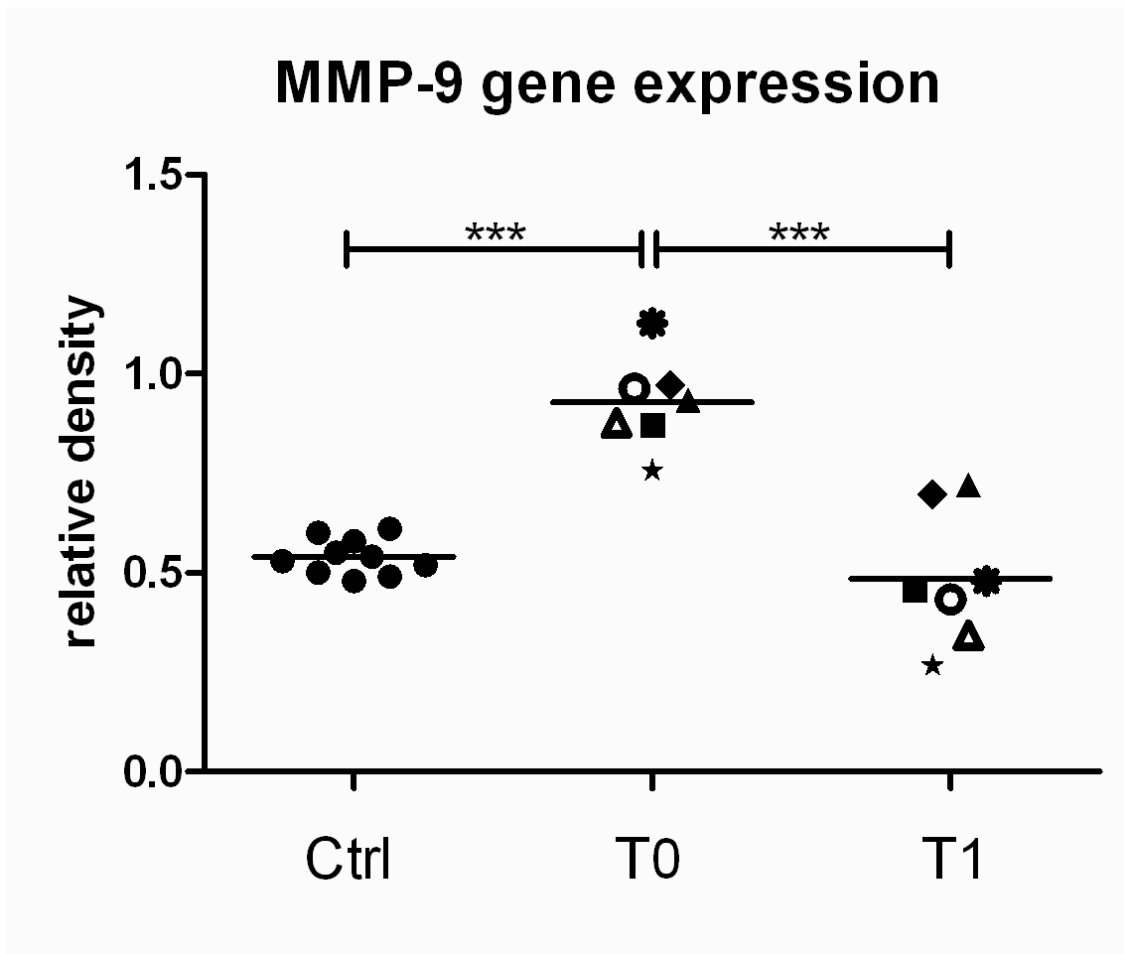


Figure 3. MMP-9 gene expression in PBMC in healthy controls (Ctrl) and psoriatic patients before (T0) and after 24 weeks of therapy with anti-TNF α drugs (T1); $P < 0.001$ (***)).

