

-Università del Piemonte Orientale-

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**PPAR γ EXPRESSION IN HUMAN
MONOCYTE/MACROPHAGES AS A BIOMARKER
OF DISEASE ACTIVITY AND THERAPY EFFICACY
IN RHEUMATOID ARTHRITIS**

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INTRODUCTION

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a destructive, inflammatory, polyarticular joint disease with an aetiology that remains to be fully elucidated. RA is characterized by massive synovial proliferation and subintimal infiltration of inflammatory cells, followed by the destruction of cartilage and bone (Feldmann *et al.*, 1996). Although the aetiology of RA remains elusive, susceptibility factors are evident. Thus, the threefold predominance of RA in women may be attributable to hormonal factors, and the clear-cut genetic contribution in this disease is contained predominantly within the HLA class II locus (Solomon *et al.*, 2003; Aho *et al.*, 1986).

A number of cellular responses are involved in the pathogenesis of RA, including activation of inflammatory cells and expression of various cytokines. The regulation of cell migration and invasion is a critical process throughout the development of RA, since the number of inflammatory cells (including monocyte/macrophages) that infiltrate into the joints increases along with time. Macrophages, T cells, B cells and neutrophils migrate into synovial tissue and activate local cells to produce both inflammatory and degradative mediators that break down the extracellular matrix of cartilage. Synovial cells undergo hyperplasia, and angiogenesis occurs in synovial tissues further promoting inflammation. Inflammatory mediators such as interleukin-6 (IL-6), interleukin-1 (IL-1) and tumour necrosis factor- α (TNF- α) play important roles in the pathogenesis of RA. These cytokines are abundant in synovial tissues and fluid from RA patients and over-expression of the cytokines mentioned above promote chronic inflammation and joint destruction (Hopkins *et al.*, 1988). These cytokines have emerged not only as dominant pro-inflammatory mediators but as important molecular targets for therapy too (Okamoto *et al.*, 2008). TNF- α and IL-1 has been reported to stimulate synovial cells to release VEGF which has important role in the angiogenesis observed in RA pathology (Koch AE, 1998; Koch AE *et al.*, 1994).

Several evidences suggest that in RA pathology there is an imbalance between Th1 and Th2 immune response (Feldmann *et al.*, 1996). Classically, immune responses are regulated by two subtypes of CD4+ T helper (Th) cells, designated Th1 and Th2. Th1 cells produce interferon- γ (IFN- γ) and interleukin-2 (IL-2), which are known to be important mediators of organ-specific autoimmune disorders. On the other hand, Th2 cells produce the cytokines IL-4, IL-5, IL-9, and IL-13, which are responsible for promoting the development of atopic allergy. RA has been reported to be a Th1 and not a Th2 associated disorder.

Moreover, proliferative fibroblast-like synoviocytes play important roles in both joint damage and the propagation of inflammation, because they produce many mediators of inflammation, such as cytokines and metalloproteinases (MMPs), which contribute to cartilage degradation in the joints (Mor *et al.*, 2005; Weinberg *et al.*, 1993; Feldmann *et al.*, 1996). MMPs are a large family of

proteolytic enzymes involved in a range of physiological processes (e.g., development, morphogenesis, reproduction, wound healing, and aging) and pathological processes (e.g., inflammation, angiogenesis, neurological disorders, cancer cell invasion, and metastasis) (Muroski *et al.*, 2008). Among various MMPs (MMP-2, MMP-3, MMP-7, and MMP-9), only MMP-2 (also called gelatinase A) and MMP-9 (also called gelatinase B) were strongly associated with total MMP-13 in the progression of RA (Ahrens *et al.*, 1999; Yoshida *et al.*, 2009). Importantly, TNF- α and IL-1 are potent inducer of MMPs enzyme (Dayer *et al.*, 1985; McCachren *et al.*, 1990; Gravallesse *et al.*, 1991; Martel-Pelletier *et al.*, 1994).

Traditionally RA has been treated with disease modifying anti-rheumatic drugs (DMARDs) but in the last 15 years the introduction of biological response modifiers has revolutionized the treatment of RA. Among these, anti-TNF- α agents were the first to be successfully used in treating RA. The goal in treating RA is to induce remission or very low disease activity; remission is now accepted as the ultimate therapeutic goal by adoption of a "treat to target" strategy to achieve tight disease control. Therefore early diagnosis, as well as immediate intervention, is of the utmost importance. DMARDs, such as methotrexate and glucocorticoids, not only improve the joint pain and swelling associated with RA, but also slow down the joint damage associated with the disease. The efficacy of biologic drugs, such as adalimumab, anakinra, infliximab, rituximab, has been unequivocally established (Turkstra *et al.*, 2011).

Rheumatoid arthritis and Cardiovascular disease

RA is associated with an increased susceptibility to cardiovascular diseases and related mortality, recent estimates suggesting around a 50 % higher cardiovascular mortality risk (Thomas *et al.*, 2003; Ozbalkan *et al.*, 2010; Roifman *et al.*, 2011). In fact, several studies have demonstrated that the patients with RA had higher rates of ischemic heart disease (IHD) and were more likely to be hospitalized for acute MI compared to control subjects (Maradit-Kremers *et al.*, 2005; Han *et al.*, 2006).

This may be due to complex interactions between traditional CVD risk factors, systemic rheumatoid inflammation and the vasculature (Sandoo *et al.*, 2011). As described before, in RA, the primary site of inflammation is the synovial tissue, from which cytokines can be released into the systemic circulation. These circulating cytokines are in a position to alter the function of distant tissues, including adipose, skeletal muscle, liver, and vascular endothelium, to generate a spectrum of proatherogenic changes that includes insulin resistance, a characteristic dyslipidemia, prothrombotic effects, pro-oxidative stress, and endothelial dysfunction. These individual pathway perturbances, linked at many sites, in turn converge to promote accelerated atherogenesis in RA patients (Sattar *et al.*, 2003).

