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Peroxisome Proliferator-Activated Receptor-γ (PPARγ) expression in human monocytes and monocyte-derived macrophages: possible gender differences and drug response in Coronary Artery Disease (CAD) and Rheumatoid Arthritis (RA)

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ABBREVIATIONS:

CAD: Coronary Artery Disease; CIA: collagen-induced arthritis; COX2: cyclooxygenase 2; CRP: C reactive protein; CVD: Cardiovascular diseases; CYP: Cytochrome P; DAS28: 28-joint Disease Activity Score-28; DMARDs: disease-modifying anti-rheumatic drugs; ELAM: endothelial–leukocyte adhesion molecule; ESR: erythrocyte sedimentation rate; GC: glucocorticoids; GD: gender difference; GRF: Glomerular Filtration Rate; HCQ: hydroxycloroquine; HDLs: High-Density Lipoprotein; HMG-CoA: 3-hydroxy-3-methylglutaryl-coenzyme A reductas; HRT: hormone replacement therapy; ICAM-1: intercellular adhesion molecules; IFN-γ: interferon-γ; IL-1β: Interleukin-1β; LDL: Low Density Lipoprotein; LEF: leflunomide; LPS: lipopolysaccharide; MCP-1: monocyte chemotactic protein-1; MMPs: metalloproteinases; MP: methylprednisolone; MTX: methotrexate; NF-κB: nuclear factor- kappa B; NK: natural killer; NO: Nitric Oxide; NO^{2-}: Nitrite; NOS: NO synthase; NO^{2-}FA: Nitro derivatives of unsaturated fatty acids; PPARs: Peroxisome Proliferator-Activated Receptor; RA: Rheumatoid Arthritis; ROS: reactive oxygen species; RXRα: 9-cis retinoic acid receptor; SRA: scavenger receptor; SSZ: sulfasalazine; TF: Tissue Factor; TGF-β: Transforming Growth Factor β; TNF-α: Tumor Necrosis Factor-alpha; TZDs: Thiazolidinediones; VCAM-1: vascular adhesion molecule-1; VEGF: vascular endothelial growth factor; T2DM: diabetes mellitus type 2; 15-D-PGJ2: 15-deoxy$\Delta^{12,14}$ prostaglandin J_2.

INTRODUCTION

GENDER DIFFERENCES (GD)

The World Health Organization (WHO) supports the concept that the word *"gender"* is used to describe the characteristics, roles and responsibilities of women and men, boys and girls, which are socially constructed. Gender is related to how we are perceived and expected to think and act as women and men because of the way society is organized, not because of our biological differences. However no one should be sick or die because of gender inequality.

Women and men differ in terms of biological make-up, power, status, norms and roles in society. WHO Member States and international agreements stress that these differences must be acknowledged analysed and addressed through gender analysis and actions. Without due attention to gender equality, health services, programmes, laws and policies will have limited effects. Women and men will not achieve their full health potential over the life-course.

But what does it mean by "sex" and "gender"?

Sometimes it is hard to understand exactly what is meant by the term *"gender"*, and how it differs from the closely related term *"sex".*

"Sex" refers to the biological and physiological characteristics that define men and women.

"Gender" refers to the socially constructed roles, behaviours, activities, and attributes that a given society considers appropriate for men and women.

Aspects of sex will not vary substantially between different human societies, while aspects of gender may vary greatly.

Why gender and health?

The distinct roles and behaviours of men and women in a given culture, dictated by that culture's gender norms and values, give rise to gender differences. Not all such differences between men and women imply inequity - for example, the fact that in many western societies men generally wears trousers while women often wear skirts and dresses is a gender difference (GD) which does not, in itself, favour either group. Gender norms and values, however, also give rise to gender inequalities - that is, differences between men and women, which systematically empower one group to the detriment of the other. The fact that, throughout the world, women on average have lower cash incomes than men is an example of a gender inequality.

The gender bias in health care research and clinical practice was documented in 1985 when the US Public Health Service Task Force issued its report on women's health issues. But the knowledge gap was not addressed in any comprehensive way until 1990 when the National Institutes of Health (NIH) established the Office for Research on Women's Health. As part of this new policy directive, in 1993 the NIH developed the women's health initiative (WHI) a 20-year study to examine the major causes of death and disability among 163.000 postmenopausal women (Roberts, 1990; Roberts, 1992; Bird et al., 1999). Until 1993, these gendered practices were

complemented by rigid protectionist policies of the Federal Food and Drug Administration, which were intended to prevent the abuse of women as research subjects and to reduce the risk of foetal exposure to experimental treatments (Mastroianni et al., 1994). However, it is not valid to assume that treatments developed by studying men are directly generalizable to women. Furthermore, single sex studies fail to provide a complete picture of the similarities in men's and women's health and morbidity. Exclusion of women from these studies lead to a lack of information on the effectiveness of the treatments in women and of their risk of iatrogenic and other side effects (Hamilton, 1995; Bird, 1999). The inclusion of women as subjects in clinical research provides additional information not only on women's health but also on possible interventions that would benefit men's health as well. The advancement of human health and health-related knowledge requires research which includes both sexes and leads to a more integrated understanding of diseases and health problems that affect both men and women (Bird, 1999). Up to 5% of all hospital admissions and up to 7000 deaths annually in the United States are the results of adverse drug reactions (ADRs) (del Carmen Carrasco-Portugal & Flores-Murrieta, 2011). Identifying those factors that may predispose to ADRs is essential for risk management. Amongst the known risk factors for ADRs are increasing age, polypharmacy, liver and renal disease as well as being female. In fact, females have been shown to have major risk factor for clinically relevant ADRs with a 1.5 to 1.7-fold compared to male patients (Kando et al., 1995).

However, the reasons for this increased risk in female patients are not entirely clear but include gender related differences in pharmacokinetics, pharmacodynamics, immunological and hormonal factors**,** as well as differences in the use of medications by women compared with men. One of the first medications to be gender analyzed for pharmacokinetic differences was antipyrine in 1971. This drug is eliminated entirely by hepatic metabolism, and the study showed that the half-life of antipyrine was shorter in women (Berg et al; 1999). The next drug to be analyzed was acetaminophen, its clearance resulting faster in men than in women (del Carmen Carrasco-Portugal M & Flores-Murrieta, 2011).

Gender-related variations in pharmacokinetics have been frequently considered relevant determinants for the clinical effectiveness of therapeutic agents.

Differences in the four major determinants of pharmacokinetic variability – bioavailability, distribution, metabolism and elimination – are theorized to stem from variations in different factors between the sexes such as body weight, plasma volume, gastric emptying time, plasma protein levels, Cytochrome P (CYP) activity, drug transporter function and clearance activity.

Changes in bioavailability will depend on the route of drug administration and differences in site of absorption. In fact for a drugs assumed by the oral route, gastrointestinal motility has been shown to be affected by sex hormones (Huston et al., 1989; Singer et al., 1991), with the transit time reported slower in females than in males (Sadik et al., 2003; Mearadji et al., 2001).

Gastrointestinal enzymes responsible for drug metabolism also differ by sex. For example, GDs in gastric alcohol dehydrogenase activity have been described with higher levels occurring in males compared to females, so that more elevated alcohol concentrations may be found in women than in men, also following an equivalent drink (Frezza et al., 1990).

The distribution of a drug is influenced by numerous factors such as body mass index and body composition, plasma volume and the extent of plasma protein binding of the drug. Since protein binding affects drug distribution volume, GDs in the binding might in theory lead to different pharmacokinetics for some compounds. Concentrations of albumin, the major plasma protein involved in reversible drug binding, do not consistently vary with the gender, whereas (as demonstrated in animals) α-1 acid glycoprotein and α–globulins have been reported variation in oestrogens (Succari et al., 1990; Tuck et al., 1997; Brinkman-Van der Linden et al., 1996). The clinical relevance of these observations has not been fully defined in humans yet, even if their practical impact has been questioned by investigations that failed to observe GDs in free fractions of highly bound drugs (Rowland et al., 1995).

It is commonly believed that the most prominent factor in adapting medication dosages between the sexes is to tailor for body size. Therefore, at steady state**,** some of the pharmacokinetic sex differences, due to different body weight and composition, can be corrected by normalizing the dose for body weight or surface (Wilson et al., 1984), and such corrections are particularly proper when drugs with a narrow therapeutic index are administered. However, it is not obvious that adjustments for body size automatically optimize the therapy, since there are differences in drug metabolism that remain also after these corrections have been performed.

Discrepancies in drug metabolism between sexes are currently thought to play a leading role in determining GDs in pharmacokinetic parameters.

The CYP450 superfamily (enzyme involved in phase I metabolism) is one of the major drug metabolizing systems in humans and significant GDs in some key CYP450 subtypes have been demonstrated. For example the activity of CYP3A4, responsible for the metabolism of about 50% of the current used drugs (Zhou et al., 2005), is higher in women than in men, whereas CYP1A2 and CYP2D6 activity is higher in men.

Metabolism phase II reactions involve glucuronidation, sulfation, acetylation or methylation of the parent drug or its phase I metabolites to generate polar conjugates for renal excretion; some findings support the occurrence of GDs in the enzyme involved in phase II metabolism. For example**,** a gender effect has been demonstrated for both paracetamol and diflunisal glucuronidation, being higher in men than in women (Bock et al., 1994; Court et al., 2001), whereas glucuronidation of zidovudine was found as not gender-dependent (Pacifici et al., 1996).

Renal excretion of compounds that are non-actively secreted or reabsorbed is determined by the Glomerular Filtration Rate (GRF) that is known to be proportional to body weight. Since average GRF is higher in men than women, it has been demonstrated that a possible GD disappear after

adjustment for weight. However, it is noteworthy that population kinetic analysis of methotrexate reported a gender effect on kidney excretion even after normalization for body weight (Godfrey et al., 1998), suggesting that, in some circumstances, sex-adjusted dosages are required, mainly for renally eliminated compounds with narrow therapeutic index.

Moreover, medications actively secreted by the kidney have been found to display more pronounced GDs. For amantadine, an organic cation with renal clearance, a significantly higher excretion has been observed in men (Guadry et al., 1993). These findings agree with studies carried out in rats, showing that sex hormone differences are responsible for gender disparities in kidney clearance for organic ions (Reyes et al., 1998; Kudo et al., 2001). However, additional investigations on sex differences in renal excretion are needed to better understand the real contribution of this factor in humans.

All these observations explain GD in drug pharmacokinetic, but important GD has been demonstrated in some inflammatory diseases such as Coronary Artery Disease (CAD) and Rheumatoid Arthritis (RA).

CORONARY ARTERY DISEASE (CAD)

Cardiovascular diseases (CVD) are the leading cause of mortality and admission in hospital for women, accounting for a third of all deaths of women worldwide and half of all deaths of women over 50 years of age in developing countries (Leuzzi et al., 2010).

One of the most important risk factor in atherosclerosis is high plasma concentration of cholesterol, particularly in the form of LDL (Low Density Lipoprotein). As elevated circulating lipids have long been established as the principal risk factor for the development of atherosclerosis, it was originally thought to be a process mainly consisting of the accumulation of lipids within the artery wall. However, it is now known to be a much more complex and multifaceted disease**;** in fact atherosclerosis, as a major cause of mortality in diabetes mellitus type 2 (T2DM) patients, involves many risk factors and complex changes of the vascular components in its progression. These include perturbation/injury of the endothelium, adhesion to and transmigration of monocytes/macrophages into the intima, foam cell formation, and the migration and proliferation of medial smooth muscle cells (SMCs).

Endothelial activation is the first step in atherogenesis and it's characterized by the increased expression of pro-inflammatory adhesion molecules and chemokines such as intercellular adhesion molecules (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin and monocyte chemotactic protein-1 (MCP-1). Therefore, all these mediators attract monocytes to the arterial intima, where they differentiate to macrophage undergoing to a series of changes that lead ultimately to foam cell formation. In fact, monocytes increase expression of scavenger receptors, such as the scavenger receptor A (SRA) and CD36, and then internalize modified lipoproteins, such that cholesteryl esters accumulate into cytoplasmic droplets (Libby, 2002).

The foam cells secrete pro-inflammatory cytokines, e.g., Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β) and IL-6**,** that amplify the local inflammatory response in the lesion, as well as reactive oxygen species (ROS).

There has been great interest in macrophage heterogeneity in atherosclerotic lesions, particularly regarding macrophages involved in pro-inflammatory processes called (M1) versus those involved in resolution and repair called (M2), but a clear picture has not yet emerged from these studies (Johnson and Newby, 2009). Much of the theory in this area has been driven by in vitro studies exploring gene/protein expression patterns and functional attributes of monocytes or macrophages subjected to various treatments, including growth/differentiation factors; cytokines derived from type 1 versus type 2 helper T cells; transcription factors, notably Peroxisome Proliferator-Activated Receptor (PPARs)**,** and even atherogenic lipoproteins and lipids (Johnson and Newby, 2009; Kadl et al., 2010). A chronic inflammation and cell infiltration causes progressive enlargement of the plaque, which protrudes into the arterial lumen blocking normal blood flow. Eventually, the plaque

ruptures, due to degradation by macrophage-induced matrix metalloproteinases (MMPs) and hydrolytic enzymes, result in thrombus formation and tissue infarction (Libby et al., 1996; Ross et al., 1999).

GENDER DIFFERENCES IN CORONARY ARTERY DISEASE (CAD)

Men and women differ in some aspects of cardiovascular system in terms of anatomy, physiology and ageing (Legato, 2004). In fact, women have a smaller heart, higher resting heart rate (three to five beats higher than in men) and the cardiac cycle length is prolonged during menstruation (Jochmann et al., 2005). GDs have been demonstrated for the coronary left main and left anterior descending arteries that are smaller in women, independent of their body size (Sheifer et al., 2000). Simply by virtue of their smaller diameter vessels, women may be more prone to coronary occlusion than men. Moreover, there is initial intriguing epidemiological evidence that the inflammatory process associated with plaque development may differ in women and men. Interestingly, C reactive protein (CRP) appears to be enhanced in the presence of increased oestrogen levels, as evidenced by clinical trials of hormone replacement therapy (HRT) (Ridker et al., 1999; Cushman et al., 1999). Together, these findings suggest that oestrogens may be involved in altering plaque stability, via inflammatory mechanisms.

Recent data show that a greater incidence of plaque erosion rather than plaque rupture occurs in women compared to men (Rossi et al., 2000).

Traditional risk factors differ between men and women. Although in the past the difference was attributed to the presence of oestrogens in the premenopausal period, one of the most significant differences to be considered is diabetes mellitus, which is associated with a three-to seven-fold increased coronary artery disease (CAD) risk in women, compared to a two-to three-fold elevation in CAD risk in men. Although younger-aged diabetic women (i.e., <45 years) have an equally low prevalence of atherosclerosis (Hoff et al., 2003), numerous studies have reported a significantly higher cardiovascular mortality for diabetic women when compared with diabetic men (Barrett-Connor et al 1991; Raggi et al., 2004). The latter results is probably related to the fact that premenopausal diabetes eliminates the ‘female advantage’ of a predominately lower CAD prevalence and outcome risk that exists for the female population in general (Barrett-Connor et al 1991). Notably, the age-adjusted prevalence of CAD is nearly twofold higher in diabetic versus non-diabetic women (Shaw et al., 2006).

The reason for this GD is not known (Mosca et al., 2002). In women older than 65 years, dyslipidemia may also put women at a greater risk than men. High levels of triglycerides and low levels of high-density lipoproteins (HDLs) are strongly correlated with CAD in women (Elsaesser et al., 2004).

Population studies have noted that total cholesterol measurements are higher in men until the fifth decade of life but, beyond this age, women have greater values (Shaw et al., 2006). Furthermore, GDs in HDL values diminish with advancing age. Women typically experience a relatively mild decline in HDL cholesterol at the time of menopause (Association A.H. Heart Disease and Stroke

Statistics: 2004; Lemer et al., 1986). In a comprehensive review of 25 population studies, Manolio et al. (1992) reported that HDL cholesterol inversely predicted CAD in younger women and men as well as older (65 years) women. Hypertriglyceridemia is also a more potent independent risk factor for CAD in women when compared with men (Shaw et al., 2006) as described by a meta-analysis (Hocanson et al., 1996).

Moreover one of the most significant differences to be considered is diabetes mellitus, which is associated with a three- to seven-fold increased CAD risk in women, compared to a two- to three-fold in men (Mosca et al., 2002).