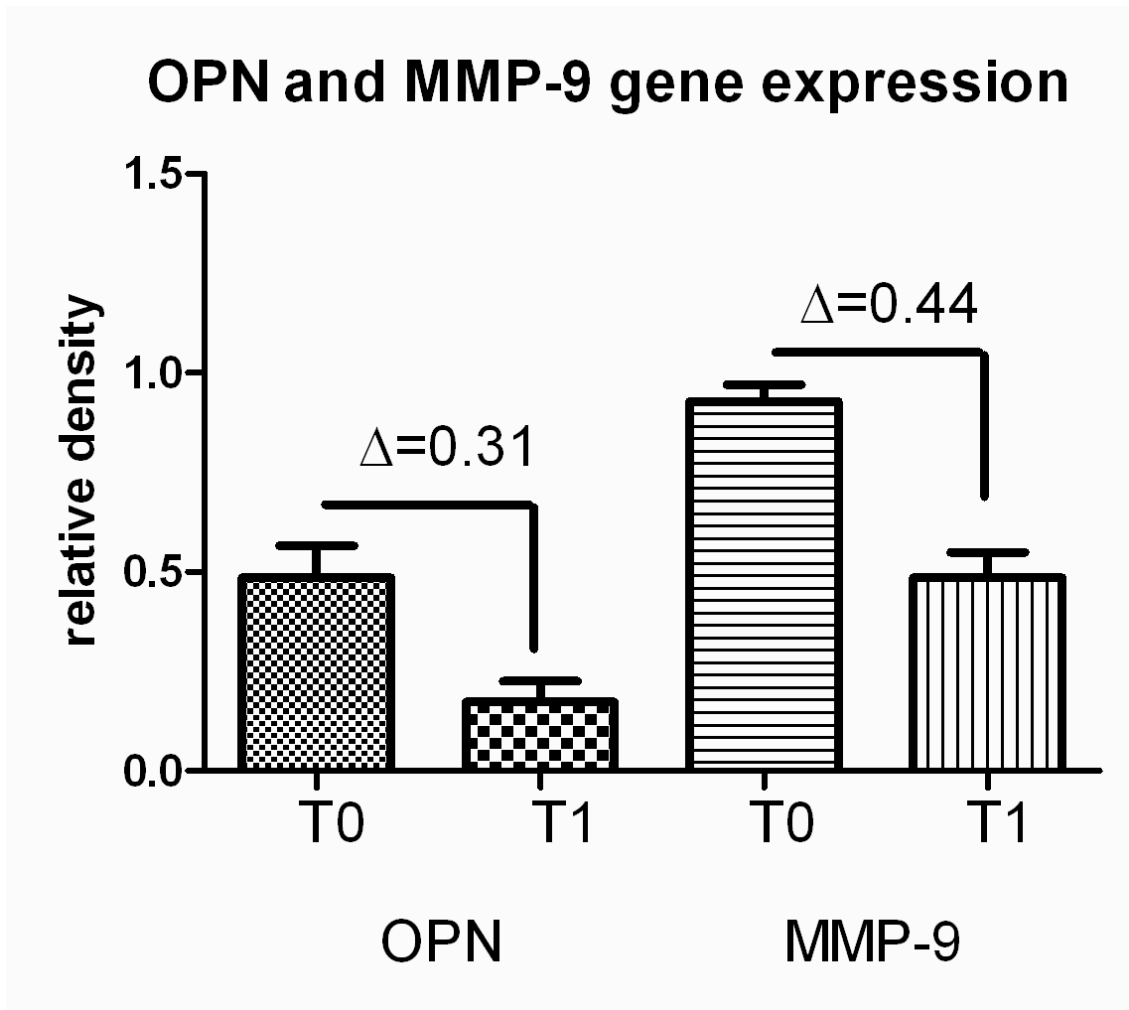


Figure 4. Difference in PBMC gene expression of all psoriatic subjects between T0 and T1 for OPN and MMP-9.

Conclusions

We have previously showed that OPN was increased in PBMC and skin samples of psoriatic subjects (31), whereas in the present study we have analysed the effect of anti-TNF- α therapy on OPN. In trying to understand if a change in OPN may be associated with TNF- α and involved in psoriasis pathogenesis, we focused our attention on its level in PBMC and in serum of psoriatic subjects where TNF- α is increased. TNF- α antagonists (etanercept and adalimumab) were able to decrease OPN either in PBMC or in plasma. A relationship between OPN and TNF- α has been previously assessed in tumour cell line in rat and human models, but not in psoriasis. Expression of OPN gene has been reported to be elevated by several factors, including TNF- α in ROS17/2.8 cells (rat osteosarcoma cells) (35) and in HN-22 cells (human head and neck squamous cell carcinoma cell line) (36). Our findings suggested a possible link between OPN and TNF- α in an inflammatory disease as psoriasis. This finding was supported by the fact that anti-TNF- α treatment was able to downregulate OPN. Consistent with this notion, OPN has been shown to be induced in other inflammatory conditions with TNF- α associated fibro-inflammatory process like human biliary atresia (37) and mice pulmonary fibrosis (38).

Our findings showed that anti-TNF- α treatment reduced MMP-9 expression in PBMC of psoriatic patients. These data fitted to Cordiali-Fei et al who showed that therapeutic approaches based on anti-TNF- α were significantly associated to the decrease of serum levels of TNF- α , angiogenic molecules

and MMP-9 (34,25). MMP-9 contributes to the chronic inflammatory process in the skin of the psoriatic patients either directly, by sustaining the inflammatory process and the tissue destruction or indirectly, by allowing the traffic of inflammatory cells and enhancing the activity of inflammatory cytokines. Experimental evidences indicated that MMPs can mediate the proteolytic process leading to the release of the soluble, active molecule of TNF- α from a cell membrane-achored molecular form (39). In addition, due to its proteolytic activity, MMP-9 could contribute to the generation of immunogenic fragments of normal proteins that may offer the basis for the initiation of local autoimmune cellular responses. The inhibitory effect of anti-TNF- α therapy may thus offer a two-fold efficacy through the reduction of MMP-9 levels and the inhibition of processing of TNF- α precursor into its active molecular form. Our findings confirm the existence of a direct relationship between anti-TNF- α therapy and decrease of MMP-9 expression.

Since it has been reported that OPN is a matricellular protein that plays an important role in tumour cell invasion due to its capacity to regulate the activity of at least two extracellular matrix degrading proteins: MMP-2 and urokinase plasminogen activator (40) and since it has been showed that OPN contributes to the increased amounts of MMP-9 in cardiac and skeletal muscle of mice (30), we wanted to analyse if there was a relationship between OPN and MMP-9 in psoriasis. A similar decreasing trend for OPN and MMP-9 was obtained by anti-TNF- α treatment, suggesting a possible link among the 2 molecules.

For the wide spectrum of biological activities, different names have been assigned to OPN through time, indicating a role in processes that are not confined to bone development, remodeling, and resorption. In summary our findings showed that OPN and MMP-9 are increased in psoriasis and anti-TNF- α treatment reduced them in a comparable fashion, so that we can hypothesize that they are involved in psoriasis pathogenesis. Our results suggest that OPN could be not only a key regulator of inflammatory processes of psoriasis, but also a biomarker for the efficacy of a treatment of this cutaneous disorder.

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