As well known, atherosclerosis is believed to be a complex disease resulting from changes in lipid

metabolism (hyperlipidaemia and/or hypercholesterolemia) and affecting the walls of arteries. Many factors could be responsible, from high fat diet to infectious agents causing inflammation in the artery wall with accumulation of immune cells and fatty deposits. T cells are responsible for orchestrating further inflammatory responses in plaque formation and progression in a Th1 type manner. The migration of monocytes into the arterial wall results in the activation and in the differentiation of monocytes into macrophages. Activated macrophages in the intima of the wall express high levels of receptors – scavenger receptor A, CD36 and CD68 – capable of uptaking lipids leading to lipid accumulation. These receptors are different from the normal LDL receptor in that they are not regulated by the cholesterol content within the macrophage. As a result, oxidized LDL is continuously taken up via these receptors, loading the macrophages with cholesterol. The lipid-engorged macrophages have a foamy appearance and are called 'foam cells'; they are the hallmark of the atherosclerotic lesion. Ultimately, the cholesterol-loaded macrophages in the artery wall die and release their cholesterol, causing pools of cholesterol to accumulate (Navab *et al.*, 2006). Activated macrophages also have an immunological role in chronic inflammation of the wall by secreting cytokines and interacting with lymphocytes and other cells involved in plaque formation like vascular smooth muscle cells.

So, by this very streamlined elucidation of atherosclerosis, some common denominators are present in the pathogenesis of RA and atherosclerosis. Unfavourable lipid profiles, including higher total cholesterol/HDL-cholesterol ratio, or the so-called atherogenic lipoprotein phenotype (characterized by decreased HDL, raised triglycerides and increased levels of small dense LDL) are associated with RA. Hurt-Camejo *et al.* (2001) reported that patients with long-term RA had significantly higher levels of small dense LDL (Superko *et al.*, 2008; White *et al.*, 2006). Atherosclerotic plaque and the inflamed synovium show marked similarities in the accumulation of inflammatory macrophages, monocytes and T cells, and share a similar profile of both systemic and local immune activation: mast and T-cell activation, TNF- α and IL-6 production, increased MMP and leukocyte adhesion molecules expression. Furthermore, both RA and atherosclerosis are associated with up-regulation of the Th1 related immune response (Libby, 2008).

Peroxisome Proliferator-Activator Receptor (PPAR γ) in Atherosclerosis and Rheumatoid arthritis

PPAR γ was first linked to atherosclerosis by the findings that, when activated, this receptor regulates lipid uptake and foam cell formation, and that oxidized LDL (oxLDL) are activators of the receptor. Activation of PPAR γ by its ligands leads to up-regulation of CD36, suggesting the existence of a positive feed back loop and providing a possible explanation of how lipid laden macrophages are formed in atherosclerotic plaques (Nagy *et al.*, 1998; Tontonoz *et al.*, 1998). Cells derived from the infected joints of patients with RA were found to express PPAR γ : in macrophages

the expression was at high levels and at moderate levels in synovial cells, endothelial cells and fibroblasts. In the *in vitro* cultures of synoviocytes both 15d-PGJ and troglitazone were found to inhibit proliferation of synoviocytes and induced apoptosis at higher doses (Kawahito et al., 2000). The role for PPAR γ in the control of the inflammatory responses of macrophages, such as reducing the production of IL-1 β , IL-6 and TNF α that are key mediators in RA, suggests that application of PPAR γ agonists might have multiple targets in RA. This is supported by studies on female Lewis rats where adjuvant induced arthritis was ameliorated by intraperitoneal administration of 15d-PGJ or troglitazone with a reduced number of inflammatory cells and pannus formation in the affected joints (Kawahito et al., 2000), in other different animal models (Cuzzocrea et al., 2003; Koufany et al., 2008) and in several inflammatory cells (Jiang et al., 1998; Ricote et al., 1998; Amoruso et al., 2007). Previous reports have also documented that PPAR γ is expressed at both mRNA and protein levels by major cell populations in joints; in these cells, 15d-PGJ and thiazolidinediones (TZD) inhibit the transcriptional induction of genes that contribute to joint pathology, e.g., TNF- α , IL-1, gelatinase B, iNOS and MMP-13 (Jang et al., 1998; Fahmi et al., 2001; Ji et al., 2001; Amoruso et al., 2007; Giaginis et al., 2009; Amoruso et al., 2009). Interestingly, up-regulation of innate immune pathways, including IL-6, Toll-like receptor/IL-1 receptor and PPAR signalling, was observed in peripheral blood mononuclear cells from patients with systemic juvenile idiopathic arthritis (Barnes et al., 2009). In addition, we previously documented a constitutively enhanced PPAR γ expression in both monocytes and macrophages from patients affected by coronary artery disease (CAD) as compared to healthy subjects (Amoruso et al., JPET 2009; Amoruso et al., BJP 2009), with an important gender difference in the CAD group. Furthermore, PPAR γ ligands have been shown to induce apoptosis in T lymphocytes and macrophages (Yessoufou et al., 2010; Shahin et al., 2011).

Therefore, the potential anti-inflammatory properties of PPAR γ ligands on RA activity have been investigated in several models of arthritis (Shahin et al., 2011). PPAR γ ligand treatment was shown to reduce a wide variety of inflammatory markers in several animal models of OA, RA, sepsis, pancreatitis, atherosclerosis, ulcerative colitis, chronic asthma, as well as Parkinson and Alzheimer's disease (Moraes et al., 2006; Rizzo et al., 2006).

In addition, CLX-090717, a novel synthetic PPAR γ agonist, significantly inhibited spontaneous TNF- α release by RA synovial membrane cells, as well as LPS-induced TNF- α release from human and murine monocytic cells. Inhibition of TNF- α in monocytes was mediated partially through a NF- κ B-dependent pathway, as judged by sustained levels of I κ B α in cytosolic extracts and a reduced level of LPS-induced NF- κ B activity in nuclear extracts (Ishino et al., 2008; Sumariwalla et al., 2009).

In line with the project of the PhD, the aim of my last year was to evaluate the expression of PPAR γ (both as protein and mRNA) in monocytes/MDM isolated from RA patients, and compare

the results to cells of healthy donors. Additionally, we measured the MMP-9 activity and together with the PPAR level, the results were correlated to the disease's severity, according to the scale DAS28.