It is known that blood pressure is typically lower in premenopausal women than men; however, post menopause increases it to levels similar to or higher than age-matched men (Coylewright et al., 2008; Reckelhoff et al., 2010). Approximately 75% of women over 60 years of age are hypertensive (Barton et al., 2009). Comparison of cohorts from the National Health and Nutrition Examination Survey (NHANES) III (1988–1994) with NHANES IV (1999–2002) showed that over the time period from 1994 to 2002, the percentage of hypertensive patients decreased among men but increased among women (Kim et al., 2006). Indeed, the percentage of individuals with uncontrolled hypertension was also higher in women, despite the fact that a higher percentage of women than men reported having their blood pressure measured within the previous 6 months (Kim et al., 2006). It is not clear why hypertension is less well controlled in women than men despite more frequent blood pressure monitoring, but this observation suggests the mechanism responsible may differ in men and women. Premenopausal women also have a much lower incidence and prevalence of heart and renal disease compared to men of the same age (Rosano et al., 2007; Silbierg et al., 2008; Wake et al., 2009; Reckelhoff et al., 2010). This sex difference in favour of women also gradually disappears after menopause; indeed cardiovascular risk becomes even higher in older women (Kim et al., 2009; Anderson et al., 2007). The recent Nurse's Health Study (Parker et al., 2009) and the WISE Study (Bairey Merz et al., 2003; Rivera et al., 2009) have demonstrated that early menopause in young women due to ovarian dysfunction or bilateral oophorectomy is associated with increased risk of CAD compared to women with normal endogenous oestrogen levels. In animal models of CAD, females exhibited a lower mortality, less vascular injury, better preserved cardiovascular function and slower progression to decompensated heart failure, the differences being narrowed or abolished by ovariectomy or deficiency of endogenous oestrogen (Wang et al., 2007; Javeshghani et al., 2009; Dent et al., 2010; Lagranha et al., 2010). Endogenous oestrogen may have a cardioprotective effect in men as well. In men, significant amounts of oestrogen can be produced via conversion of C19 androgenic steroids to 17β- estradiol by the enzyme aromatase. Therefore has been demonstrated that**,** in healthy young men**,** inhibition of aromatase lowers plasma 17β-estradiol, and is associated with decreased flow-mediated dilatation of the brachial artery (Lew et al., 2003). Similarly, aromatase knockout mice demonstrated impaired endothelial function (Kimura et al., 2003). Supplemental

oestrogen in men attenuated volume overload-induced structural and functional remodelling (Gardner et al., 2010) and slowed the progression of left ventricular dysfunction to heart failure post-myocardial infarction (MI) (Cavasin et al., 2006). Therefore taken together, the evidence suggests that the differences in cardioprotection between men and women may be attributable largely to the protective effect of oestrogen in women.

RHEUMATOID ARTHRITIS (RA)

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 pathway, 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).

Different 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 IL-6, IL-1 TNF-α play important roles in the pathogenesis of RA. These cytokines are abundant in synovial tissues and fluid from RA patients and their over-expression promotes 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 have been reported to stimulate synovial cells to release vascular endothelial growth factor (VEGF) which has an 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 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 inducers of MMPs enzyme (Dayer et al., 1985; McCachren et al., 1990; Gravallese et al., 1991; Martel-Pelletier et al., 1994). (Fig.1)

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, sulfasalazine, hydroxycloroquine and leflunomide, 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).

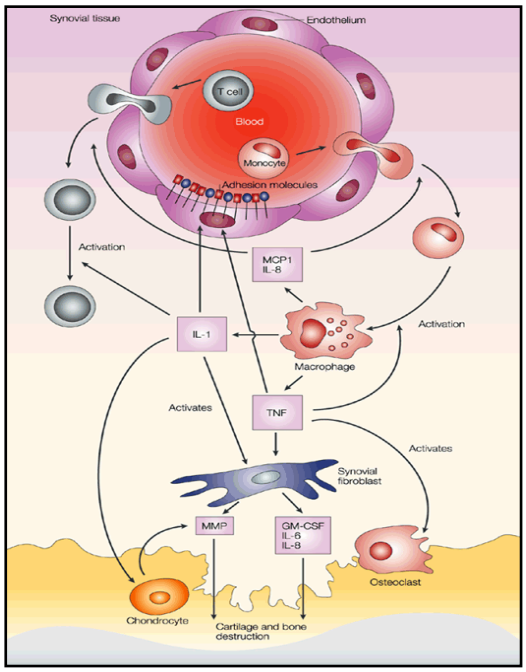

Fig.1 Monocytes are attracted to the rheumatoid arthritis (RA) joint, where they differentiate into macrophages and become activated. They secrete tumour-necrosis factor (TNF) and interleukin-1 (IL-1). TNF increases the expression of adhesion molecules on endothelial cells, which recruit more cells to the joint. Chemokines, such as monocyte chemotactic protein 1 (MCP1) and IL-8, are also secreted by macrophages and attract more cells into the joint. IL-1 and TNF induce synovial fibroblasts to express cytokines (such as IL-6), chemokines (such as IL-8), growth factors (such as granulocyte–macrophage colony-stimulating factor; GM-CSF) and matrix metalloproteinases (MMPs), which contribute to cartilage and bone destruction. TNF contributes to osteoclast activation and differentiation. In addition, IL-1 mediates cartilage degradation directly by inducing the expression of MMPs by chondrocytes. Nature Reviews Immunology 2, 527-535 (July 2002)

GENDER DIFFERENCES IN RHEUMATOID ARTHRITIS (RA)

Most human autoimmune diseases such as RA, systemic lupus erythematosus (SLE), multiple sclerosis (MS) and autoimmune thyroid disease, have an increased incidence and prevalence in females, but a few others such as autoimmune diabetes, the Guillain Barré Syndrome (GBS) and psoriasis are increased in males (McCombe et al., 2009).

Several findings indicate the involvement of sex hormones in RA. For example, the female to male incidence ratio is 5:1 before 50 years of age and 2:1 for patients with a later onset (McCombe et al., 2009); the peak incidence in women coincides with menopause (Goemaere et al., 1990). It has been shown that oestrogens can affect the disease course of RA in humans (Ostensen et al., 1983) and in animal models (Holmdahl et al., 1986; Jansson and Holmdahl, 1989; Yamasaki et al., 2001), as already demonstrated in CAD.

Mice subjected to ovariectomy (OVX) have decreased levels of oestrogens and display higher frequency and increased severity of collagen-induced arthritis (CIA), as compared to OVX mice treated with oestrogen or sham-operated mice with intact levels of oestrogen (Holmdahl et al., 1986;).

Oestrogens are also main regulators of skeletal growth and maintenance, as demonstrated in both experimental and human studies (Sinigaglia et al., 2000; Vidal et al., 2000; Riggs et al., 2002; Forsblad-D'Elia et al., 2003b; Vanderschueren et al., 2004).

Previous studies have demonstrated that oestrogen deprivation, such as after OVX in animal models and after menopause in women, reduces trabecular bone mineral density (BMD) as well as cortical BMD, while oestrogen substitution restores both bone compartments (Recker et al., 1999; Turner, 1999; Windahl et al., 1999). There are several skeletal manifestations in RA, including joint erosions and both periarticular and generalized bone loss, due to excess bone resorption by osteoclasts (Hayward and Fiedler-Nagy, 1987).

In many RA women, the disease activity diminishes during pregnancy when the levels of female sex hormones are high (Ostensen et al., 1983; Barrett et al., 1999). In contrast, the disease is often aggravated after delivery (Ostensen et al., 1983; Barrett et al., 1999, 2000).

The frequency of generalized osteoporosis in postmenopausal patients with RA has been reported to be approximately 50% (ForsbladD'Elia et al., 2003a).

Furthermore oestrogen affects cells of both the innate and adaptive immune system (Straub, 2007). For example it inhibits neutrophil function and adhesion to endothelium, and the number of neutrophils in peripheral blood (Buyon et al., 1984; Josefsson et al., 1992; Geraldes et al., 2006; Bekesi et al., 2007). Also natural killer (NK) cell activity is decreased (Nilsson and Carlsten, 1994).

Oestrogen induces apoptosis in human monocytes, and also modulates the pro-inflammatory cytokine release from activated monocytes and macrophages (Mor et al., 2003; Kramer et al.,

2004). Serum levels of IL-1, IL-6 and TNF-α are increased after menopause and decreased by HRT (Pfeilschifter et al., 2002; Rachon et al., 2002).

CORONARY ARTHERY DISEASE AND RHEUMATOID ARTHRITIS

The increase in mortality in RA is predominantly due to accelerated coronary artery and cerebrovascular atherosclerosis, as well as to other cardiovascular (CV) complications including heart failure (Rise et al., 2001; Nicola et al., 2006).

In RA patients of both sexes with disease onset in the 1980s and 1990s, CAD mortality was significantly increased. However, standardized admission rates for CV complications were not increased in these patients, suggesting either that vascular disease in RA has a higher case fatality than in the general population or that it often goes unrecognized before the fatal event (Goodson et al., 2005). Patients with RA also have substantially increased 30-day mortality from all causes and from CAD following a first acute vascular event (Van Doornum et al., 2006), as well as more frequent recurrent ischemic events after acute coronary syndrome (Douglas et al., 2006). RA extra-articular manifestations, usually related to uncontrolled inflammation, are also associated with increased CAD mortality (Van Doornum et al., 2002), suggesting that processes intrinsic to RA pathogenesis play important roles in CAD damage and its clinical consequences.

Previous studies have suggested that traditional CAD risk factors do not fully account for the increased propensity to vascular complications in RA (del Rincon et al., 2001) and that immune dysregulation, inflammation, and metabolic disturbances observed in RA could play an important role in accelerated atherogenesis and mortality. Indeed, histological examination of coronary arteries in RA has revealed less atherosclerosis but greater evidence of inflammation and instability (Aubry et al., 2007). Recent evidence indicates that there is a close temporal correlation between inflammation and morphologic features of rapidly progressive carotid atherosclerosis, which suggests that elevations in inflammatory biomarkers might help in predicting the presence of atherosclerosis (Schillinger et al., 2005) (Fig.2).

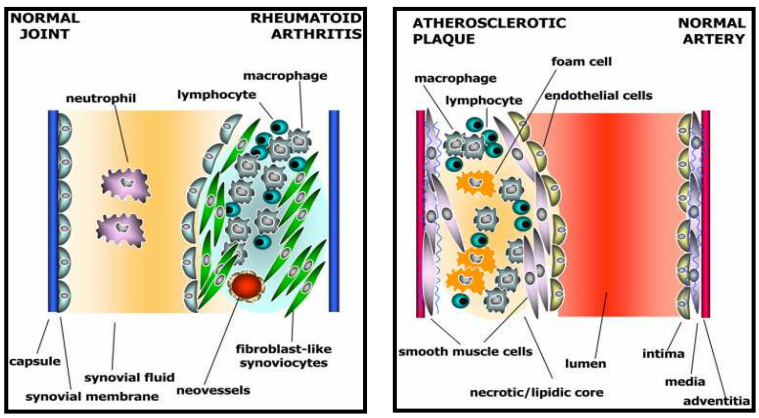

Fig.2 Similarities between the atherosclerotic plaque and rheumatoid arthritis joint. The atherosclerotic plaque has many features in common with rheumatoid arthritic synovium. First, in both diseases, blood-borne mononuclear cells are recruited to sites that are devoid of any significant inflammation in physiological conditions. Second, upregulation of cytokines and matrix-degrading enzymes is central to the pathogenesis of both diseases. Third, both in rheumatoid arthritis and atherosclerosis, immune cells do not target resident cells in the same way that diabetogenic T cells directly destroy pancreatic islets. Instead, immune cells begin complex interactions with the resident cell types, which proliferate, change their properties and phenotype, and contribute to the inflammatory process and tissue destruction. Full *et al. Arthritis Research & Therapy* 2009 **11**:217

Markers of systemic inflammation confer a statistically significant additional risk for CV death among patients with RA, even after controlling for traditional CV risk factors and comorbidities (Solomon et al., 2004; Maradit-Kremers et al., 2005). Increased levels of pro-inflammatory mediators including TNF-α, IL-6, IL-17 and others could be detrimental to the endothelium and myocardium and promote insulin resistance. Levels of these cytokines are increased in RA (Svenson et al., 1988; Hurlimann et al., 2002; Sattar et al., 2003). The C-reactive protein (CRP) concentration at baseline is an important predictor of subsequent death from CVD in patients with new onset inflammatory polyarthritis, and is independent of other factors of disease severity (Goodson et al., 2005). High levels of CRP also correlate with carotid intima media thickness (Gonzalez-Gay et al., 2005). High sensitivity CRP and lower glomerular filtration rate have been independently predictive of endothelial dysfunction in RA (Dessein et al., 2005).

In a recent study comparing patients with RA and controls, TNF-α and IL-6 were significantly associated with the severity of coronary artery calcification in RA, independent of Framingham risk score (Rho et al., 2009). Enhanced arterial stiffness in RA correlates with high CRP and IL-6 levels (Roman et al., 2005). This indicates that an enhanced inflammatory process may promote the development of heart dysfunction in inflammatory arthritis (Maradit-Kremers et al., 2007). The magnitude and chronicity of the inflammatory response, as measured by circulating levels of inflammatory markers, correlates with carotid atherosclerosis development in RA (Gonzalez-Gay et al., 2005). Levels of adhesion molecules linked to vascular damage including VCAM-1, ICAM-1, and endothelial–leukocyte adhesion molecule (ELAM) were higher in RA. VCAM-1 levels have been associated with carotid atherosclerosis in RA (Dessein et al., 2005).

While the exact role of IL-17 in premature vascular damage in RA remains to be determined, recent work indicates that this cytokine may play a role in atherosclerosis development in murine models of vascular disease (Xie et al., 2009; van Es et al., 2009) and elevated circulating levels of IL-17 have been reported in patients with acute coronary syndromes (Liang et al., 2009). Therefore IL-17 is produced concomitantly with IFN-γ by coronary artery-infiltrating T cells and these cytokines act synergistically to induce pro-inflammatory responses in vascular smooth muscle cells (Eid et al., 2009). IL-17 accelerates myocardial fibrosis in animal models of heart injury (Feng et al., 2009). However, there is recent evidence that IL-17 may also play a regulatory role in atherosclerosis (Taleb et al., 2009) and future studies should determine whether this cytokine plays or not a pivotal role in vascular damage in RA.

A recent study indicated that prolonged exposure to various DMARDs, including methotrexate (MTX), leflunomide and sulfasalazine, was associated with a reduction of CAD risk in RA, and similar trends were observed with corticosteroid use (Naranjo et al., 2009). Further supporting a beneficial effect of MTX treatment in CAD prevention, this drug reduced the incidence of vascular disease in veterans with psoriasis or RA (Prodanovich et al., 2005), low to moderate cumulative

doses being more beneficial than higher doses. In addition, a combination of MTX and folic acid led to a further reduction in the incidence of CAD, suggesting that the latter did not decrease the efficacy of MTX. Furthermore, MTX use was associated with a significantly lower risk for CV events in RA patients compared with patients who had never used DMARDs.

MTX use has also been associated with a decreased incidence of metabolic syndrome, while corticosteroids or other DMARDs did not show a protective effect (Toms et al., 2009). Adding additional DMARDs**,** such as sulfasalazine and hydroxychloroquine**,** appears to provide additional CV protection (van Halm et al., 2006). In a Canadian study, DMARDs use was associated with a reduction in myocardial infarction risk in patients with RA, while corticosteroids showed an increased risk and coxibs did not change risk (Suissa et al., 2006). In a recent cross-sectional analysis, drugs used to treat RA did not have major adverse effects on CV risk factors and use of antimalarials was actually associated with beneficial lipid profiles and lower blood pressure (Rho et al., 2009). As a potential antiatherogenic mechanism of MTX, Reiss et al. (2008) have shown that MTX promotes reverse cholesterol transport and limits foam cell formation in macrophages through adenosine A2A receptor activation (Reiss et al., 2008).

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTORγ (PPARγ) IN CAD AND RA

Peroxisome proliferators-activated receptors (PPARs) belong to a superfamily of the nuclear hormone receptors that consists of 48 members. PPARs have 3 isoforms, α, β/δ, and γ (Kliewer et al., 2001). PPARα is mainly expressed in liver and involved in fatty acid oxidation (Isseman et al., 1990); PPARβ/δ is ubiquitously expressed with a higher level in gut, epidermis, placenta, skeletal muscles and adipose tissue (Michalik et al., 2003). PPAR γ is predominantly expressed in adipose tissues and plays a role in adipogenesis and glucose homeostasis; it is also expressed in cardiovascular tissues, such as vascular endothelial cells (ECs), SMCs, macrophages and cardiomyocytes (Spiegelman et al., 1997, 1998).

Differential promoter usage and alternative splicing of the gene generate 4 mRNA isoforms: PPAR γ 1, γ 2, γ 3 and γ 4. While the latter 2 transcripts encode the same protein as PPAR γ1, the PPAR γ2 protein has an additional 28 amino acid residues at its N-terminus and is exclusively expressed in adipose tissue (Lehrke et al., 2005). PPARs bind to specific DNA responsive elements as heterodimers with the 9-cis retinoic acid receptor (RXRα) (Fig.3). In the absence of the cognate ligands, PPAR:RXR heterodimers bind a number of co-repressors, including nuclear receptor co-repressor and the silencing mediator of retinoid and thyroid hormone receptor, to suppress the target genes (Nolte et al., 1998; Powell et al., 2007; Yu et al., 2007).