MATERIALS AND METHODS

Patients

We enrolled 30 consecutive adult patients with established RA (20 females and 10 males, aged 60.1 ± 2.6 years), attending the tertiary level immuno-rheumatology clinic of an Academic hospital from January 2010 till January 2011, and 15 healthy donors (10 females and 5 males). Only patients with established RA who gave their consent were included in the study. Healthy subjects were age-matched to RA patients, had no history of inflammatory/immune or other chronic diseases and were drug-free (including paracetamol or aspirin) at the time of the study. RA was diagnosed according to the 1987 criteria of the American College of Rheumatology (Arnett *et al.*, 1988). All patients were on current RA therapy, some receiving weekly methotrexate (MTX), others receiving different disease-modifying anti-rheumatic drugs (DMARDs), e.g., sulfasalazine (SSZ), hydroxychloroquine (HCQ) or leflunomide (LEF), in single or combination therapy. Some patients were also receiving low-dose oral corticosteroids: 16 patients assumed prednisone < 5 mg and 4 patients had methylprednisolone (MP) < 4 mg daily. None of them received monoclonal antibodies or TNF- α inhibitors. For each patient, a complete medical history was obtained and a full physical examination, including joint assessment, was performed. Baseline demographic data were collected for all patients, including sex, age, anti-rheumatic therapy, and are shown in Table 1. Clinical assessment included the 28-joint Disease Activity Score, DAS28 (20), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level. Blood samples were obtained from each participant at 9.00 a.m. at fasting. The Research Protocol was approved by the Ethic Committee of Azienda Ospedaliera Maggiore della Carità, Novara; informed written consent was obtained from all RA patients and healthy volunteers.

Isolation of monocytes and macrophages (MDM) from RA patients and healthy donors

Human monocytes were isolated from heparinised venous blood by standard techniques of dextran sedimentation, Hystopaque (density= 1077g/cm^3) gradient centrifugation (400g, 30 min, room temperature) and recovered by thin suction at the interface, as described (Amoruso *et al.*, 2007; Amoruso *et al.*, BJP 2009; Amoruso *et al.*, JPET 2009). Cells were re-suspended in RPMI 1640 medium, supplemented with 5% heat-inactivated foetal bovine serum (FBS), 2 mM glutamine, 50 $\mu\text{g/ml}$ streptomycin, 5 U/ml penicillin, and 2.5 $\mu\text{g/ml}$ amphotericin B; purified monocyte populations were obtained by adhesion (90 min, 37°C, 5% CO_2), non-adherent cells (mainly lymphocytes) being removed by gentle washings. Cell viability (trypan blue dye exclusion) was usually >98%; expression of surface markers was analyzed by flow cytometry, yielding > 90 % pure monocyte populations (Amoruso *et al.*, 2007). Monocyte-derived macrophages (MDM) were prepared from monocytes by culture (8–10 days) in a 5% CO_2 incubator at 37°C in RPMI 1640 medium containing

20% FBS, glutamine and antibiotics (Amoruso *et al.*, 2007; Amoruso *et al.*, JPET 2009). MDM were defined as macrophage-like cells by evaluating surface markers, as described (Amoruso *et al.*, 2007; Amoruso *et al.*, BJP 2009).

PPAR γ protein expression and semi-quantitative analysis

The constitutive expression of PPAR γ protein was evaluated in monocytes and MDM, as described (Amoruso *et al.*, 2007; Amoruso *et al.*, BJP 2009; Amoruso *et al.*, JPET 2009). Cells (2×10^6) were scraped off in RIPA buffer and lysed by sonication; when necessary, cell lysates were stored at -80°C . The determination of protein concentration was done with a BCA assay. Protein samples (20 μg) were analysed by SDS-PAGE (10% acrylamide) and electro-blotted on nitrocellulose membrane (Protran, Perkin Elmer Life Sciences, USA). Immunoblots were performed using polyclonal rabbit anti-human PPAR γ (Abcam, UK), and monoclonal mouse anti-human β -actin (Sigma, Italy) antibodies; anti-mouse and anti-rabbit secondary antibodies were coupled to horseradish peroxidase. Chemiluminescence's signals were analysed under non-saturating conditions with an image densitometer (Versadoc, Bio-Rad, USA). Semi-quantitative evaluation of PPAR γ protein was performed by calculating the ratio between its total expression and the expression of the reference housekeeping protein, β -actin (Amoruso *et al.*, 2007). We also evaluated the ability of the endogenous PPAR γ agonist 15-deoxy-D^{12,14}-prostaglandin J₂ (15d-PGJ), and of two anti-rheumatic drugs largely used in our RA patients, methotrexate (MTX) and methylprednisolone (MP), to affect PPAR γ protein expression *in vitro*. In these experiments, cells from healthy donors were treated with the drugs for 6 hr; this incubation time was previously shown to represent the optimal challenge period to induce PPAR γ protein expression (Amoruso *et al.*, 2007; Amoruso *et al.*, BJP 2009; Amoruso *et al.*, JPET 2009).

PPAR γ mRNA evaluation and Real-Time PCR

Total RNA was extracted with the GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich), according to the manufacturer's instructions. The amount and purity of total RNA were spectrophotometrically quantified by measuring the optical density at 260 and 280 nm. cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions. Real-time PCR was carried out in a volume of 20 μl per well in a 96-well optical reaction plate (Applied Biosystems) containing 1 μl of TaqMan Expression Assay (PPAR- γ), 5 μl of RNase-free water, 10 μl of TaqMan Universal PCR MasterMix (2x) (without AmpErase UNG), and 4 μl of cDNA template, as described (Amoruso *et al.*, JPET 2009). The plate was run on the 7000 ABI Prism system (Applied Biosystems). To compensate for variations in cDNA concentrations and PCR efficiency between tubes, an endogenous gene control (β -glucuronidase) was included for each sample and used for

normalization; results were analyzed by the comparative cycle threshold method, as described (Amoruso *et al.*, JPET 2009).

Evaluation of matrix metalloproteinase-9 (MMP-9) activity

We used gelatin zymography to detect changes in MMP-9 activity in human monocytes and MDM of RA patients and healthy donors. Cells (1×10^6) were incubated for 6 hr in RPMI 1640 medium; the supernatants were then mixed with Laemmli sample buffer (Bio-Rad) in a ratio 2:1 and analyzed by electrophoresis with a 10% Novex zymogram gel (Invitrogen). The gel was developed according to the manufacturer's instructions, with Colloidal Blue Staining Kit (Invitrogen) and analyzed with densitometer in non-saturating conditions (Versadoc, Bio-Rad, USA). We also evaluated the ability of 15d-PGJ, MTX and MP to affect MMP-9 activity in monocyte/MDM. In these experiments, cells (isolated from healthy donors) were pre-treated with the drugs for 1 hr and then stimulated by lipopolysaccharide (LPS) 100 ng/ml for 6 hr, that is the same time as for the determination of PPAR γ protein expression.

Statistical analysis

Statistical analyses were performed using SPSS statistical software (version 15.0, SPSS Inc., Chicago, USA). All data are mean \pm SEM of 'n' independent experiments on monocyte/macrophages from different patients or healthy donors. Variables were compared among groups by ANOVA and Bonferroni correction. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Characteristics of the patients enrolled in the study.

The clinical and demographic features of the 30 RA patients enrolled in this study are shown in Table 1. All patients were on current DMARDs therapy, most of them also assuming oral corticosteroids; in this study population, the DAS28 (Arnett *et al.*, 1988) score ranged from 0.63 to 5.97, with a mean value of 3 ± 0.2 (Table1).

Table 1. Main demographic and clinical features of the studied population

Characteristics	RA Patients (n = 30)	Healthy Donors (n = 15)
Age, years	60.1 \pm 2.6	59.8 \pm 1.9
CRP, mg/dl	0.6 \pm 0.1	-
ESR, mm/h	19.4 \pm 2.7	-
DAS28	3 \pm 0.2	-
Gender (M/F)	10/20	5/15
Immunosuppressive Treatment:		NO
Glucocorticoids [§]	20/30	-
DMARDs ^{§§}	30/30	-
MTX	22/30	-
Other DMARDs ^{§§§}	11/30	-

Data are means \pm SEM. RA = rheumatoid arthritis; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; DAS28 = Disease Activity Score in 28 joints; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate. § indicates prednisone \leq 5 mg/day or equivalent; §§ includes MTX, sulfasalazine (SSZ), hydroxychloroquine (HCQ) and leflunomide (LEF); §§§ includes SSZ, HCQ and LEF.

Expression of PPAR γ protein and mRNA in monocyte/macrophages.

As depicted in Figure 1a, monocytes and MDM obtained from RA patients present a significantly ($P < 0.01$) enhanced constitutive expression of PPAR γ protein as compared to healthy donors. In fact, the PPAR γ / β -actin ratio amounts to 0.21 ± 0.02 and 0.46 ± 0.05 in monocytes from healthy donors ($n=15$) and RA patients ($n=30$), respectively. Similar results are obtained in MDM, PPAR γ / β -actin ratio being 0.67 ± 0.08 and 1.8 ± 0.19 in healthy donors ($n=15$) and RA patients ($n=30$), respectively (Figure 1a). Representative Western blots for PPAR γ protein expression are shown in Figure 1b. In RA patients, also PPAR γ mRNA levels are increased (4-fold in monocytes and about 3-fold in MDM), compared to healthy donors (Figure 1c).

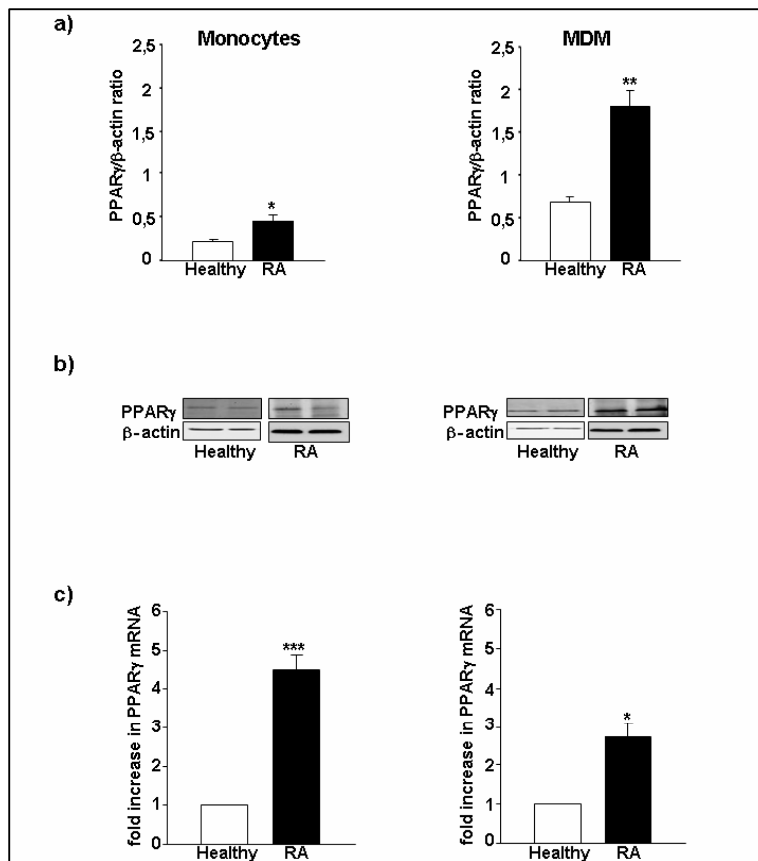


Figure 1. Enhanced PPAR γ expression in monocytes and monocyte-derived macrophages (MDM) from rheumatoid arthritis (RA) patients. **a**, PPAR γ protein expression in healthy donors ($n=15$) and RA patients ($n=30$). Semi-quantitative evaluation of PPAR γ protein was performed by calculating the ratio between its total expression and β -actin. Data are mean \pm SEM; * $P < 0.05$ vs healthy donors; ** $P < 0.01$ vs healthy donors. **b**, representative blots for PPAR γ protein expression. **c**, PPAR γ mRNA in cells from healthy donors ($n=9$) and RA patients ($n=15$ for monocytes; $n=14$ for MDM). PPAR γ mRNA level in healthy donors represents the “calibrator” level = 1. Data are mean \pm SEM; * $P < 0.05$ vs healthy donors; *** $P < 0.001$ vs healthy donors.