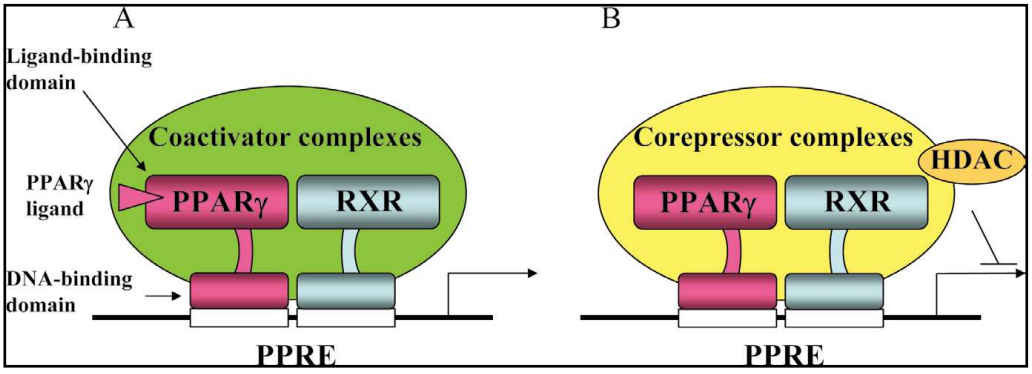

Fig.3 Transactivation and active repression. PPARγ functions as a heterodimer with RXR. (**A**) In the presence of ligand, PPARγ binds to coactivator complexes, resulting in the activation of target genes. (**B**) In the absence of ligand, PPARγ binds to the promoters of several target genes and associates with corepressor complexes, leading to active repression of target genes. HDAC, histone deacetylase; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR responsive element; RXR, retinoid X receptor (Takano et al., 2003).

Upon binding to their selective ligands, PPARs undergo a conformational change that facilitates the dissociation of the co-repressors and the recruitment of co-activators, leading to the transcriptional activation of the target genes. Following the first report that eicosanoid 15-

deoxy$\Delta^{12,14}$ prostaglandin J_2 (15-D-PGJ2) was an endogenous ligand for PPARγ, a number of naturally occurring fatty acid metabolites, were also found to activate PPARγ.

Nitro derivatives of unsaturated fatty acids (NO^{2-}FA) are endogenous products of nitric oxide (NO) and nitrite (NO^{2-})-mediated redox reactions that activate PPARγ at nanomolar concentrations (Schopfer et al., 2010). Thiazolidinediones (TZDs) or glitazones, including troglitazone (Rezulin, known as Noscal in Japan; never registered in Italy), rosiglitazone (Avandia) and pioglitazone (Actos), were discovered as selective ligands for PPARγ (Ibrahimi et al., 1994; Lehmann et al 1995).

PPARγ was first linked to atherosclerosis by the findings that it regulates lipid uptake and foam cell formation, and that oxidized LDL (oxLDL) are activators of the receptor. Activation of PPARγ by its ligands leads to up-regulation of CD36, suggesting the existence of a positive feedback loop and providing a possible explanation of how lipid laden macrophages are formed in atherosclerotic plaques (Nagy *et al*., 1998; Tontonoz *et al*., 1998).

Anyway, as described in Figure 4, anti-atherosclerotic effects of PPARγ have been extensively recognised (Fig.4).

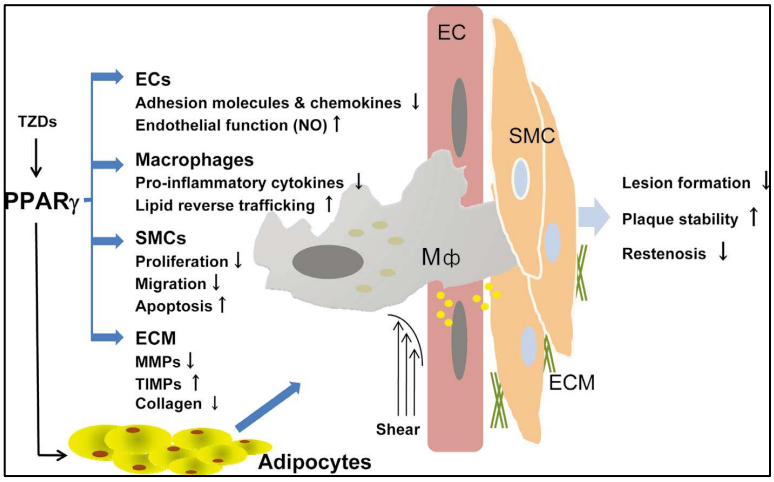

Fig.4 Anti-atherosclerotic effects of peroxisome proliferator-activated receptor-γ (PPARγ). In the vasculature, PPARγ is activated by both synthetic ligands, such as thiazolidinediones, and endogenous ligands under flow shear stress. In vitro and in vivo data using PPARγ -specific ligands or genetic manipulation have demonstrated that PPARγ suppresses the pro-inflammatory activation of monocytes/macrophages (Mφ) and endothelial cells (ECs), promotes the reverse trafficking of lipids, and modulates both the proliferation, migration, apoptosis of smooth muscle cells (SMCs) and the composition of extracellular matrix (ECM). Alternatively, PPARγ may also play an atheroprotective role through its metabolic effects. Overall, PPARγ activation reduces the formation and vulnerability of atherosclerotic plaques and attenuates neointimal hyperplasia and restenosis after arterial injury. (Wang et al., 2011)

Interestingly, cells derived from the inflamed joints of RA patients were found to express PPARγ: in macrophages the expression was at high levels, and in synovial cells, endothelial cells and fibroblasts at moderate levels. In the *in vitro* cultures of synoviocytes, both 15d-PGJ_2 and troglitazone were found to inhibit proliferation and induced apoptosis at higher doses (Kawahito et al., 2000). The role for PPARγ in the control of the inflammatory responses of macrophages, such as reducing the production of IL-1β, IL-6 and TNF-α that are key mediators in RA, suggests that

application of PPARγ 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_2 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 PPARγ is expressed at both mRNA and protein levels by major cell populations in joints; in these cells, 15d-PGJ_2 and TZD inhibit the transcriptional induction of genes that contribute to joint pathology, e.g., TNF-α, IL-1, gelatinase B, iNOS and MMP-13 (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).

Therefore, the potential anti-inflammatory properties of PPARγ ligands on RA activity have been investigated in several models of arthritis (Shahin et al., 2011). PPARγ ligand treatment was shown to reduce a wide variety of inflammatory markers in several animal models of osteoarthritis (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 PPARγ agonist, significantly inhibited spontaneous TNF-α release by RA synovial membrane cells, as well as lipopolysaccharide (LPS)-induced TNF-α release from human and murine monocytic cells. Inhibition of TNF-α in monocytes was mediated partially through a NF-κB-dependent pathway, as judged by sustained levels of IκBα in cytosolic extracts and a reduced level of LPS-induced NF-κB activity in nuclear extracts (Ishino et al., 2008; Sumariwalla et al., 2009).

STATINS AND NITRIC OXIDE (NO)

Nitric oxide (NO) was first discovered in 1772 by J. Priestly as a colourless gas. In 1980, Furchgott demonstrated that endothelial cells produce a factor capable of inducing vascular relaxation: endothelium-derived relaxing factor (EDRF) and in 1987, in two separate studies, Moncada et al. and Ignarro et al. showed that this factor was NO (Ignarro et al., 1987; Palmer et al., 1987), synthesized in cell from L-arginine by NO synthase (NOS) (Palmer et al., 1988). In 1992, the cover of the journal Science proclaimed NO "molecule of the year" (Koshland, 1992). In the same year, the importance of NO was recognized with the awarding of the Nobel Prize in Physiology and Medicine to Robert F. Furchgott, Louis J. Ignarro and Ferid Murad "for their discoveries concerning nitric oxide as a signalling molecule in the cardiovascular system".

Since 1993, NO has been assigned a key role in the pathogenesis of many diseases, septic shock hypertension and dementia (Moncada et al., 1993).

It is difficult to find a cardiovascular disease that is not associated with an alteration of NO homeostasis and the term "endothelial dysfunction" (originally coined in 1983 by Catravas) has now become synonymous of reduced biological activity of NO. This may reflect an absolute deficit of NO, reduced availability of biologically active NO or increased inactivation of NO.

A key aspect of NO regulatory role is the transcription of inducible genes such as iNOS and cyclooxygenase 2 (COX2). NO, which has a short half-life, has a stimulatory or inhibitory action depending of the concentration of NO released in the specific tissue that mimics the effects of both constitutive and inducible isoforms of NOS (Colasanti et al., 2000).

The pharmacological approach of cardiovascular medicine has been to replace or increase the levels of endogenous NO by administration of donor molecules.

Among the various NO donors, nitroglycerine and sodium nitroprusside are the drugs used for the longest time, even before the discovery of NO and its role in cardiovascular physio-pathology. Over the past twenty years, the interest in these molecules has grown significantly and has reached the development of different molecules of NO donors that offer additional advantages over conventional ones.

The redox form of NO that is released (i.e. the anion [N^-] nitroxil anion, the radical NO [$NO^\cdot$] Radical nitrosodium or [NO +]) defines the reactivity of NO donor to other biomolecules, the profile of the product and the bioactivity of the donor (Ignarro et al., 2002).

The so-called "direct NO donors" are agents that have both a functional group and nitrous nitroxil anion, that spontaneously release NOx. To this class belong the gas NO donor, sodium nitroprusside and sodium trioxodinitrate.

To the class of "donors requiring metabolism" belong the nitrovasodilatators that is esters of nitrates and nitrites, including nitroglycerine, amyl nitrite, isosorbide dinitrate, isosorbide

mononitrate, and nicorandil. All nitrate esters are prodrugs that require enzymatic metabolism to generate bioactive NO and are in use for many years in the treatment of cardiovascular diseases. Their primary action is vasodilatation mediated by activation of guanylate cyclase and direct inhibition of cationic non-specific channels on vascular smooth muscle cells (VSMCs). These agents represent the prototype of NO replacement therapy. The limitations of this class are well known and include potential hemodynamic side effects, drug tolerance, loss of selectivity and limited bioavailability. In any case, the prudent use of these drugs is an important aspect of the therapy for angina pectoris.

A new class of NO donors (the so called “bifunctional donor") is represented by agents that have been modified in their structure, by the addition of an ester or a nitrate or S-nitrosothiols, in order to obtain the beneficial effects of NO and minimize the effects of the native drug compound. An example is nitro-aspirin: NCX4016 and NCX4215.

NCX4016 (Wallace et al., 1997) is a stable compound that requires enzymatic hydrolysis to release NO, and the kinetics of this metabolic step leads to a sustained production of NO released at a constant rate from the site of metabolism (Del Soldato et al., 1999; Minuz et al., 2001). The biological activity of this molecule has been evaluated in different experimental models to characterize its anti-inflammatory and anti-thrombotic effects (Lechi et al., 1996; Lechi et al., 1996b; Wallace et al., 1999).

In addition to NO donors, there are other cardiovascular pharmacological agents that modulate the bioactivity of endogenous NO. These include i) ACE inhibitors, which, by increasing bradykinin, promote endothelial release of NO (Mombouli et al., 1999; Vanhoutten, 1998), ii) the dihydropyridine calcium antagonists that increase the availability of NO by a mechanism not yet clarified (Muraki et al., 2000), iii) Beta-blockers (Cleopas, 1998; Gao et al., 1991) and iiii) statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA). These are the most widely used class of drugs in the treatment of hypercholesterolemia, a condition associated with the development of atherosclerosis (Shepherd et al., 1995). Several clinical studies have demonstrated the efficacy of statins in reducing cholesterol levels, LDL-C and the incidence of coronary heart disease (Heart Protection Study Collaborative Group, 2002). In addition, the results of several studies have emphasized the therapeutic impact of the so-called "pleiotropic effects" of statins, involving endothelial cells, smooth muscle cells, monocytes-macrophages and T lymphocytes, which may be responsible for the beneficial effects of statins in atherosclerotic patients (Marz et al., 2003; Weitz-Schmidt, 2002). Regardless of the inhibition of cholesterol synthesis, statins seem to exert anti-inflammatory effects in endothelial cells probably mediated by NO and it also recalls the capacity of NO to inhibit the proliferation of vascular muscle cells (Weitz-Schmidt, 2002).

On these bases, new NO donor have been synthesized, combining the properties of statins with those of a compound capable to release NO slowly, in order to obtain the therapeutic benefits

especially for those diseases, like diabetes and atherosclerosis, in which an impairment of endothelial function induce a blockade of endogenous NO production and plays an important role in the disease progression.

In 2004 Ongini et al described the nitric ester derivatives of pravastatin, NCX 6550, and fluvastatin, NCX 6553, which not only present the effect of native statin, but also possess the ability to release functional NO, which in turn produces additional pharmacological effects such as increased anti-inflammatory activity.

The nitropravastatin (NCX 6550) has been demonstrated to exert an important anti-inflammatory action in RAW 264.7 cells, a murine macrophage cell line (Ongini et al., 2004), to have an anti-thrombotic effect and to inhibit the expression of TF (tissue factor), to reduce the procoagulant potential of mouse peritoneal macrophages (Rossiello et al., 2005), to reduce ROS production in mouse splenocytes (both normal and atherosclerotic), demonstrating higher anti-inflammatory actions than pravastin (Dever et al., 2006). Moreover**,** it stimulates healing and angiogenesis and improves recovery from limb ischemia in both normoglycemic and diabetic mice (Emanueli et al., 2007).

MONOCYTES AND MONOCYTE-DERIVED-MACROPHAGE (MDM)

As already described, monocytes and MDM play a relevant role in RA and CAD. Therefore we choose these cells as model for our investigation.

Monocytes originate in the bone marrow from a common myeloid progenitor and are then released into the peripheral blood, where they circulate for several days before entering tissues and replenishing the tissue macrophage populations (Volkman et al., 1965). Mature monocytes constitute about 5–10% of peripheral-blood leukocytes in humans and represent a heterogeneous population. In fact, they vary in size and have different degrees of granularity and varied nuclear morphology (Gordon et al., 2005). As long ago as 1939, Ebert and Florey (Ebert et al., 1939) observed that monocytes migrate from blood vessels and develop into macrophages in the tissues. Pro-inflammatory, metabolic and immune stimuli all elicit increased recruitment of monocytes to peripheral sites (van Furth et al., 1973), where differentiation into macrophages occurs, contributing to host defence, tissue remodelling and repair. Monocytes are identified by their expression of large amounts of co-receptor CD-14 (which is part of the receptor for lipopolysaccharide). The activation state and functions of mononuclear phagocytes are profoundly affected by different cytokines and microbial products. While Th1 cytokines (e.g., IFN-γ, IL-1β) and LPS induce a ‘‘classical’’ activation profile (M1), Th2 cytokines, such as IL-4 and IL-13, induce an ‘‘alternative’’ activation program (M2) in macrophages. Moreover, macrophages are plastic cells because they can switch from an activated M1 state to M2, and vice versa, upon specific signals (Porcheray et al., 2005). M1 macrophages are potent effector immune cells that kill microorganisms and produce primarily pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-12 (Gordon, 2003). In contrast, M2 macrophages dampen these inflammatory and adaptive Th1 responses by producing anti-inflammatory factors (IL-10, Transforming Growth Factor β [TGF-β], and IL-1Ra), scavenging debris, and promoting angiogenesis, tissue remodelling and repair (Gordon, 2003; Mantovani et al., 2001).

MATERIALS AND METHODS

The methods used in the two published papers can be seen in the specific “Material and Method” section of the papers.
In this section, I detail the methods used in the unpublished work on RA.

Patients. Thirty consecutive adult RA outpatients (20 females and 10 males) attending the local Immuno-Rheumatology clinic in the period January 2010-January 2011, and 15 healthy donors (10 females and 5 males) were enrolled; only patients with established RA who gave their consent were included in 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 with disease-modifying anti-rheumatic drugs (DMARDs) and/or low-dose oral corticosteroids; 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. Clinical assessment included the 28-joint Disease Activity Score, DAS28 (Prevoo et al., 1995), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) level. Blood samples were obtained from each participant at fasting at 9.00 a.m. The Research Protocol was approved by the Ethic Committee of Azienda Ospedaliera Maggiore della Carità, Novara; informed written consent was obtained from both RA patients and healthy volunteers.

Isolation of monocytes and macrophages (MDM) from RA patients and healthy donors. Human monocytes were isolated from heparinized venous blood by standard techniques of dextran sedimentation, Hystopaque (density= 1077g/cm^3) gradient centrifugation (400*g*, 30 min, room temperature) and recovered by thin suction at the interface**.** Cells were re-suspended in RPMI 1640 medium, supplemented with 5% heat-inactivated foetal bovine serum (FBS), 2 mM glutamine and antibiotics; 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., 2009). 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, and defined as macrophage-like cells by evaluating surface markers, as described (Amoruso et al., 2009).

PPARγ protein expression and semi-quantitative analysis. The constitutive expression of PPARγ protein was evaluated in monocytes and MDM, as described (Amoruso et al., 2009). Cells were scraped off in RIPA buffer and lysed by sonication; 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 PPARγ (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 PPARγ protein was performed by calculating the ratio between its total expression and the expression of the reference housekeeping protein, β-actin (Amoruso et al., 2009). We also evaluated the ability of the endogenous PPARγ agonist 15d-PGJ, and of two anti-rheumatic drugs largely used by our RA patients, methotrexate (MTX) and methylprednisolone (MP), to affect PPARγ 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 PPARγ protein expression (Amoruso et al., 2009).