However, by pooling the data obtained into two series according to the DAS28 score (<3.2 or >3.2 score) of each single patient, we show that monocytes and MDM from patients with low disease activity (DAS28 <3.2) present a significantly ($P < 0.01$) higher PPAR γ protein expression than healthy donors (Figure 2a). On the contrary, PPAR γ protein expression in cells from patients with moderate-high disease activity (DAS28 >3.2), although enhanced (as compared to healthy donors), does not reach statistical significance (Figure 2a). By evaluating PPAR γ mRNA levels in monocyte/MDM, we found a significant increase ($P < 0.001$ vs healthy donors in monocytes, and $P < 0.05$ in MDM) in all the RA patients, with no statistically significant difference according to the disease activity (Figure 2b).

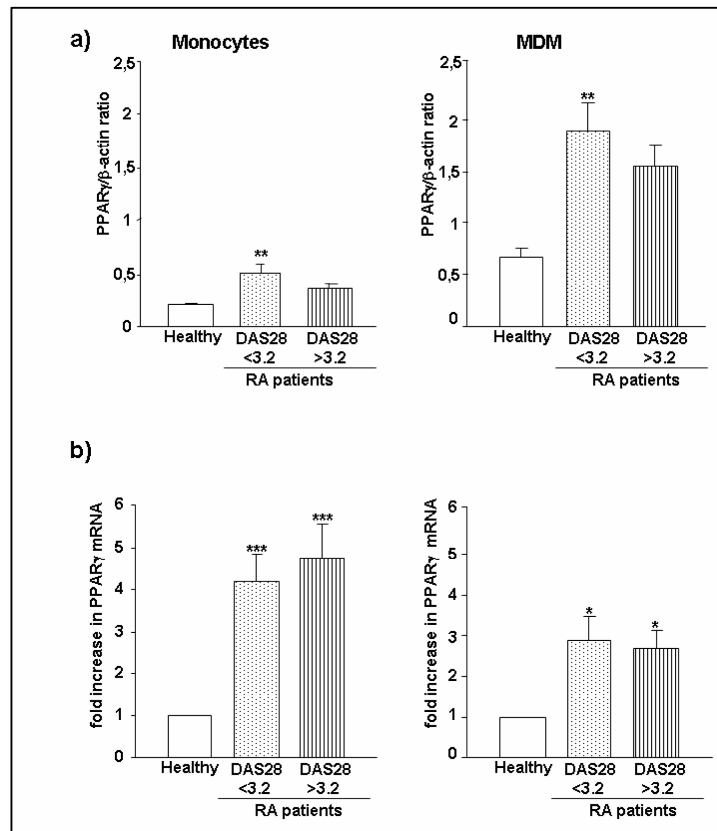


Figure 2. PPAR γ expression in monocytes and MDM from RA patients, in relation to DAS28 score. **a**, PPAR γ protein expression in healthy donors (n=15), RA patients with DAS28 score < 3.2 (n=20) or > 3.2 (n=10). Data are mean \pm SEM; ** $P < 0.01$ vs healthy donors. **b**, PPAR γ mRNA expression in healthy donors (n=9), RA patients with DAS28 score < 3.2 (n=9 for monocytes; n=7 for MDM) or > 3.2 (n=6 for monocytes; n=7 for MDM). Data are mean \pm SEM; * $P < 0.05$ vs healthy donors; ** $P < 0.01$ vs healthy donors; *** $P < 0.001$ vs healthy donors.

MMP-9 activity in monocyte/macrophages.

As known, MMP-9 activity is critical for RA progression and is inhibited by PPAR γ agonists (Ricote *et al.*, 1998; Shu *et al.*, 2000; Marx *et al.*, 2003). In monocyte/MDM from RA patients, the basal MMP-9 activity is significantly higher ($P < 0.001$) than in healthy volunteers, a more than 6-fold increase being observed in MDM (Figure 3a). Representative gelatin zymographies are provided in Figure 3b. Interestingly, by evaluating MMP-9 activity in relation to DAS28 score, we observed that monocyte/MDM isolated from patients with a more active disease (DAS28 > 3.2) present the highest increase (about 6-fold in monocytes and > 7 -fold in MDM; $P < 0.01$ vs healthy donors) in gelatinolytic activity (Figure 3c)

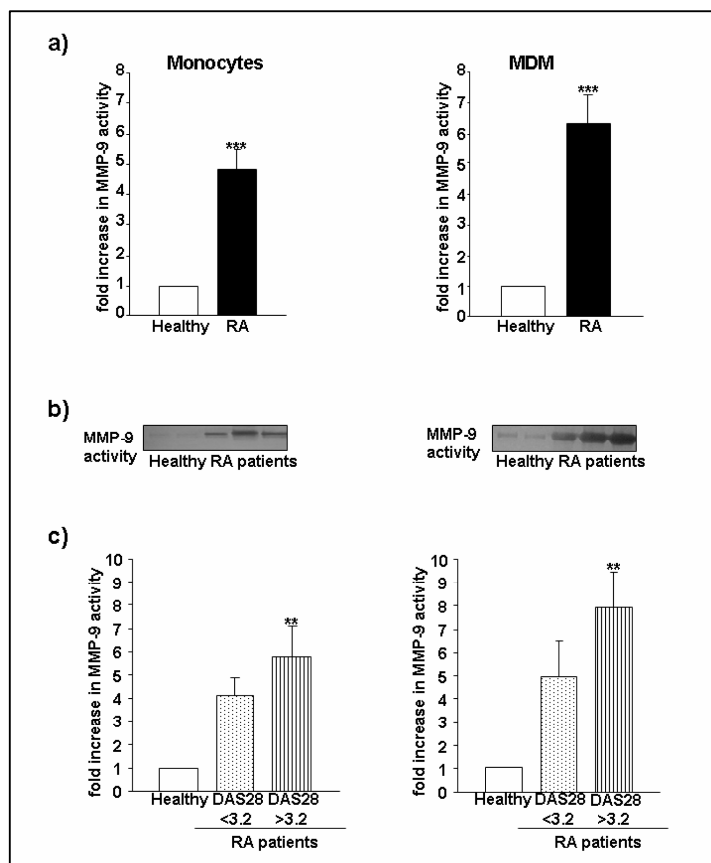


Figure 3. Enhanced matrix metalloproteinase-9 (MMP-9) activity in monocytes and MDM from RA patients. **a**, fold-increase in MMP-9 activity in cells from RA patients ($n=18$ for monocytes; $n=15$ for MDM) as compared to healthy donors ($n=8$). MMP-9 activity in healthy donors is assessed as =1. Data are mean \pm SEM; *** $P < 0.001$ vs healthy donors. **b**, representative gelatin zymographies for MMP-9 activity. **c**, MMP-9 activity in cells from healthy donors ($n=8$), RA patients with DAS28 score < 3.2 ($n=10$ for monocytes; $n=9$ for MDM) or > 3.2 ($n=8$ for monocytes; $n=6$ for MDM). Data are mean \pm SEM; ** $P < 0.01$ vs healthy donors.