PPARγ 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 (PPARγ), 5 µl of RNase-free water, 10 µl of TaqMan Universal PCR MasterMix (2×) (without AmpErase UNG), and 4 µl of cDNA template, as described (Amoruso et al., 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., 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 analysed 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 analysed 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 LPS 100 ng/ml for 6 hr that is the same time as for the determination of PPARγ 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

PAPER n.1

Angela Amoruso, Claudio Bardelli, Luigia Grazia Fresu, Alessandra Palma, Matteo Vidali, Valeria Ferrero, Flavio Ribichini, Corrado Vassanelli and Sandra Brunelleschi

"Enhanced Peroxisome Proliferator-Activated Receptor-γ Expression in Monocyte/Macrophages from Coronary Artery Disease Patients and Possible Gender Differences"

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Peroxisome proliferator-activated receptor (PPAR) activation reduces inflammation and atherosclerosis, but recent evidence raised concerns about its beneficial clinical effects. However, the effects of gender on PPAR expression and basal cytokine release have not been investigated. In the present study, we evaluated PPARγ and α expression, as well as cytokine release, in monocyte/macrophages from 15 male and 15 female patients with coronary artery disease (CAD) in comparison with healthy controls. Both expression and activation of PPAR-α and PPARγ proteins were evaluated by Western blot and electrophoretic mobility shift assay. Gene expression was evaluated by real-time polymerase chain reaction; cytokine release was measured by enzyme-linked immunosorbent assay. Monocyte/macrophages of CAD patients yielded a constitutively enhanced (approximately 10-fold; $p < 0.001$) protein expression of PPARγ, but not PPARα, compared with healthy controls. Evaluation of PPARγ gene expression showed a 60-fold increase in monocytes from CAD patients, compared with healthy donors. Moreover, monocytes spontaneously released higher amounts of pro-inflammatory cytokines than macrophages. It is interesting to note that monocytes from CAD females expressed significantly higher levels of PPARγ protein compared with male patients ($p < 0.05$) and showed the lowest basal release of TNF-α. These results indicate that the expression of PPARγ 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 demonstrated the highest PPARγ expression and the lowest cytokine release. Such differences may, in part, modulate the response to PPARγ activators.

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Enhanced Peroxisome Proliferator-Activated Receptor-γ Expression in Monocyte/Macrophages from Coronary Artery Disease Patients and Possible Gender Differences

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ABSTRACT

Peroxisome proliferator-activated receptor (PPAR) activation reduces inflammation and atherosclerosis, but recent evidence raised concerns about its beneficial clinical effects. However, the effects of gender on PPAR expression and basal cytokine release have not been investigated. In the present study, we evaluated PPAR-γ and -α expression, as well as cytokine release, in monocyte/macrophages from 15 male and 15 female patients with coronary artery disease (CAD) in comparison with healthy controls. Both expression and activation of PPAR-α and PPAR-γ proteins were evaluated by Western blot and electrophoretic mobility shift assay. Gene expression was evaluated by real-time polymerase chain reaction; cytokine release was measured by enzyme-linked immunosorbent assay. Monocyte/macrophages of CAD patients yielded a constitutively enhanced (approximately 10-fold; $p < 0.001$) protein expression of PPAR-γ, but not PPAR-α, compared with healthy controls. Evaluation of PPAR-γ gene expression showed a 60-fold increase in monocytes from CAD patients, compared with healthy donors. Moreover, monocytes spontaneously released higher amounts of proinflammatory cytokines than macrophages. It is interesting that monocytes from CAD females expressed significantly higher levels of PPAR-γ protein compared with male patients ($p < 0.05$) and showed the lowest basal release of tumor necrosis factor-α. These results indicate that 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 demonstrated the highest PPAR-γ expression and the lowest cytokine release. Such differences may, in part, modulate the response to PPAR-γ activators.

Peroxisome proliferator-activated receptors (PPARs) are members of the superfamily of nuclear receptors and regulate metabolic pathways involved in glucose and lipid homeostasis. PPAR-α is the molecular target for fibrates, frequently used to treat dyslipidemia, whereas the insulin-sensitizing thiazolidinediones (TZDs) are selective ligands for PPAR-γ (Li and Palinski, 2006; Brown and Plutzky, 2007). PPAR-α, preferentially expressed in tissues exhibiting high catabolic rates of fatty acids, is also present in cells involved in inflammatory/immune processes, including monocytes and macrophages (Mandard et al., 2004). Instead, relevant levels of PPAR-γ mRNA can be found in lung, skeletal muscle, colon, and macrophages, with PPAR-γ being recognized as a regulator of macrophage differentiation (Vidal-Puig et al., 1997; Ricote et al., 1998; Amoruso et al., 2007).

A large array of experimental results, especially in animal models and isolated cells, indicate that PPAR-α and PPAR-γ regulate inflammatory processes and are involved in vascular disorders, including atherosclerosis (Vidal-Puig et al., 1997; Ricote et al., 1998; Duval et al., 2002; Mandard et al., 2004; Li and Palinski, 2006; Amoruso et al., 2007; Brown and Plutzky, 2007). In murine models of atherosclerosis, the role of PPAR-α is debated, even though most studies demonstrate

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ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; TZD, thiazolidinedione; LDL, low-density lipoprotein; Rgtz, 5-[[4-(2-methyl-2-pyridinylamino)ethoxyphenylmethyl]-2,4-thiazolidinedione, rosiglitazone; TNF, tumor necrosis factor; MDM, monocyte-derived macrophage; CAD, coronary artery disease; FBS, fetal bovine serum; M 4d, partially differentiated macrophage; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; EMSA, electrophoretic mobility shift assay; IL, interleukin; ACE, angiotensin-converting enzyme; AT1, angiotensin type 1 receptor; HDL, high-density lipoprotein; C, cholesterol.

beneficial effects for PPAR-α agonists and suggest a sexual dimorphism (Tordjman et al., 2001; Duez et al., 2002; Zambon et al., 2006; Babaev et al., 2007). In low-density lipoprotein (LDL)-deficient mice of both sexes, PPAR-α expression in macrophages was shown to exert protective antiatherogenic effects in vivo, by modulating cholesterol trafficking and inflammatory activity (Babaev et al., 2007). Moreover, PPAR-γ ligands inhibit the development of atherosclerosis in LDL-deficient mice, antiatherogenic effects correlating with improved insulin sensitivity, and inhibition of TNF-α and gelatinase B expression (Li et al., 2000); in this case, too, a significant gender difference was reported, because female mice had no reduction of atherosclerosis in response to 5-[[4-(2-methyl-2-pyridinylamino)ethoxy]phenylmethyl]-2,4-thiazolidinedione (rosiglitazone; Rgtz).

Despite extensive evidence supporting the anti-inflammatory properties for TZDs, recent clinical trials raised concerns about the increased risk of peripheral edema, myocardial infarction, and heart failure in diabetic patients treated with the PPAR-γ agonist rosiglitazone (Home et al., 2007; Nissen and Wolski, 2007; McGuire and Inzucchi, 2008). Whether PPAR-γ agonists promote or inhibit atherosclerosis is a question of clinical relevance, because many type 2-diabetes patients are currently treated with TZDs to control hyperglycemia.

We have recently demonstrated that monocyte-derived macrophages (MDM) from healthy donors constitutively express more PPAR-γ protein than monocytes (Amoruso et al., 2007), but there is no information regarding patients with coronary artery disease (CAD). Therefore, we have planned to investigate the constitutive expression of PPAR-α and PPAR-γ proteins, as well as the basal release of proinflammatory cytokines, in monocytes and MDM isolated from male and female CAD patients, to evaluate possible differences according to gender and disease characteristics. To this aim, only nonsmoker CAD patients were enrolled in this study, because tobacco smoke could act as a confounding factor (Amoruso et al., 2007).

Materials and Methods

Patient Selection. Thirty Caucasian nonsmoker patients with CAD (15 men and 15 women) and 15 healthy nonsmoker controls (seven males and eight females) were prospectively included in this investigator-initiated observational pilot study. The research protocol was approved by the Ethical Committee of Ospedale Civile Maggiore (Verona, Italy); informed written consent was obtained by all participants. Patients enrolled were symptomatic for CAD (either stable or unstable angina), had angiographic evidence of significant coronary artery disease (diameter stenosis >70%) in at least one major epicardial coronary vessel, and required revascularization. All patients were on current medical therapy, none receiving TZDs or fibrates (which increase PPARs). Complete clinical and laboratory data were prospectively collected and inserted in a dedicated database. Healthy subjects were age-matched to CAD patients, had no history of cardiopulmonary or other chronic diseases, had no diagnosed lung disease, and were drug-free at the time of the study.

Experimental Protocol. Human monocytes were isolated from heparinized venous blood by standard techniques of dextran sedimentation, Hystopaque (density, 1.077 g/cm^3; Sigma-Aldrich, St. Louis, MO) gradient centrifugation (400*g* for 30 min at room temperature), and recovery by thin suction at the interface, as described previously (Amoruso et al., 2007, 2008). Cells were resuspended in RPMI 1640 medium, supplemented with 5% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml streptomycin, 5 U/ml penicillin, and 2.5 μg/ml amphotericin B; purified monocyte populations were obtained by adhesion (90 min at 37°C in 5% CO_2). Cell viability (trypan blue dye exclusion) was usually >98%; expression of surface markers was analyzed by flow cytometry, yielding >85% pure monocyte populations (Amoruso et al., 2007). 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, 2008). Medium was exchanged every 2 to 3 days, and MDM were defined as macrophage-like cells by evaluating surface markers CD14, CD68, and major histocompatibility class II, as described previously (Amoruso et al., 2008). The absence of CD1a expression demonstrated that no differentiation toward dendritic cells occurred in our MDM preparations (Amoruso et al., 2008). Partially differentiated macrophages (M 4d), in addition to monocytes cultured as described above for 4 days, were also assessed (Amoruso et al., 2007).

Semiquantitative PPAR Protein Expression. Monocytes, M 4d, and MDM from CAD patients and healthy donors were used to evaluate their constitutive expression in PPAR-α and PPAR-γ proteins, as described previously (Amoruso et al., 2007). Cells, seeded in six-well plates were washed twice with ice-cold phosphate-buffered saline and scraped off in lysis buffer; when necessary, cell lysates were stored at −80°C. Protein samples (20 μg) were analyzed by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and electroblotted on nitrocellulose membrane (Protran; PerkinElmer Life and Analytical Sciences, Boston, MA). Immunoblots were performed using the following antibodies: monoclonal mouse anti-human PPAR-γ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-human PPAR-α (3B6/PPAR; Alexis Corporation, Lausen, Switzerland), and anti-human β-actin (Sigma-Aldrich). Anti-mouse secondary antibody was coupled to horseradish peroxidase (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK); chemiluminescence signals were analyzed under nonsaturating conditions with an image densitometer (Versadoc; Bio-Rad Laboratories, Hercules, CA). Semiquantitative evaluation of PPAR-α and -γ protein was performed as described previously (Amoruso et al., 2007) by calculating the ratio between the expression of each PPAR isoform and the expression of the reference housekeeping protein β-actin. In some experiments, we also evaluated the ability of two selective PPAR-γ agonists, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and rosiglitazone, to induce receptor expression; in this case, cells were stimulated (6 h at 37°C in 5% CO_2) with the ligands, according to Amoruso et al. (2007).

RNA Isolation and Real-Time PCR. Total RNA was extracted from monocytes 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, Foster City, CA) 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 plates (Applied Biosystems) containing 1 μl of TaqMan Expression Assay (PPAR-γ), 5 μl of RNase-free water, 10 μl of TaqMan Universal PCR MasterMix (2×) (without AmpErase UNG), and 4 μl of cDNA template. 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 (Livak and Schmittgen, 2001). Results were analyzed by the comparative cycle threshold method, according to Livak and Schmittgen (2001).

Electrophoretic Mobility Shift Assay. The activation of PPAR-γ was evaluated by measuring the nuclear migration by EMSA (Bardelli et al., 2005), with cells being stimulated for 1 h with 10 μM 15d-PGJ_2 or 5 μM rosiglitazone. Nuclear extracts (5 μg) were incubated with 2 μg of poly(dI-dC) and [^{32}P]ATP-labeled oligonucleotide probe (100,000–150,000 cpm; Promega, Madison, WI) in bind-

ing buffer for 30 min at room temperature. The PPAR consensus oligonucleotide (5′-CAAAACTAGGTCAAAGGTCA-3′) was from Santa Cruz Biotechnology, Inc. The nucleotide-protein complex was separated on a polyacrylamide gel, the gel was dried, and radioactive bands were detected by autoradiography (Bardelli et al., 2005). Supershift assays were performed by commercial monoclonal antibodies for PPAR-γ (Santa Cruz Biotechnology, Inc.) at a final concentration of 1 μg/ml.

Cytokine Release. Cells were maintained in a 5% CO_2 incubator at 37°C for 6 h; supernatants were collected and stored at −80°C. TNF-α, IL-6, and IL-10 (the latter was evaluated as the most relevant anti-inflammatory cytokine) in the samples were estimated by enzyme-linked immunosorbent assay (Pelikine Compact human enzyme-linked immunosorbent assay kit), following the manufacturer's instructions (CLB/Sanquin, Amsterdam, The Netherlands). Results are expressed in picograms per milliliter.

Materials. FBS was from Invitrogen (Paisley, UK). Phosphate-buffered saline, Hystopaque, RPMI 1640 medium, glutamine, HEPES, streptomycin, penicillin, amphotericin B, protease inhibitors, and monoclonal mouse anti-human β-actin antibodies were obtained from Aldrich Chemical Co. (Milwaukee, WI). The monoclonal mouse anti-human PPAR-γ (E-8) antibody and the PPAR consensus oligonucleotide were from Santa Cruz Biotechnology, Inc.; anti-human PPAR-α (3B6/PPAR) antibody, rosiglitazone, and 15d-PGJ_2 were from Alexis Corporation. Poly(dI-dC) was from Promega. Tissue culture plates were from NUNC A/S (Roskilde, Denmark); all cell culture reagents, with the exception of FBS, were endotoxin-free according to details provided by the manufacturer.

Statistical Analysis. Data are expressed as mean ± S.E.M. of *n* independent experiments; statistic analyses were performed by SPSS statistical software, version 15.0 (SPSS Inc., Chicago, IL). Differences between groups were estimated by nonparametric Mann-Whitney *U* test or *t* test. Normality distribution was assessed preliminarily by q-q plot, Kolmogorov-Smirnov, and Shapiro-Wilk tests. In the presence of a substantial deviation from normality hypothesis, correction was performed by logarithmic transformation. Spearman rank correlation coefficient and Pearson parametric coefficient were used as appropriate. Main effects of differentiation, group, and their interaction were evaluated by General Linear Model for repeated measures.

Results

Characteristics of CAD Patients and Healthy Controls. Baseline demographic, clinical, and laboratory characteristics of CAD patients (n = 30; 15 males and 15 females) and healthy donors (n = 15; seven males and eight females) are summarized in Table 1. No gender-related differences were observed in our study population. All the females enrolled in this study were postmenopausal women, with none receiving hormonal replacement therapy. All CAD patients were on current medical therapy, with none receiving TZDs or fibrates (which increase PPAR-γ and PPAR-α protein expression, respectively). A similar number of patients in each subgroup received statins and ACE-inhibitors; only four patients (two males and two females) were also treated with the angiotensin type 1 receptor (AT1) blocker losartan. As already known, at relevant in vitro concentrations, statins (Yano et al., 2007), ACE-inhibitors (Storka et al., 2008), and AT1 blockers (Schupp et al., 2004) stimulate PPAR-γ activity, with losartan acting only at very high concentrations (Schupp et al., 2004, 2006). The most relevant clinical parameters were in a normal or near normal range (Table 1). The mean value of HDL-C in CAD females (1.13 ± 0.08 mM) was lower than the desired HDL-C level indicated by the National Cholesterol Education Program (2002), whereas the mean value (1.13 ± 0.06 mM) in CAD males was optimal (Table 1). Fifteen healthy donors were also recruited: they were age-matched with CAD patients, presented normal laboratory parameters, and were drug-free at the time of the study (Table 1).

Expression of PPAR-γ and PPAR-α in Monocytes and MDM. As depicted in Fig. 1, PPAR-γ expression is confirmed to be up-regulated during differentiation to MDM, as reported previously (Amoruso et al., 2007). Furthermore, compared with healthy subjects, monocytes, partially differentiated (M 4d), and fully differentiated macrophages (MDM) obtained from CAD patients presented a significantly higher constitutive expression of PPAR-γ protein (Fig. 1A). In con-

TABLE 1
Demographic and clinical characteristics of CAD patients and healthy controls
Values are mean ± S.E.M. No significant differences were observed between males (M) and females (F) in both CAD patients and healthy controls. Only two patients in each subgroup of CAD patients received losartan, an AT1 blocker.