Effects of PPAR γ agonists and DMRDs on monocyte/macrophages from healthy donors.

Since all the patients enrolled in this pilot study were treated with the conventional RA therapy that combines DMARDs and, most of the times, corticosteroids (Table 1), we also evaluated the *in vitro* ability of methylprednisolone (MP) and methotrexate (MTX) to directly affect PPAR γ expression and MMP-9 activity in monocyte/macrophages of healthy donors. Both drugs were used at concentrations that can be found in RA patients and their effects were compared with those elicited by the PPAR γ agonist 15d-PGJ. MTX was used at 1 μ M, since Godfrey *et al.* (Godfrey *et al.*, 1998), in a large pharmacokinetic study in RA patients, reported serum MTX concentrations in the range 0.3 -1.5 μ M. Moreover, C_{max} values of 1.1 μ M for MTX and 180 ng/ml for MP are also reported (Goodman&Gilman's, 2001). As shown in Figure 4a, MTX and MP, used at 1 μ M and 180 ng/ml, respectively, induce PPAR γ protein expression in both monocytes and MDM; the effects elicited by 15d-PGJ, which at 10 μ M ensures maximal activity (Amoruso *et al.*, 2007; Amoruso *et al.*, JPET 2009), are shown for comparison (Figure 4a). Representative Western blots are also provided (Figure 4b).

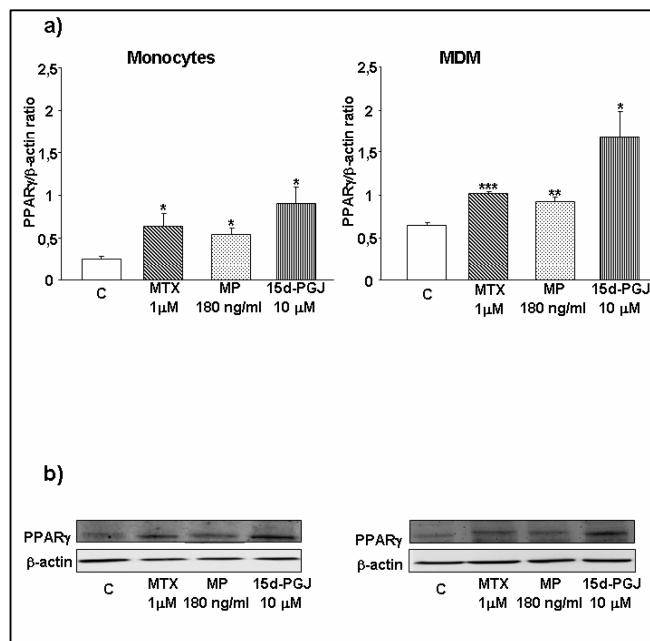


Figure 4. The PPAR γ agonist 15-deoxy-D^{12,14}-prostaglandin J₂ (15d-PGJ), methotrexate (MTX) and methylprednisolone (MP) increase PPAR γ protein expression in cells isolated from healthy donors. **a**, PPAR γ / β -actin ratio protein in monocytes and MDM from healthy donors. Cells were challenged with fixed concentrations of drugs for 6 hr. Data are mean \pm SEM; n= 4. *P < 0.05 vs un-stimulated (control, C) cells; ** P < 0.01 vs control; ***P < 0.001 vs control. **b**, representative blots for PPAR γ protein expression.

As known, MMP-9 exerts a crucial role in RA progression and joint destruction (Ahrens *et al.*, 1999; Yoshida *et al.*, 2009); therefore, inhibition of the gelatinolytic activity can contribute to the overall clinical efficacy of a given drug in RA therapy. In human monocytes and MDM, LPS (100 ng/ml, 6 hr) stimulates about 2-fold MMP-9 activity (5892 \pm 968 arbitrary intensity units in monocytes, n = 4, P < 0.01 vs control; and 9753 \pm 310 arbitrary intensity units in MDM, n = 4, P < 0.01 vs control; data not shown). Interestingly, 15d-PGJ, MTX and MP (used at 10 μ M, 1 μ M and 180 ng/ml, respectively) potently inhibit LPS-induced MMP-9 activity, the endogenous PPAR γ ligand and MTX being more effective than MP (P < 0.001; Figure 5a) in monocytes. On the contrary, the three drugs inhibited similarly LPS-induced MMP-9 activity in MDM (Figure 5a). Representative gelatin zymographies are provided in Figure 5b.

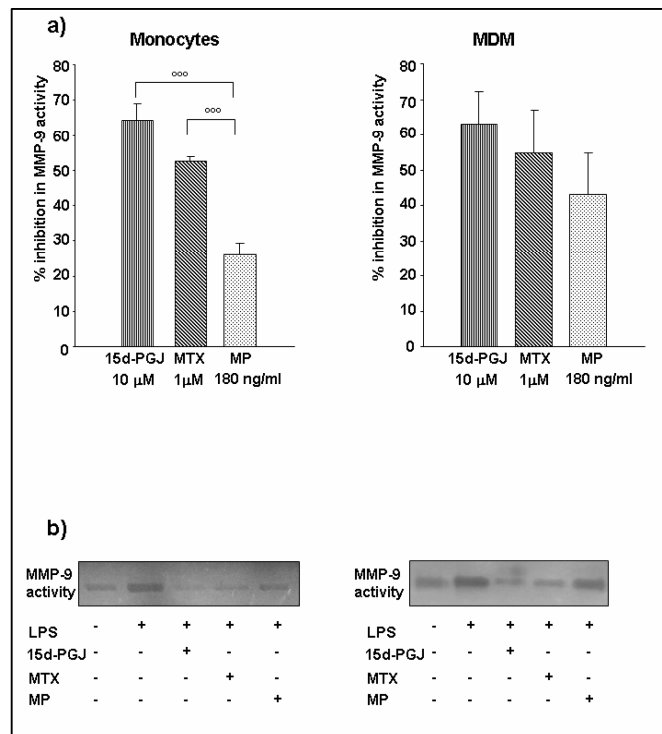


Figure 5. 15d-PGJ, MTX and MP inhibit LPS (lipopolysaccharide)-stimulated MMP-9 activity in human monocytes and MDM isolated from healthy donors. **a**, % inhibition of LPS-stimulated MMP-9 activity. Cells were pre-treated with the drugs for 1 hr and then stimulated by LPS 100 ng/ml for 6 hr. Data are mean \pm SEM; n = 4. ∞ P < 0.001. **b**, representative gelatin zymographies for MMP-9 activity.