	CAD, F (n = 15)	CAD, M (n = 15)	Healthy, F (n = 8)	Healthy, M (n = 7)
Age (year)	66 ± 2.8	65.5 ± 2	63 ± 3	64.2 ± 3
BMI (kg/m²)	26.1 ± 1.1	28.4 ± 0.8	25.8 ± 0.9	27 ± 1.1
Dyslipidemia	10/15	8/15		
Hypertension	12/15	11/15		
Diabetes mellitus	5/15	4/15		
Family history of CHD	5/15	4/15		
Unstable angina	8/15	7/15		
Multivessel disease	5/15	5/15		
Glucose (mM)	7.15 ± 0.7	6.99 ± 0.4	5.9 ± 0.9	6.1 ± 0.7
Total C (mM)	4.55 ± 0.28	4.93 ± 0.23	4.6 ± 0.2	4.7 ± 0.2
LDL-C (mM)	2.76 ± 0.22	3.29 ± 0.2	2.8 ± 0.2	3.1 ± 0.2
HDL-C (mM)	1.13 ± 0.08	1.13 ± 0.06	1.36 ± 0.1	1.1 ± 0.05
TG (mM)	1.27 ± 0.14	1.16 ± 0.12	1.3 ± 0.1	1.3 ± 0.1
Nitrates	11/15	11/15		
Calcium antagonists	2/15	4/15		
ACE-inhibitor ± AT1 blockers	10/15	12/15		
β-Blockers	13/15	8/15		
Statins	9/15	7/15		
Aspirin	13/15	14/15		
Antidiabetics	5/15	5/15		

BMI, body mass index; CHD, coronary heart disease; TG, triglycerides.

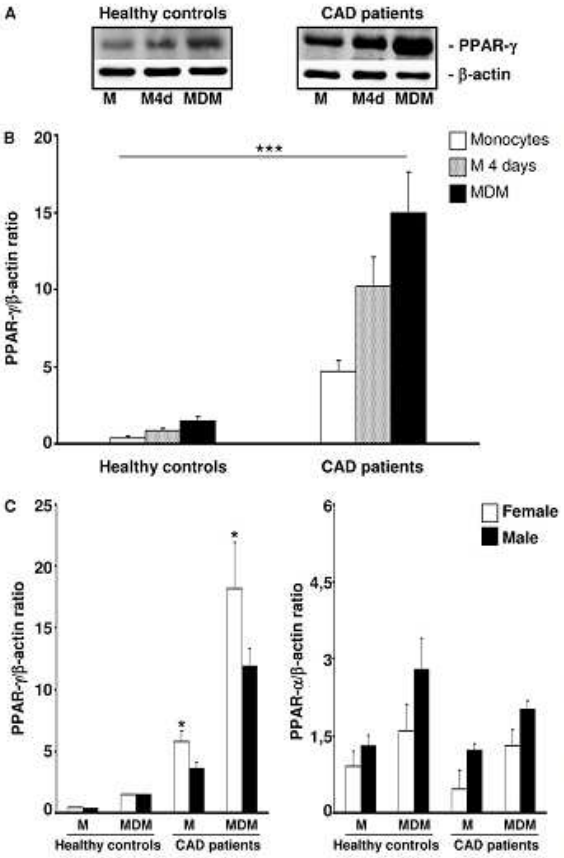

Fig. 1. Constitutive PPAR-γ and PPAR-α protein expression in monocytes (M) and fully differentiated macrophages (MDM) from CAD patients and healthy controls. Fifteen healthy individuals (seven males and eight females) and 30 CAD patients (15 males and 15 females) were evaluated. A, representative blots for PPAR-γ and β-actin expression in M, partially differentiated macrophages (M 4 days) and MDM. B, semiquantitative expression of PPAR-γ protein. Results are mean ± S.E.M. of n = 15 healthy individuals and n = 30 CAD patients. ***, $p < 0.001$ versus healthy controls. C, semiquantitative expression of PPARs proteins according to gender. Left, PPAR-γ expression; right, PPAR-α expression. Healthy donors (seven males and eight females) and CAD patients (15 males and 15 females) were evaluated. Females, □; males, ■. Results are means ± S.E.M. *, $p < 0.05$ versus CAD males.

trols and CAD patients, PPAR-γ/β-actin ratios were 0.37 ± 0.08 and 4.71 ± 0.7 ($p < 0.001$), respectively, in monocytes; 0.86 ± 0.15 and 10.2 ± 1.9 ($p < 0.001$) in M 4d; and 1.50 ± 0.25 and 15.00 ± 2.6 ($p < 0.001$) in MDM (Fig. 1B). These data were further analyzed by General Linear Model analysis, using differentiation from monocytes to MDM as "within-subjects" factor and group (controls versus CAD) as "between-subjects" factor. The main effect of group yielded an F ratio = 24.04 ($p < 0.001$), indicating that the PPAR-γ/β-actin ratios were significantly higher in CAD patients than in healthy subjects. The main effect of differentiation yielded an F ratio = 23.53, $p < 0.001$, indicating that the PPAR-γ/β-actin ratios were significantly increased along with differentiation. The interaction effect between differentiation and group was significant (F = 5.7; $p < 0.001$), suggesting that the PPAR-γ/β-actin ratio response to differentiation depended upon which of the two groups (control or CAD patient) is considered. Analysis by gender in CAD patients revealed that monocytes and MDM from females expressed significantly higher levels of PPAR-γ protein than males (PPAR-γ/β-actin ratios in female and male monocytes were 5.80 ± 0.85 and 3.62 ± 0.46 (n = 15; $p < 0.05$) and in female and male MDM were 18.15 ± 3.82 and 11.85 ± 1.43 (n = 15; $p < 0.05$), whereas no gender difference was observed in healthy volunteers (Fig. 1C, left). By evaluating PPAR-α expression in monocytes and MDM, no major differences were found between cell types, or between CAD patients and healthy donors (Fig. 1C, right). In fact, in spite of a trend toward a higher expression in cells from men, no statistical significance was attained. Therefore, monocytes and MDM of CAD patients seemed to express more PPAR-γ than PPAR-α protein (Fig. 1C), and this was particularly relevant in women. In monocytes and MDM from CAD females, PPAR-γ/β-actin ratio was >12-fold higher than PPAR-α/β-actin ratio, whereas in male CAD cells there was a 3- to 6-fold increase (monocytes and MDM, respectively).

It is interesting that, as reported in Table 2 and in keeping with our previous observations (Amoruso et al., 2007), selective PPAR-γ agonists up-regulated receptor expression in both cell types (>2-fold for 15d-PGJ_2; >4-fold for rosiglitazone), with no major differences being detected between monocytes and MDM in healthy donors and CAD patients.

By real-time PCR, we also evaluated PPAR-γ gene expression in monocytes from selected healthy donors (n = 8; four females and four males) or CAD patients (n = 15; six females and nine males). As reported in Fig. 2, mRNA level in monocytes from CAD patients was approximately 60-fold higher than in healthy individuals ($p < 0.001$ versus healthy controls), with no significant gender difference. These CAD patients had no diabetes and were not treated with AT1 blockers.

PPAR-γ Is Functionally Active in Monocytes. Taking into consideration that PPAR expression may be directly linked to its activation, we analyzed the DNA binding capacity of PPAR-γ using EMSA. As shown in Fig. 3 (lane 1), nuclear extracts isolated from unstimulated monocytes of healthy donors revealed ow DNA binding of PPAR. To prove the assumption that PPAR-γ binds DNA in response to selective agonists, cells were challenged with the natural ago-

TABLE 2

Selective ligands enhance PPAR-γ protein expression in monocytes and MDM

Monocytes and MDM from five different donors per group were stimulated (6 h at 37°C in 5% CO_2) with the natural ligand 15d-PGJ_2 (10 μM) or the synthetic agonist Rgtz (5 μM). PPAR-γ/β -actin ratio was measured as described in text, and results are expressed as -fold increase over control, unstimulated cells. Values are mean ± S.E.M. p value, not significant between cell types and donors.

	-Fold Increase in PPAR-γ/β-Actin Ratio	
	+ 10 μM 15d-PGJ_2 (n = 5)	+ 5 μM Rgtz (n = 5)
Monocytes, healthy, M	2.5 ± 0.3	4 ± 0.4
Monocytes, healthy, F	2.6 ± 0.4	3.9 ± 0.3
Monocytes, CAD, M	2.7 ± 0.3	4 ± 0.3
Monocytes, CAD, F	2.8 ± 0.3	4.2 ± 0.4
MDM, healthy, M	2.6 ± 0.2	4 ± 0.3
MDM, healthy, F	2.7 ± 0.3	4.2 ± 0.4
MDM, CAD, M	2.7 ± 0.2	4.1 ± 0.4
MDM, CAD, F	2.8 ± 0.4	4.4 ± 0.3

CAD, patients with coronary artery disease; F, females; M, males.

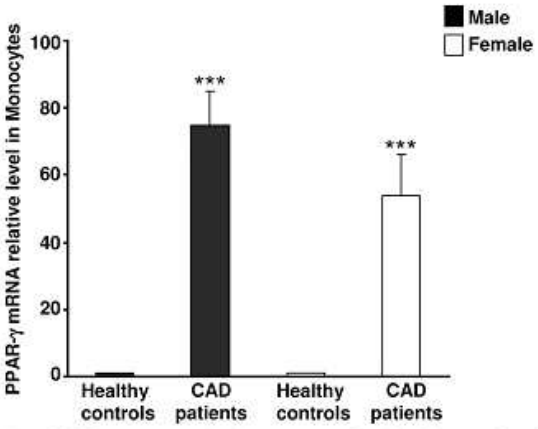

Fig. 2. PPAR-γ gene expression in monocytes from healthy controls and CAD patients. mRNA from healthy controls (n = 8; four females and four males) represents the "calibrator" (as named by the 7000 System JDS software; Applied Biosystems) value = 1. No significant gender differences were detected between healthy females (□) and males (■). Monocytes from 15 CAD patients (six females, □; nine males, ■) were evaluated, with mRNA level being significantly increased (***, $p < 0.001$ versus healthy controls). Values are mean ± S.E.M.; p value not significant between CAD males and females.

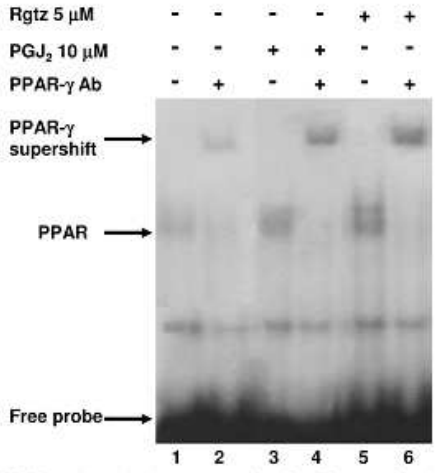

Fig. 3. PPAR-γ activation in monocytes from healthy controls. Activation of PPAR-γ was analyzed by EMSA using a specific PPAR consensus oligonucleotide, as described under *Materials and Methods*. Cells were stimulated with 10 μM 15d-PGJ_2 (PGJ_2; lanes 3 and 4) or 5 μM Rgtz (lanes 5 and 6) for 6 h. Untreated cells are shown in lanes 1 and 2. Data are representative of three similar experiments.

nist 15d-PGJ_2 (10 μM; lane 3) and the synthetic agonist Rgtz (5 μM, lane 5). As demonstrated in Fig. 3, both ligands enhanced binding of PPAR-γ to the labeled oligonucleotide. To provide unequivocal proof for the involvement of PPAR-γ in DNA binding, we conducted a supershift analysis: as evidenced in Fig. 3, PPAR-γ was always supershifted (lane 2, untreated monocytes; lane 4, 15d-PGJ_2; and lane 6, Rgtz). The same experiment was also performed in MDM from healthy donors, as well as in both cell types from CAD patients, with similar effects (data not shown). No significant differences were observed among the groups for PPAR-α (data not shown).

PPAR-γ Expression in Monocytes from CAD Patients and Correlation with Clinically Relevant Parameters. Possible relationships between PPAR-γ expression and clinically relevant parameters were sought in CAD patients. An inverse correlation was found between PPAR-γ expression in monocytes and LDL-C levels ($r = -0.467$; $p = 0.009$) in CAD patients, both males and females (Fig. 4A). It is noteworthy that 12 of 15 CAD women presented optimal or near optimal LDL-C levels, whereas only seven of 15 CAD men had optimal or near optimal LDL-C levels (Fig. 4A). No significant correlations were detected between PPAR-γ expression and body mass index, glucose, triglycerides, total cholesterol, and HDL-C (data not shown). It is worth recalling that the study population was under medical therapy, the most relevant clinical parameters being in a normal range (Table 1). Therefore, we analyzed PPAR-γ expression in monocytes according to disease characteristics (e.g., unstable angina, multivessel disease, hyperten-

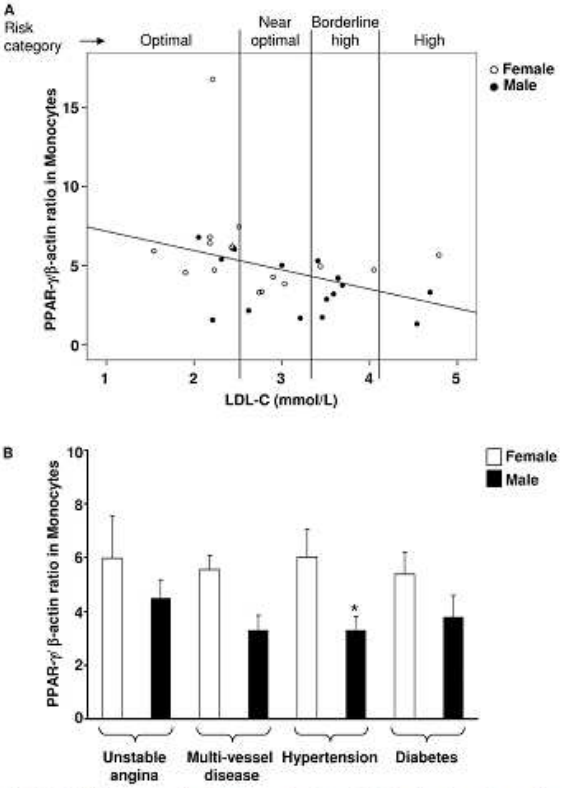

Fig. 4. PPAR-γ expression in monocytes from CAD patients and correlation with clinically relevant parameters. A, inverse correlation between PPAR-γ expression and LDL-C levels ($r = -0.467$; $p = 0.009$) in female (○) and male (●) CAD patients. See text for further details. B, PPAR-γ expression in monocytes from CAD patients according to disease characteristics and gender. Fifteen CAD females (□) and 15 CAD males (■) were evaluated. Each disease subgroup deals with different numbers of patients. Unstable angina, n = 8 females and 7 males; multivessel disease, n = 5 females and 5 males; hypertension, n = 12 females and 11 males; and diabetes, n = 5 females and 4 males (also see Table 1). *, $p < 0.05$ versus female monocytes.

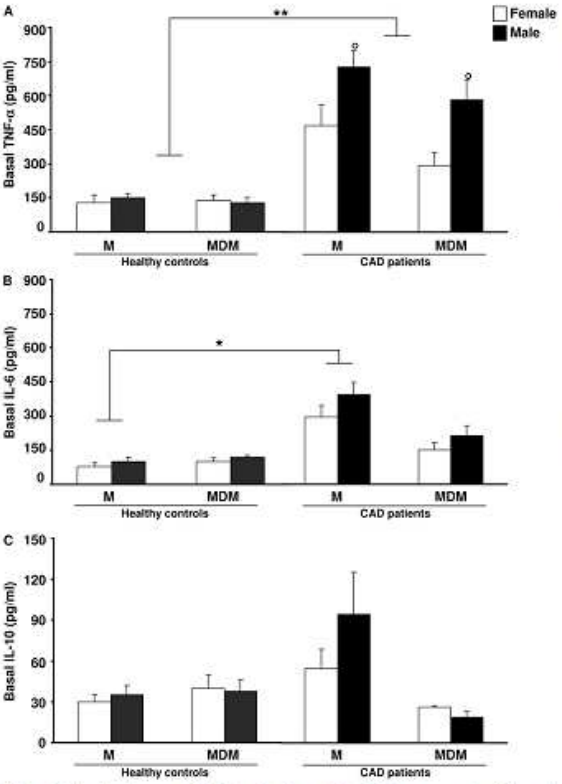

Fig. 5. Basal release of TNF-α, IL-6, and IL-10 in monocytes (M) and macrophages (MDM) from CAD patients and healthy controls. Fifteen CAD males (■) and 15 CAD females (□) were evaluated and compared with healthy females (n = 8; □) and males (n = 7; ■). A, TNF-α. B, IL-6. C, IL-10. Results are mean ± S.E.M. and are expressed as picograms per milliliter. **, $p < 0.01$, CAD patients versus healthy controls; *, $p < 0.05$, CAD monocytes versus healthy control monocytes; and °, $p < 0.05$ versus female cells.

sion and diabetes). Although monocytes from CAD females in all subgroups usually had higher levels of PPAR-γ/β-actin ratio than CAD males, a significant gender difference was found only for hypertension ($p < 0.05$; Fig. 4B).

Basal Cytokine Release in CAD Patients and Healthy Controls. Because monocyte/macrophages spontaneously release measurable amounts of inflammatory cytokines (Amoruso et al., 2007) and PPARs modulate cytokine release (Jiang et al., 1998; Duval et al., 2002; Amoruso et al., 2007; Brown and Plutzky, 2007), we evaluated possible gender-related differences in cells from CAD patients and healthy donors. It is interesting that monocytes and MDM from CAD patients secreted more TNF-α ($p < 0.01$; Fig. 5A) than healthy donors. Although no significant gender difference was observed in healthy donors for both cell types, monocytes and MDM from CAD females spontaneously released less TNF-α than the corresponding males ($p < 0.05$; Fig. 5A). IL-6 release, although not reaching statistical significance, was slightly higher in cells from CAD males compared with CAD females (Fig. 5B); moreover, monocytes from CAD patients secreted more IL-6 ($p < 0.05$; Fig. 5B) than healthy controls. Conversely, no gender difference occurred in IL-10 secretion by monocytes/MDM from CAD patients and healthy donors (Fig. 5C). In addition, no significant correlation was found between PPAR-γ expression levels in monocyte/MDM and TNF-α, IL-6, or IL-10 concentrations (data not shown).