DISCUSSION

In line with the project of my PhD, which was focused on the role of PPARs in inflammation and gender difference, I have concentrated my project in two important diseases, such as coronary artery disease (CAD) and rheumatoid arthritis (RA).

The results obtained in the second year demonstrated that in monocytes/macrophages the expression of PPAR γ is significantly higher in CAD patients than in healthy donors and that, together with cytokine release, it seems to be gender-related. In fact, CAD women showed the highest PPAR γ expression and the lowest cytokine release (Amoruso *et al.*, JPET 2009; Amoruso *et al.*, BJP 2009). Because of the striking similarity between atherosclerosis and RA, I moved my attention to the latter disease, yet considering the different therapy to which the patients are exposed.

This study indicates that monocytes and macrophages isolated from RA patients, under pharmacological treatment (MTX and corticosteroids, mainly), present an increased PPAR γ expression (both as protein and mRNA) as compared to cells of healthy donors, in good agreement with previous findings in experimental arthritis and patients. In fact, an increase in PPAR γ (but not adiponectin) mRNA levels was observed in a model of adjuvant-induced arthritis, and TZD reduced bone erosions and inflammatory bone loss but did not exert protective effects on cartilage (Koufany *et al.*, 2008). Moreover, Jiang *et al.* (Jiang *et al.*, 2008), by evaluating PPAR γ gene expression in bone marrow cells obtained from patients with traumatic femoral neck fracture, osteoarthritis (OA) or RA, observed that RA patients (but not OA patients) had significantly higher PPAR γ mRNA levels than fractured subjects. Increased PPAR γ mRNA levels were also detected in macrophages from patients with active systemic lupus erythematosus (SLE), as compared to patients with inactive SLE or infectious diseases and healthy donors (Oxer *et al.*, 2011).

The fact that PPAR γ protein and/or mRNA might be enhanced in inflammatory/immune diseases with a relevant involvement of monocyte/macrophages is not surprising, since PPAR γ has been widely demonstrated to be a key modulator of macrophage differentiation (Amoruso *et al.*, 2007; Moore *et al.*, 2001; Tontonoz *et al.*, 1998) and participates in different inflammatory and autoimmune disorders (see Szeles *et al.*, 2007; Szanto and Nagy 2008, for review).

It has also been suggested that M1 (the classically activated, inflammatory macrophages that secrete higher levels of pro-inflammatory genes and likely contribute to inflammation) and M2 macrophages can switch from one phenotype to the other (Gordon, 2003). Interestingly, PPAR γ is required for promoting the less inflammatory, alternatively activated M2 phenotype of macrophages (Odegard *et al.*, 2007) and rosiglitazone has been shown to up-regulate markers (e.g., arginase 1, IL-10) characteristic of the M2 phenotype (Stienstra *et al.*, 2008).

Therefore, the local environment created by the activation of PPAR γ might induce a switch from M1 toward M2 activated macrophages, thus contributing to the anti-inflammatory effect. Interestingly, glucocorticoids represent another stimulus that favours the M2 phenotype (Mantovani

et al., 2002) and this switching could be relevant for their anti-inflammatory action at the macrophage level (Hamilton and Tak, 2009).

As largely demonstrated (Hamilton and Tak, 2009, for review), the number of macrophages in the synovium of RA patients correlates with the degree/severity of joint erosions. Previous observations also indicated that a reduced macrophage infiltration is strictly related to successful response to RA treatment with methotrexate (Dolhain *et al.*, 1998) or corticosteroids (Gerlag *et al.*, 2004). Although most of our RA patients were treated with oral prednisone, we used methylprednisolone as a prototype of corticosteroids for *in vitro* studies, since its characteristics - bioactivity *per se* and higher liposolubility - make it more suitable for a 6hr challenge, as for the evaluation of PPAR γ protein expression and MMP-9 activity. The results show that MTX and MP, besides reducing MMP-9 activity, enhance about 2-fold PPAR γ protein expression in monocytes and MDM, as the endogenous ligand 15d-PGJ does.

It is well known that different drugs may affect PPAR γ , since indomethacin and other selected non-steroidal anti-inflammatory drugs, as well as telmisartan and some statins, have been described to act as PPAR γ agonists at relatively high concentrations, this property contributing to their overall therapeutic activity (Jiang *et al.*, 1998; Lehmann *et al.*, 1997; Jaradat *et al.*, 2001; Xue *et al.*, 2010; Yano *et al.*, 2007; Amoruso *et al.*, 2010; Matsumura *et al.*, 2011). Given that MTX and MP increase PPAR γ expression at therapeutic concentrations in our *in vitro* experiments, we suggest that this activity might contribute to their clinical efficacy in RA. On this regard, it is worth noting that we measured a different PPAR γ expression according to disease activity. In fact, RA patients with a DAS28 score < 3.2, that reasonably identifies those patients in which MTX and corticosteroids are effective in controlling disease severity (see also Table 1) express higher PPAR γ protein levels than patients with DAS28 score >3.2, that could be assimilated to "inadequate-responding" patients. Interestingly, the highest PPAR γ protein expression (PPAR γ / β -actin ratio = 0.72 + 0.1 in monocytes and 2.1 + 0.2 in MDM; data not shown) is documented in the 9 RA patients with a score < 2.6, which, in keeping with Felson *et al.* (2011), represents minimal disease activity rather than remission. It should be noted that PPAR γ mRNA levels, even if significantly enhanced as compared to healthy donors, are similar in both DAS28 cohorts. Although we have no definite explanation for this fact, this is in accordance with previous results by Amoruso *et al.* (JPET 2009) in patients with CAD: by evaluating PPAR γ expression in monocyte/MDM from CAD patients, no significant difference in PPAR γ mRNA levels between CAD males and females was found, despite a clear gender difference at the protein level. Therefore, it seems conceivable to suggest PPAR γ expression in human monocyte/macrophages as a predictor of DAS28 remission and/or therapy efficacy.