Discussion

Despite that both PPAR-α and PPAR-γ are largely indicated as anti-inflammatory and antiatherogenic mediators, no information on their quantitative expression is available, and only a few investigations have evaluated their relevance in patients with CAD. This observational investigator-initiated pilot study was conceived to analyze, in a real-life scenario, the expression of PPAR-α and PPAR-γ in monocyte/macrophages of healthy volunteers and patients with CAD, by enrolling male and female patients with similar baseline characteristics and medical therapy. Given the fact that tobacco smoke could behave as a confounding factor, at least in healthy volunteers (Amoruso et al., 2007), smoker CAD patients were excluded from this study.

As a first goal of our study, by a semiquantitative evaluation, we demonstrated that monocytes (whose role in the initiation of atherosclerosis is well established; Osterud and Bjorklid, 2003) from CAD patients express significantly higher levels of both PPAR-γ protein (approximately 10-fold) and mRNA (approximately 60-fold) compared with healthy donors. This is in good agreement with Teupser et al. (2008) who showed that another important marker, CD36, has an mRNA increase in circulating monocytes from CAD patients. CD36 is a multiligand scavenger receptor present on the surface of monocyte/macrophages; it plays a critical role in the development of atherosclerotic lesions, by its capacity to bind and endocytose oxidized LDL, and it is implicated in the formation of foam cells (Collot-Teixeira et al., 2007). Moreover, CD36 expression is increased by transcriptional activation of PPAR-γ as well as in vitro PPAR-γ agonists (both 15d-PGJ_2 and TZD) (Tontonoz et al., 1998; Nicholson, 2004). Therefore, our finding of an increased PPAR-γ expression in monocyte/macrophages from CAD patients, compared with healthy donors, further extends the information reported above and reflects the local inflammatory milieu in which CAD develops, prolonged recruitment/activation of monocytes leading to differentiation into macrophages and foam cells. However, as largely established, the accumulation of cholesterol esters in macrophages, and subsequent foam cell formation, depends on the balance between the uptake of LDL and the efflux of free cholesterol that is mainly controlled by the transporter ATP-binding cassette transporter A1 (Young and Fielding, 1999). PPAR-γ has been indicated to exert a dual role, either pro- (Tontonoz et al., 1998) and antiatherogenic (Chawla et al., 2001; Chinetti et al., 2001), but, according to Lazar (2001), a net antiatherogenic can be envisaged. In fact, activation of PPAR-γ enhances CD36 expression (by itself, this effect accelerates atherosclerosis), but the increased CD36 expression is counteracted by the reduced expression of class A scavenger receptor and by the increased expression of liver X receptor, a nuclear receptor that induces the reverse cholesterol transporter ATP-binding cassette transporter A1, mediating cholesterol efflux.

In addition, we provide evidence that PPAR-γ is functionally active, responding to selective agonists in both EMSA assays and Western blots. Furthermore, PPAR-α was func-

tionally active in EMSA analysis, but its expression was unchanged between cell types and groups.

It is noteworthy that gender analysis indicates that cells from female CAD patients have a higher PPAR-γ protein content than male CAD patients. Results obtained by real time-PCR do not shown up-regulation of the gene, suggesting that the gender difference is possibly due to post-transcriptional events. The existence of a gender difference was suggested previously (Vidal-Puig et al., 1997) in subcutaneous adipose tissue from lean and obese individuals, with women presenting higher levels of PPAR-γ1 and -γ2 mRNA expression than men. A second relevant observation regards the possible correlation between PPAR-γ expression and cytokine release in monocyte/macrophages isolated from healthy controls and CAD patients.

It is largely established that cytokine release is a complex phenomenon that involves several signal pathways and is finely regulated, often in a stimulus- and cell-specific manner (Andreakos et al., 2004; Amoruso et al., 2008); that PPAR-γ agonists inhibit cytokine release (Jiang et al., 1998; Duval et al., 2002; Amoruso et al., 2007); and that TNF-α is a PPAR-γ pathway target (Brown and Plutzky, 2007). Increased plasma concentrations of TNF-α and IL-6 have been repeatedly evidenced in CAD, with levels of proinflammatory cytokines correlating with the severity of coronary artery occlusion (Ridker et al., 2000; Skoog et al., 2002), so that both cytokines have been highlighted as relevant markers of cardiovascular risk (Blake and Ridker, 2002). Our results are in keeping with these findings, because we evidence that monocyte/macrophages from CAD patients spontaneously release higher amounts of TNF-α than healthy donors, with no significant variation in the anti-inflammatory IL-10. It is interesting that, in our study, the expression of PPAR-γ protein in monocytes and MDM is inversely related to the spontaneous release of proinflammatory cytokines; in fact, CAD females, who present the highest level of PPAR-γ expression, have the lowest basal release of TNF-α. As far as IL-6 is concerned, only monocytes from CAD patients present a significantly enhanced release compared with healthy donors. We have no definite explanation for this, but it is worth recalling that, in adipose cells from insulin-resistant subjects, IL-6 and TNF-α reduce PPAR-γ mRNA levels, with TNF-α exerting a greater inhibitory effect (Rotter et al., 2003). When considering clinical parameters, such as body mass index, diabetes mellitus, unstable angina, multivessel disease, and hypertension, this study documents a significant gender difference in PPAR-γ protein expression only in the hypertension subgroup. Because approximately two thirds of these CAD patients are treated with ACE-inhibitors and AT1 blockers that, at high in vitro concentrations, enhance PPAR-γ expression in differentiated adipocytes (Schupp et al., 2004, 2006; Storka et al., 2008), this observation warrants further evaluation. Currently, we can exclude that the pharmacological therapy accounts for the gender difference; in fact, a similar number (10 females and 12 males) of patients is treated with an ACE-inhibitor, and only two patients in each group also receive losartan.

We can also evidence a significant inverse correlation (but no clear gender difference) between PPAR-γ/β-actin ratio and LDL-C levels: the higher the PPAR-γ expression, the lower the LDL-C level. It has to be noted that all CAD patients were on current medical therapy (more than 50% receiving statins) and that their lipid profile was near normal, with no major differences between males and females (Table 1). This inverse correlation between PPAR-γ protein expression and LDL-C levels is observed in monocytes, which are main players in atherogenesis (Osterud and Bjorklid, 2003) and are readily prepared from circulating blood, but is less evident for MDM. We suggest that this could be related, at least partially, to the experimental procedures and longer time (8–10 days) for MDM preparation. In macrophage cell lines, micromolar concentrations of five different statins were shown to activate PPAR-γ, although to a minor extent than with the TZD pioglitazone (Yano et al., 2007). We can rule out that, in our study, statin treatment could account for the enhanced PPAR-γ protein expression in CAD patients and/or gender difference. In fact, almost the same number of male and female CAD patients is given a statin, and no major differences in PPAR-γ expression are found between patients treated with statins and those not receiving these drugs (data not shown).

In keeping with the literature indicating the anti-inflammatory and antiatherogenic properties of PPAR-γ ligands, it is tempting to speculate that the enhanced PPAR-γ expression we detect in monocytes and MDM from CAD patients (and especially in females) could represent a beneficial condition, even if the enhanced cardiovascular risk in diabetic patients treated with the PPAR-γ agonist rosiglitazone (Home et al., 2007; Nissen and Wolski, 2007) needs to be carefully considered. However, more recent clinical studies have confirmed the beneficial effects of rosiglitazone in overweight individuals (Manning et al., 2008) and diabetic CAD patients after coronary angioplasty (Wang et al., 2008).

We advance the hypothesis that the administration of PPAR-γ ligands to patients with higher PPAR-γ protein expression levels might result in enhanced clinical effects, and, according to the higher PPAR-γ expression and less proinflammatory cytokine release in monocyte/macrophages from CAD women compared with CAD men, we also suggest that this gender difference should be taken into account when treating patients with PPAR-γ agonists. We are conscious that further studies, especially targeted to the concomitant evaluation of all the metabolic pathways involved in cholesterol homeostasis, are needed to definitely ascertain whether PPAR-γ is an antiatherogenic factor.

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PPARγ Expression in Monocyte/Macrophages from RA patients

As known, 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).

The PPARγ role in controlling monocyte/macrophage inflammatory response is well established (see **I**ntroduction; Jiang et al., 1998; Ricote et al., 1998; Tontonoz et al., 1998; Amoruso et al., 2007; Johnson and Newby, 2009; Kadl et al., 2010) and PPARγ agonists have been suggested as protective agents in experimental RA models (see Introduction).

Therefore, in line with the project of this PhD and in keeping with the previously documented GD in PPAR expression that we observed in CAD patients, we evaluated PPARγ expression (both as protein and mRNA) in established RA, a disease which, as reported in the Introduction, has a higher incidence in females.

We also compared these results with those obtained in cells of healthy donor of both sexes.

Additionally, we measured MMP-9 activity and**,** together with the PPAR level, the results were correlated to the disease's severity, according to the DAS28 score.

Characteristics of the patients enrolled in the study

The baseline clinical and demographic data (including sex, age, anti-rheumatic therapy) for the 30 RA outpatients (20 females and 10 males, mean age = 60.1 ± 2.6 years) enrolled in this study are shown in Table 1. All patients were on current DMARDs therapy: 22 receiving weekly MTX, 10 patients receiving other different DMARDs, in single or combination therapy. Twenty patients were also receiving low-dose oral corticosteroids: 16 patients assumed prednisone < 5 mg and 4 patients had MP < 4 mg daily. None of them received monoclonal antibodies or TNF-α inhibitors (Table 1). All RA patients were non-smokers since tobacco smoke deeply affects PPARγ expression in monocyte/macrophages (Amoruso et al., 2007), as well as the patient's response to MTX treatment (Saevarsdottir et al., 2011). In this study population, the mean values for C-reactive protein and erythrocyte sedimentation rate were: 0.6 ± 0.1 mg/dl and 19.4 ± 2.7 mm/h, respectively. The DAS28 score ranged from 0.63 to 5.97, with a mean value of 3 ± 0.2 (Table1). Also 15 non-smoker healthy donors (5 males and 10 females) were enrolled in the study; they were age-matched (mean age = 59.8 ± 1.9 years) 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 (not shown)

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

Patient	Age, yr	Sex (M,F)	CRP, mg/dl	ESR, mm/h	DAS 28	GC§	MTX	SSZ	HCQ	LEF
1	76	F	1.4	65	3.2	+	-	+	-	-
2	74	F	0.3	28	2.6	+	+	-	-	-
3	43	F	0.63	35	3.33	+	+	-	-	-
4	77	F	0.14	12	4.12	+	-	+	-	+
5	62	F	0.97	22	4.2	-	-	+	+	-
6	55	F	0.22	5	3.08	-	+	-	-	-
7	67	M	-	33	2.59	+	+	+	-	-
8	27	F	0.1	4	2.36	+	+	-	-	-
9	56	F	-	6	2.79	+	+	-	+	-
10	70	F	0.11	4	1.25	-	+	-	-	-
11	89	F	0.11	34	3.98	+	+	-	-	-
12	70	M	0.98	10	1.75	-	+	-	-	-
13	32	F	1.26	38	4.15	+	-	+	-	-
14	58	M	-	25	2.25	+	+	-	-	-
15	47	M	2.64	35	4.45	-	+	-	-	-
16	55	F	0.7	38	2.69	+	-	+	-	-
17	77	F	0.33	31	4.45	+	+	-	-	-
18	61	M	-	2	0.63	+	-	-	-	-
19	58	F	0.2	27	4.19	+	+	-	-	-
20	67	F	0.25	9	2.24	-	+	-	-	-
21	50	F	0.37	25	5.14	-	+	-	-	-
22	70	F	2.51	8	2.2	-	+	-	-	-
23	71	F	0.18	10	3.01	-	-	+	-	-
24	35	F	0.14	28	5.97	-	+	-	-	-
25	68	F	0.11	16	3.11	+	+	-	-	-
26	49	M	0.11	9	1.96	+	+	-	-	-
27	51	M	0.11	6	2.44	+	+	-	-	-
28	58	M	-	3	1.89	+	+	-	-	-
29	73	M	-	7	1.36	+	+	-	-	-
30	58	M	0.13	8	2.25	+	+	-	+	-

Data are means ± SEM. RA = rheumatoid arthritis; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; DAS 28 = Disease Activity Score in 28 joints; GC = glucocorticoids; MTX = methotrexate; SSZ = sulfasalazine; HCQ = hydroxycloroquine; LEF = leflunomide. §indicates prednisone ≤ 5 mg/day or equivalent.

Expression of PPARγ 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 PPARγ protein as compared to healthy donors (PPARγ/β-actin ratio = 0.21 ± 0.02 and 0.46 ± 0.05 in monocytes from 15 healthy donors and 30 RA patients, respectively). Similar results are obtained in MDM, PPARγ/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 PPARγ protein expression are shown in Figure 1B. In RA patients, also PPARγ mRNA levels are increased (4-fold in monocytes and about 3-fold in MDM), compared to healthy donors (Figure 1C). 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 PPARγ protein expression than healthy donors (Figure 2A). On the contrary, PPARγ 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 PPARγ mRNA levels in monocyte/macrophages, 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 severity (Figure 2B).

PPARγ protein expression and a possible gender difference in monocytes and MDM RA patients

Because the topic of my project was to evaluate a possible gender difference in the expression of PPARγ protein in inflammatory disease, we performed a gender analysis in monocytes and MDM isolated from RA patients (20 women and 10 men). As showed in the graph in monocytes there aren't a significantly gender difference (0.59 ± 0.44 and 0.39 ± 0.21 respectively men and women). In MDM there is a significant gender difference, in fact men show an increased expression of the receptor (2.32 ± 0.4 and 1.53 ± 0.167 respectively men and women) (Figure 3).

MMP-9 activity in monocyte/macrophages

As known, MMP-9 activity is critical for RA progression and is inhibited by PPARγ 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 4A). Representative gelatin zymographies are provided in Figure 4B. 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 8-fold in MDM; $P < 0.01$ *vs* healthy donors) in gelatinolytic activity (Figure 4C).

Effects of PPARγ agonists, MP and MTX 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 MP and MTX to directly affect PPARγ expression and MMP-9 activity in cells from 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 PPARγ agonist 15d-PGJ, which ensures maximal activity at 10 μM. MTX was used at 1 μM, since Godfrey et al in a large pharmacokinetic study in RA patients, reported serum MTX concentrations in the range 0.3 -1.5 μM. Moreover, C_{max} values of 1.1 μM 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 μM and 180 ng/ml respectively) induce PPARγ protein expression in both monocytes and MDM; the effects elicited by 15d-PGJ are shown for comparison (Figure 5A) and representative Western blots are provided (Figure 5B). Since MMP-9 exerts a crucial role in RA progression and joint destruction (McInness et al., 2005; Yoshida et al., 2009) 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 μM, 1 μM and 180 ng/ml respectively) potently inhibit LPS-induced MMP-9 activity, the endogenous PPARγ ligand and MTX being more effective than MP ($P < 0.001$; Figure 6A) in monocytes. On the contrary, the three drugs inhibited similarly LPS-induced MMP-9 activity in MDM (Figure 6A). Representative gelatin zymographies are provided in Figure 6B.

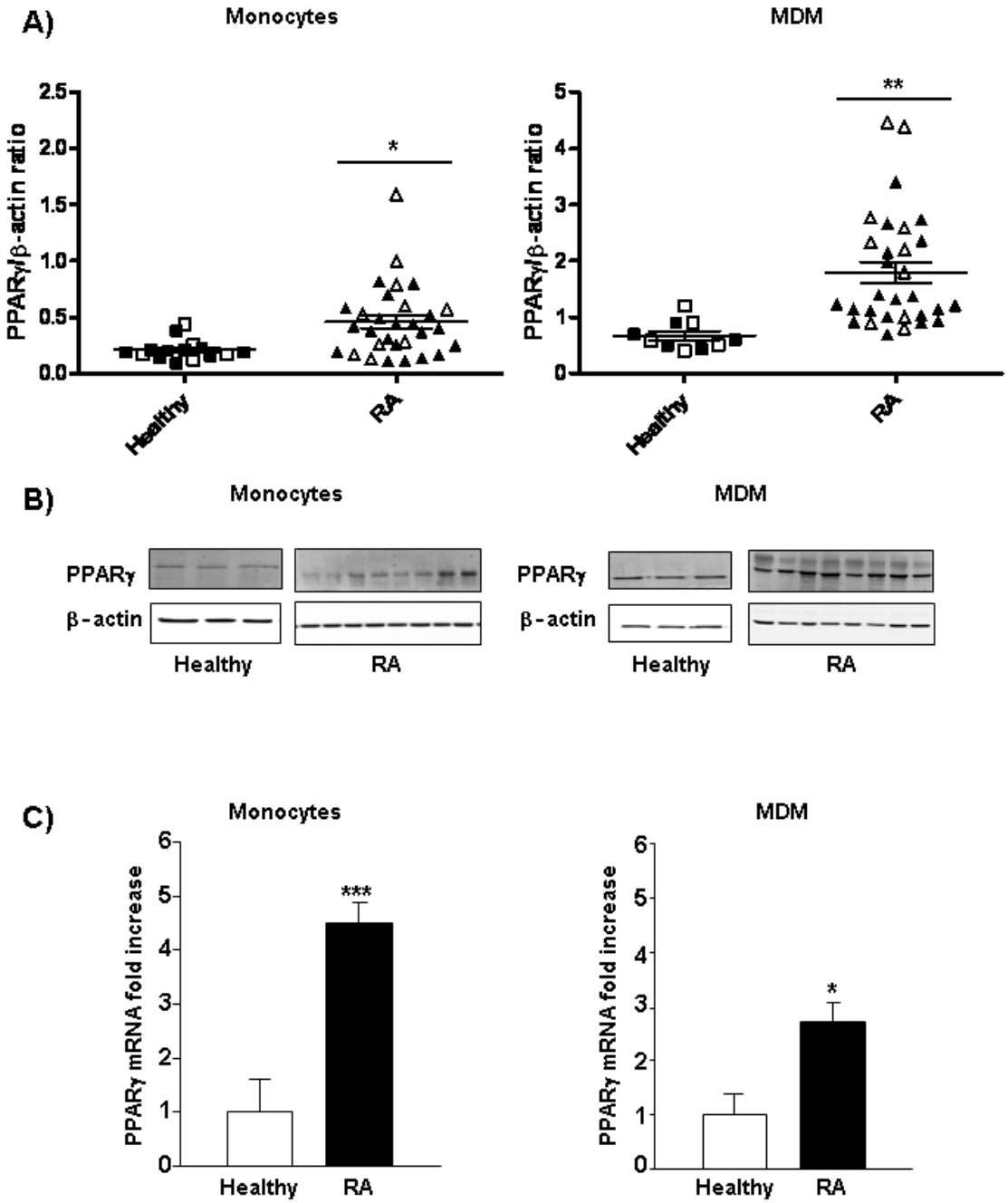

Figure 1. Enhanced PPARγ expression in monocytes and monocyte-derived macrophages (MDM) from rheumatoid arthritis (RA) patients. (**A**) PPARγ protein expression in healthy donors (n=15; ■ females, □ males) and RA patients (n=30; ▲ females, △ males). Semi-quantitative evaluation of PPARγ protein was performed by calculating the ratio between its total expression and β-actin. *P < 0.05 vs healthy donors; **P < 0.01 vs healthy donors. (**B**) Representative blots for PPARγ protein expression in healthy donors and RA patients. (**C**) PPARγ mRNA in cells from healthy donors (n=9) and RA patients (n=15 for monocytes; n=14 for MDM). Data are mean ± SEM; *P < 0.05 vs healthy donors; *** P < 0.001 vs healthy donors.

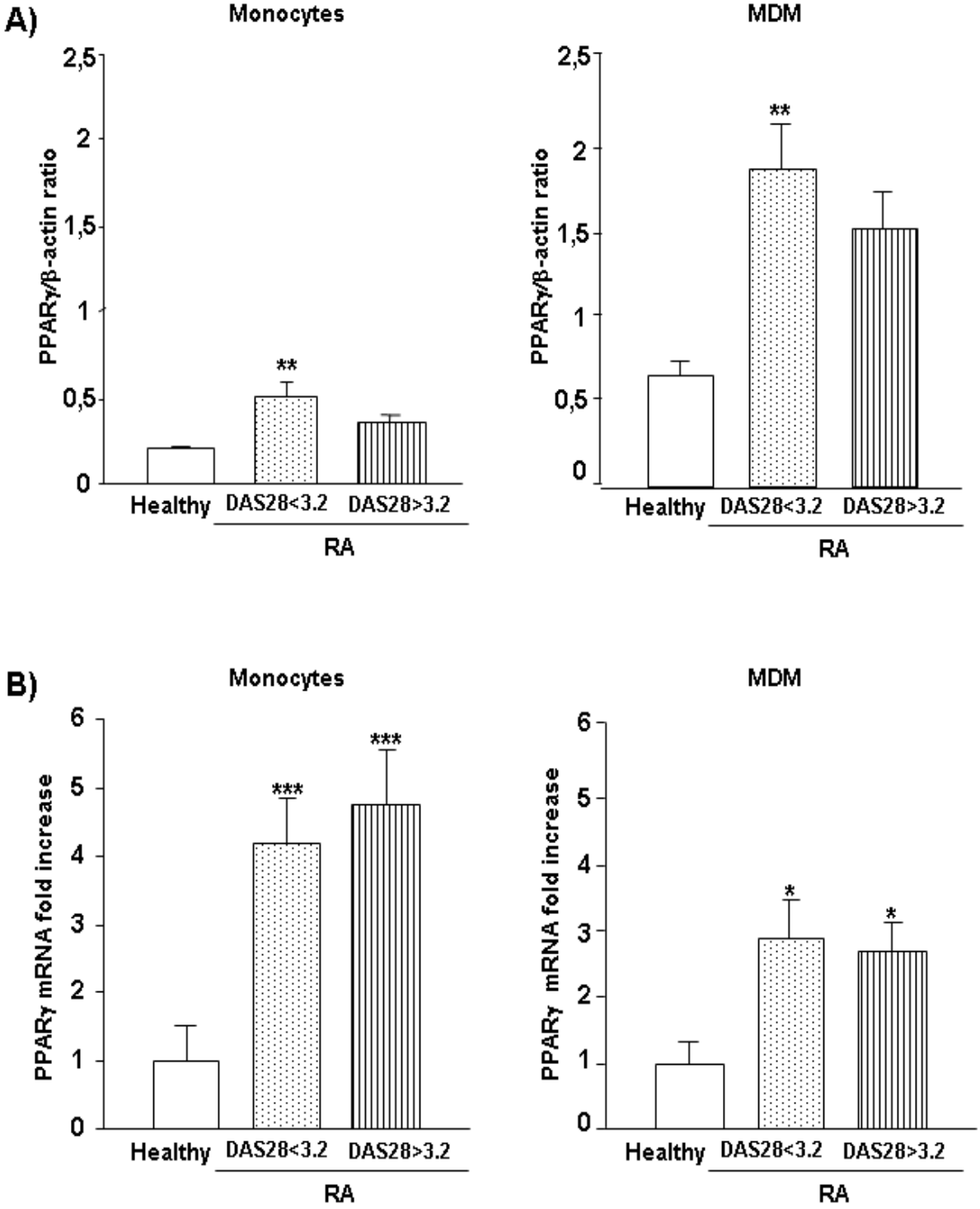

Figure 2. PPARγ expression in monocytes and MDM from RA patients, in relation to DAS28 scores. (A) PPARγ protein expression in healthy donors (n=15), RA patients with DAS28 score < 3.2 (n=20) or > 3.2 (n=10). Data are mean ± SEM; ** P < 0.01 vs healthy donors. (**B**) PPARγ mRNA expression in healthy 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 ± SEM. *P < 0.05 vs healthy donors; *** P < 0.001 vs healthy donors.

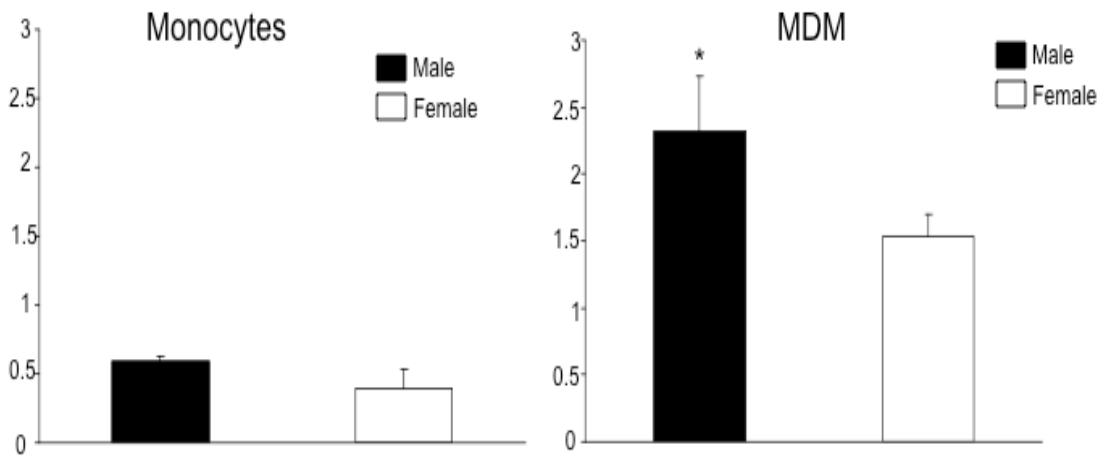

Figure 3. Gender difference in PPARγ expression in monocytes and monocyte-derived macrophages (MDM) from RA patients (20 females, 10 males). Semi-quantitative evaluation of PPARγ protein was performed by calculating the ratio between PPARγ/β-actin ratio. Data are mean ± SEM.; *P < 0.05 vs female.

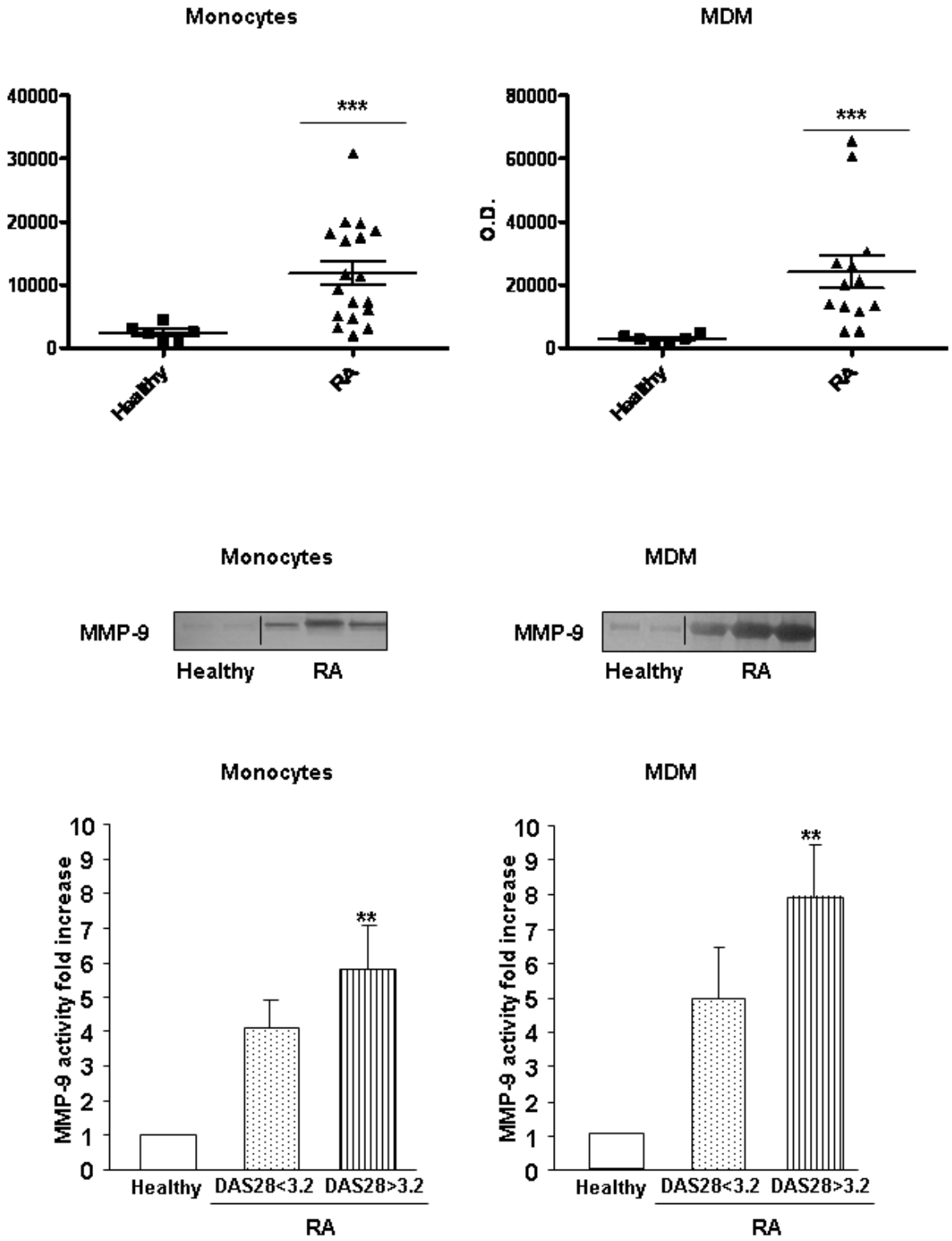

Figure 4. Enhanced matrix metalloproteinase-9 (MMP-9) activity in monocytes and MDM from RA patients. (**A**) MMP-9 activity in cells from RA patients (n=18 for monocytes; n=15 for MDM) as compared to healthy donors (n=6). ***P < 0.001 vs healthy donors. (**B**) Representative gelatin zymographies for MMP-9 activity in healthy donors and RA patients. (**C**) MMP-9 activity in cells from healthy donors (n=6), 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 ± SEM; **P < 0.01 vs healthy donors.

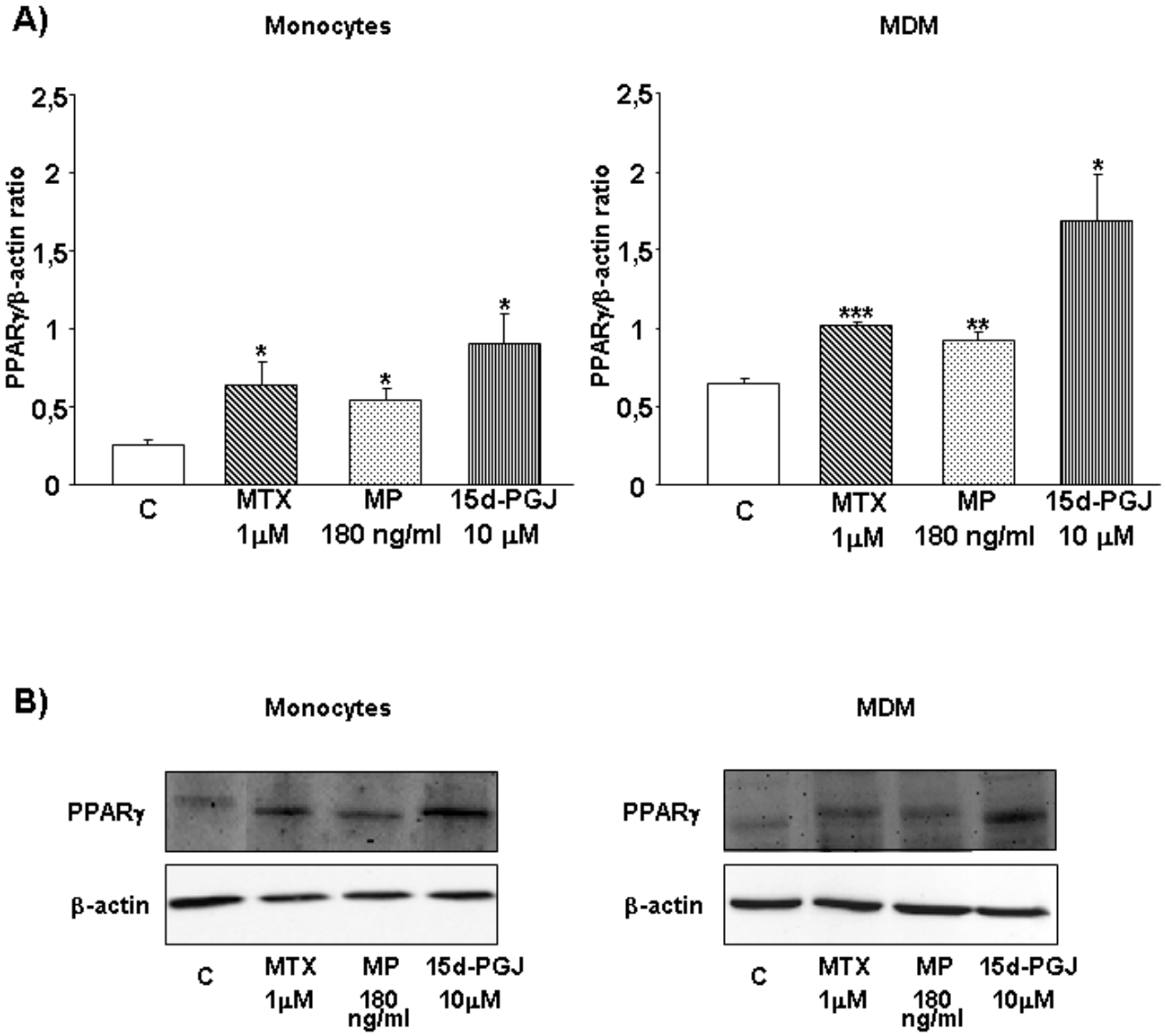

Figure 5. The PPARγ agonist 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ), methotrexate (MTX) and methylprednisolone (MP) increase PPARγ protein expression in cells isolated from healthy donors. (**A**) PPARγ/β-actin protein ratio in monocytes and MDM from healthy donors. Cells were challenged with fixed concentrations of drugs for 6 hr. Data are mean ± 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 PPARγ protein expression.