Since gelatinolytic activity of synovium is stronger in RA than in osteoarthritis (Yoshida *et al.*, 1999) and PPAR γ has been shown to inhibit MMP-9 secretion (Shu *et al.*, 2000; Marx *et al.*, 2003), we also evaluated MMP-9 activity, to better ascertain the PPAR γ involvement in RA.

Our results confirm that MMP-9 activity is potently increased in RA patients compare to healthy donor. Even more relevant is the relation between MMP-9 activity and DAS28 score. In fact, the MMP-9 activity of monocytes and MDM isolated from RA patients with DAS28 < 3.2, although increased as compared to healthy donors, did not reach statistical significance. On the contrary, MMP-9 activity in cells from patients with active disease (DAS28 > 3.2) was significantly up-regulated ($P < 0.01$ vs healthy donors), 8-fold in MDM and 6-fold in monocytes. We then evaluated the possible influence of the therapy on the LPS-induced activity of MMP-9 in monocytes/MDM isolated from healthy donors. The endogenous PPAR γ agonist, 15d-PGJ, inhibits by 60-70% MMP-9 activity. MTX and MP, evaluated *in vitro* at conceivable therapeutic concentrations (1 μ M and 180 ng/ml, respectively), also inhibit LPS-induced MMP-9 activity: MTX demonstrates the same inhibitory effect as 15d-PGJ, whereas, in human monocytes, MP exerts a significantly lower inhibition (about 25%). In our opinion, this can be largely explained by the short period (1 hr pre-incubation with drugs + 6 hr LPS challenge) of the experiment and does not imply a reduced clinical effect of the corticosteroid.

All together, the key findings of enhanced PPAR γ expression and concomitant reduction in MMP-9 activity in cells from RA patients with a less severe disease, suggest PPAR γ expression in monocyte/macrophages as a possible biomarker of disease activity and, consequently, successful RA therapy. On this regard, a recent small clinical trial in diabetic RA patients showed that the concomitant use of pioglitazone (30 mg daily) and methotrexate (15 mg/week) for 3 months, besides lowering blood glucose levels, significantly improved many RA markers, including swollen joint count, tender joint count and DAS28 score (Shahin *et al.*, 2011). However, it must be reminded that long-term use of TZDs (rosiglitazone, withdrawn from market in 2010, and pioglitazone) increases fracture rates among diabetic patients (Kahn *et al.*, 2008; Bodmer *et al.*, 2009), possibly due to PPAR γ ability to suppress osteoblastogenesis while activating osteoclastogenesis (Wei *et al.*, 2010). Although diabetes could represent by itself an independent risk factor for osteoarthritis (Berenbaum, 2011), the enhanced fracture risk needs to be taken into serious account when programming long-term treatment with TZDs, especially in women. Moreover, it has to be considered that, in animal models, higher dosages of TZDs are required to treat experimental arthritis than to restore insulin sensitivity (Koufany *et al.*, 2008).

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PUBLICATIONS

- Amoruso A, Bardelli C, Fresu LG, Poletti E, **Palma A**, Canova DF, Zeng HW, Ongini E, Brunelleschi S. The nitric oxide-donating pravastatin, NCX 6550, inhibits cytokine release and NF-kappaB activation while enhancing PPARgamma expression in human monocyte/macrophages. *Pharmacol Res* 62: 391-399 (2010).
- Amoruso A, Bardelli C, Fresu LG, **Palma A**, Vidali M, Ferrero V, Ribichini F, Vassanelli C, Brunelleschi S. Enhanced peroxisome proliferator-activated receptor-gamma expression in monocyte/macrophages from coronary artery disease patients and possible gender differences. *J Pharmacol Exp Ther*. 2009 Nov;331(2):531-8

CONGRESS PARTECIPATION

A Palma, PP Sainaghi, LG Fresu, GC Avanzi & S Brunelleschi: Expression of PPAR γ in monocyte and monocyte-derived macrophage (MDM) in patients with Rheumatoid Arthritis
35th National Congress of "Italian Society of Pharmacology" – **Bologna** (September, 14-17, 2011):
P10/8 digital abstract book

SEMINARS (Dept. Medical Science 2010-2011)

- ***Biom mineralization and preparation of biomimetic nanoapatites***, Dr. J.G. Morales
11/11/10
- ***Le cellule NK: dalla biologia alla terapia di leucemie ad alto rischio***, Prof. L. Moretta
29/11/10
- ***Linfomi cutanei primitivi***, Prof. E. Berti
02/03/11
- ***Ferritins: ancient proteins with novel unexpected features***, Dr.ssa S. Levi
12/04/11
- ***Metabolic syndrome: the gastroenterologist point of view***, Prof.ssa E. Bugianesi
13/04/11
- ***Liver fibrosis in children affected by NASH: how to investigate that***, Prof. V. Nobili
29/04/11
- ***Reverse vaccination in autoimmune diseases***, Prof. G. Filaci
03/05/11
- ***Innate immunity and the pathogenicity of inhaled microbial particles***, Prof. H. Wolff
09/05/11

- ***Farmacologia dell'aterosclerosi***, Prof. A. Corsini
13/05/11
- ***Ion torrent technology for massive parallel sequencing***, Dott. A. Di Nicola 23/05/11
- ***Iron management in the hepcidin Era***, Dr. S.R. Ellis
25/05/11
- ***Molecular pathogenesis and biomarkers of Parkinson's disease***, Prof. M. Fasano
17/06/11
- ***Importance of pathobiology in rheumatoid arthritis***, Prof. Pitzalis
24/06/11
- ***Genetics and clinical phenotypes of Frontotemporal lobar degeneration***, Dr. J. Rohrer
24/06/11
- ***Role of PET imaging in early drug development***, Dr.ssa R. Sharma
29/06/11
- ***Hypoxia, angiogenesis and liver fibrogenesis***, Prof. M. Parola
01/07/11

OTHER ACTIVITIES:

- Frontal lessons with Prof Albano, Dott.sa Corrado and Dott. Comi
- Journal Club (Dept. Medical Sciences)