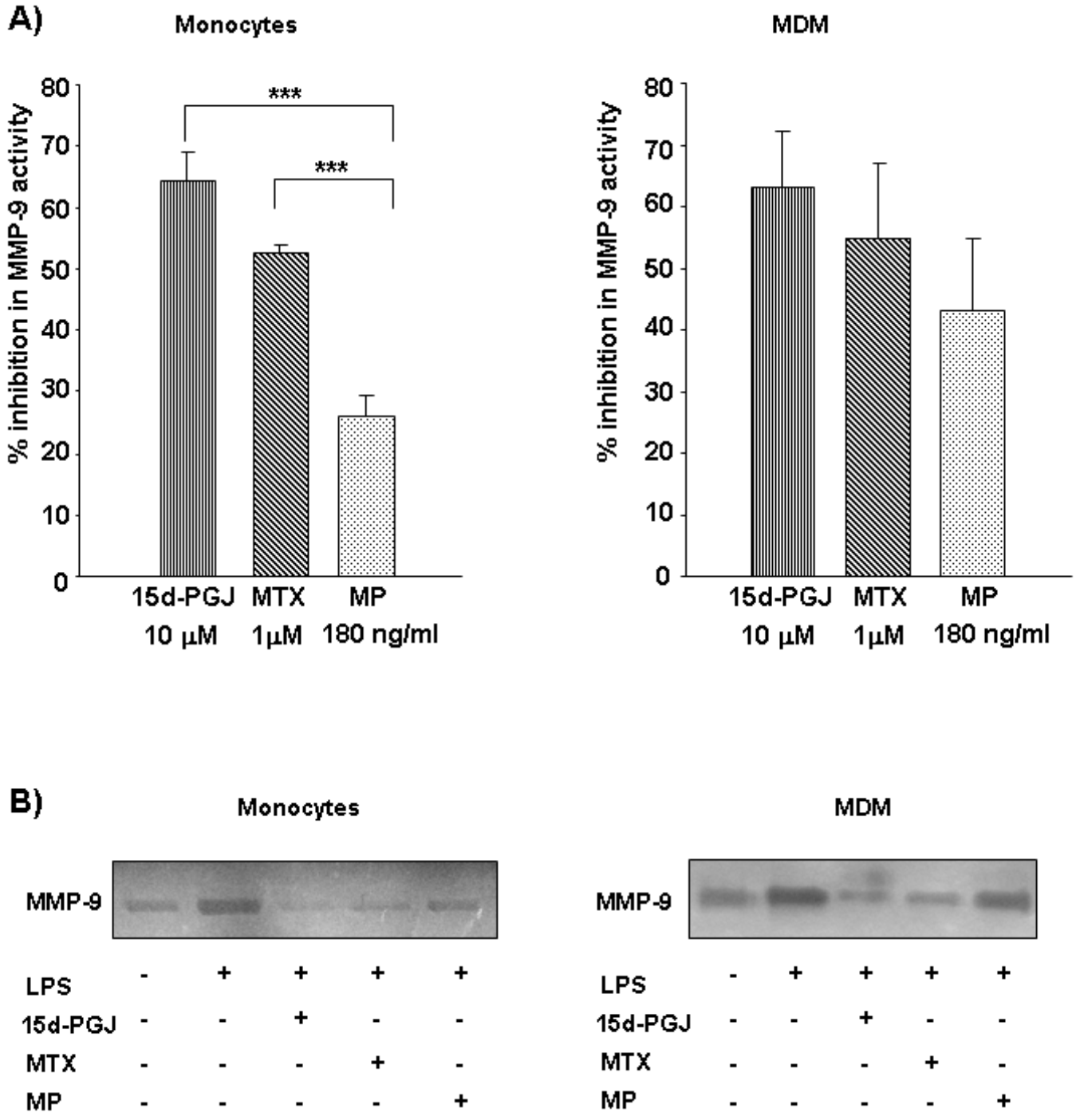

Figure 6. 15d-PGJ, MTX and MP inhibit LPS (lipopolysaccharide)-stimulated MMP-9 activity in human monocytes and MDM isolated from healthy donors. (**A**) % inhibition of LPS-stimulated MMP-9 activity. Cells were pre-treated with the drugs for 1 hr and then stimulated by LPS 100 ng/ml for 6 hr. Data are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

PAPER n.2

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-κB activation while enhancing PPARγ expression in human monocyte/macrophages"

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Previous studies have shown that NCX 6550 (NCX), a nitric oxide (NO)-donating pravastatin, induces anti-inflammatory effects in murine macrophage cell lines. Here, we have studied its activity in human monocyte/macrophages, by investigating cytokine release, NF-κB translocation and peroxisome proliferator-activated receptor γ (PPARγ) expression and function. For comparison, pravastatin, isosorbide-5-mononitrate (ISMN), sodium nitroprusside (SNP) and the PPARγ ligand 15-deoxy-Δ(12,14)-prostaglandin J(2) (PGJ) were also tested. Monocytes and macrophages (MDM: monocyte-derived macrophages) were isolated from healthy donors; cytokine release was measured by ELISA, NF-κB by electrophoretic mobility shift assay and PPARγ by Western blot and Real-Time PCR. NCX (1 nM-50 μM) dose-dependently inhibited phorbol 12-myristate 13-acetate (PMA)-induced TNF-α release from monocytes (IC_{50}=240 nM) and MDM (IC_{50}=52 nM). At 50 μM, it was more effective than pravastatin, ISMN and SNP ($P<0.05$), but less efficient than PGJ. Similar results were obtained for IL-6. Likewise, NCX was more effective than pravastatin and the other NO donors in inhibiting PMA-induced NF-κB translocation in both cell types, and, at the highest concentration, significantly ($P<0.05$) enhanced PPARγ protein expression in monocytes.

We conclude that NCX 6550 exerts a significant anti-inflammatory activity in human monocyte/macrophages that is also contributed by its NO donating properties, as the effects exerted by NCX 6550 are significantly higher than those evoked by pravastatin in many experimental assays. These data further indicate that the incorporation of a NO-donating moiety into a statin structure confers pharmacological properties which may translate into useful therapeutic benefits.

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The nitric oxide-donating pravastatin, NCX 6550, inhibits cytokine release and NF-κB activation while enhancing PPARγ expression in human monocyte/macrophages

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ABSTRACT

Previous studies have shown that NCX 6550 (NCX), a nitric oxide (NO)-donating pravastatin, induces anti-inflammatory effects in murine macrophage cell lines. Here, we have studied its activity in human monocyte/macrophages, by investigating cytokine release, NF-κB translocation and peroxisome proliferator-activated receptor γ (PPARγ) expression and function. For comparison, pravastatin, isosorbide-5-mononitrate (ISMN), sodium nitroprusside (SNP) and the PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ) were also tested.

Monocytes and macrophages (MDM: monocyte-derived macrophages) were isolated from healthy donors; cytokine release was measured by ELISA, NF-κB by electrophoretic mobility shift assay and PPARγ by Western blot and Real-Time PCR.

NCX (1 nM–50 μM) dose-dependently inhibited phorbol 12-myristate 13-acetate (PMA)-induced TNF-α release from monocytes (IC_{50} = 240 nM) and MDM (IC_{50} = 52 nM). At 50 μM, it was more effective than pravastatin, ISMN and SNP ($P<0.05$), but less efficient than PGJ. Similar results were obtained for IL-6. Likewise, NCX was more effective than pravastatin and the other NO donors in inhibiting PMA-induced NF-κB translocation in both cell types, and, at the highest concentration, significantly ($P<0.05$) enhanced PPARγ protein expression in monocytes.

We conclude that NCX 6550 exerts a significant anti-inflammatory activity in human monocyte/macrophages, that is also contributed by its NO donating properties, as the effects exerted by NCX are significantly higher than those evoked by pravastatin in many experimental assays. These data further indicate that the incorporation of a NO-donating moiety into a statin structure confers pharmacological properties which may translate into useful therapeutic benefits.

1. Introduction

There is evidence that grafting a nitric oxide (NO)-releasing moiety to well established drugs enhances their anti-inflammatory potential and confers additional properties, in a variety of models [1–4]. Human monocyte/macrophages play a key role in inflammation and chronic diseases and represent an interesting model to evaluate the effects of NO donating drugs. In fact, although human cells express the iNOS (inducible NO synthase) gene, their NO production differs markedly from that shown in murine macrophages or macrophage cell lines [5,6]. While treatment of rodent macrophages with IFN-γ, TNF-α or lipopolysaccharide (LPS) results in a rapid and abundant production of NO, the same stimuli fail to induce the appearance and accumulation of NO degradation products in cultured human monocyte/macrophages [5,6].

Abbreviations: EMSA, electrophoretic mobility shift assay; IL-6, interleukin-6; ISMN, isosorbide-5-mononitrate; MDM, monocyte-derived macrophage; NCX, NCX 6550: 1S-[1α(βS*,δS*),2α,6α,8β-(R*),8aα]-1,2,6,7,8,8a-hexahydro-β,δ,6-trihydroxy-2-methyl-8-(2-methyl-1-oxobutoxy)-1-naphtalene-heptanoic acid 4-(nitrooxy)butyl ester; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PGJ, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PMA, phorbol 12-myristate 13-acetate; PPARγ, peroxisome proliferator-activated receptor-gamma; SNP, sodium nitroprusside; TNF-α, tumour necrosis factor-alpha.

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Previous work on the NO-donating pravastatin NCX 6550 showed that the drug exerts potent anti-inflammatory activities in RAW 264.7 cells, a murine macrophage cell line [4], has anti-thrombotic activity and inhibits tissue factor expression, so reducing the generation of pro-coagulant activity by murine peritoneal macrophages [7]. It has also been reported that NCX 6550 stimulates angiogenesis and improves recovery from limb ischaemia in diabetic mice [8], reduces the generation of reactive oxygen species in normal and atherosclerotic mice and demonstrates a superior anti-inflammatory effect compared to pravastatin [9,10]. With this background, it was critical to understand whether such results could be validated in human tissues.

There is evidence that, besides lipid lowering, statins exert beneficial effects through cholesterol-independent properties, including anti-inflammatory activity [11–13]. For example, in monocyte/macrophage cell lines, statins have been shown to inhibit LPS-induced cyclooxygenase-2 (COX-2) expression, prostanoid release and Nuclear Factor-kappa B (NF-κB) translocation [14], and to increase peroxisome proliferator-activated receptor (PPAR)-γ activity [15].

The role of PPARγ in regulating inflammatory processes and atherosclerosis is widely accepted. The anti-inflammatory potential of PPARγ agonists mainly resides in their ability to inhibit monocyte/macrophage activation and the expression of inflammatory molecules, such as TNF-α, IL-6, IL-1β, iNOS, gelatinase B and COX-2 [16–18]. Therefore, PPARγ has been suggested to mediate some of the pleiotropic actions of statins [19].

Hence, we have assessed the anti-inflammatory potential of NCX 6550 and compared it with that elicited by the reference pravastatin in monocytes and macrophages (MDM: monocyte-derived macrophages) from healthy donors. We investigated the ability of NCX 6550 to modulate cytokine release, NF-κB nuclear translocation and PPARγ expression, as compared to pravastatin, sodium nitroprusside (SNP; a direct NO donor [2]), isosorbide-5-mononitrate (ISMN; a NO donor requiring metabolism [2]), and the PPARγ ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ).

Here we demonstrate that NCX 6550 enhances PPARγ protein expression and inhibits NF-κB activation and cytokine release induced by the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate (PMA). These effects are dose-dependent and, at the maximal 50 μM concentration, NCX 6550 is significantly more effective than pravastatin, SNP and ISMN.

2. Materials and methods

2.1. Cell culture

Human monocytes were isolated from heparinized venous blood (30–40 ml) of healthy donors by standard techniques of dextran sedimentation, Hystopaque (density = 1.077 g/cm^3) gradient centrifugation (400 × g, 30 min, room temperature) and recovered by thin suction at the interface, as described elsewhere [16]. Cells were resuspended in RPMI 1640 medium, supplemented with 5% heat-inactivated foetal bovine serum (FBS), 2 mM glutamine, 50 μg/ml streptomycin, 5 U/ml penicillin and 2.5 μg/ml amphotericin B; purified monocyte populations were obtained by adhesion (90 min, 37 °C, 5% CO_2). Cell viability (trypan blue dye exclusion) was usually > 98% and was not affected by the different compounds under evaluation (data not shown).

Monocyte-derived macrophages (MDM) were prepared from monocytes, by culture (8–10 days) in RPMI 1640 medium containing 20% FBS, glutamine and antibiotics; medium was exchanged every 2–3 days [16]. MDM were defined as macrophage-like cells by evaluating surface markers CD14, MHCII, CD1a and CD68 [20]. Briefly, adherent cells were detached by gentle scraping. After three washings with sterile phosphate-buffered saline (PBS), cells were resuspended at the final concentration of 1×10^5 cells/ml and fluorescent dye-labelled antibodies against the different surface markers (anti-CD14 from Becton Dickinson, Oxford, UK; anti-CD68 and anti-MHCII from Dako, Milan, Italy; anti-CD1a from Bioscence, San Diego, CA, USA) were added for 30 min on ice and the reaction was stopped with 4% paraformaldehyde, according to the manufacturer's instructions. Incubation was performed in the dark and expression of surface markers was analysed by flow cytometry.

A different number of cells was used according to the type of experiments (2×10^6 cells for Western blot experiments, 5×10^6 cells for EMSA assays and 1×10^6 cells for cytokine release). The murine macrophage RAW 264.7 cells (ATCC, Manassas, VA, USA), which were used for comparison in some experiments (see below), were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, glutamine and antibiotics.

To rule out toxic effects of the different drugs, we evaluated the cell viability by the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay, in addition to the trypan blue dye exclusion evaluations. In these experiments, monocytes (1×10^5) were challenged with the maximal concentration of the compounds for 6 or 24 h. Thereafter, the medium was replaced by the MTT solution (1 mg/ml) after 2 h incubation at 37 °C in the dark. The supernatant was removed and DMSO was added in order to dissolve the purple formazan; the absorbance of the samples was read at 580 and 675 nm. All the compounds did not reduce *per se* monocyte viability, absorbance values being always around 0.11 (MTT assay), as control cells.

2.2. Nitrite accumulation and superoxide anion (O_2^-) production

We first characterized the effects of NCX 6550 on the NO pathway and oxy-radical production. Since human monocytes are known to release very low amounts of NO [5,6], the murine macrophage cell line RAW 264.7 was used as a positive internal control. Nitrite content was determined in 100 μl of cell culture medium by the Griess colorimetric reaction, using "Griess Reagent System", according to manufacturer's instructions (Promega, Madison, USA).

Briefly, 1×10^5 RAW 264.7 cells and 1×10^5 human monocytes were pre-treated for 30 min with NCX 6550 at the maximal 50 μM concentration (see later) and then challenged with phorbol 12-myristate 13-acetate (PMA) or lipopolysaccharide (LPS) for 30 min or 24 h. PMA was used at 10^{-6} M (30 min) or 10^{-7} M (24 h); LPS was used at 100 ng/ml (30 min) or 10 ng/ml (24 h). These two time-points were selected according to the optimal times required to induce superoxide anion production (30 min) and cytokine release (24 h) in human monocyte/macrophages.

To evaluate superoxide anion (O_2^-) production, human monocytes and RAW 264.7 cells (1×10^6 cells/plate) were washed twice with PBS, and stimulated, in the absence or presence of NCX 6550, by PMA 10^{-6} M or LPS 100 ng/ml for 30 min. O_2^- production was evaluated by the superoxide dismutase (SOD)-inhibitable cytochrome *C* reduction, the absorbance changes being recorded at 550 nm in a Beckman DU 650 spectrophotometer. O_2^- production was expressed as nmol cytochrome *C* reduced/10^6 cells/30 min, using an extinction coefficient of 21.1 mM [21]. To avoid interference with spectrophotometrical recordings of O_2^- production, cells were incubated with RPMI 1640 without phenol red, antibiotics and FBS.

2.3. PPARγ protein expression in monocyte/macrophages and semi-quantitative analysis

PPARγ expression in human monocytes and MDM was evaluated by Western blot, as described [16,17,20]. Accordingly, cells

were evaluated either as they stand (i.e., "basal, constitutive PPARγ expression") or after challenge (6 h, 37 °C, 5% CO_2) with NCX 6550, pravastatin, sodium nitroprusside (SNP), isosorbide-5-mononitrate (ISMN) or the endogenous PPARγ ligand PGJ, for comparison. The 6 h challenge period was found to be the optimal one [20]. Cells were washed twice with ice-cold PBS and scraped off the wells in lysis buffer containing 3% SDS, 0.25 M Tris and 1 mM phenyl-methyl-sulfonyl fluoride (PMSF). Cells were lysed by sonication; when necessary, cell lysates were stored at −80 °C. The determination of protein concentration was done by the bicinchoninic acid assay (BCA). Protein samples (20 μg) were analysed by SDS-PAGE (10% acrylamide) and electro-blotted on nitrocellulose membrane (Protran, PerkinElmer Life Sciences). Immunoblots were performed according to standard methods using the following antibodies: monoclonal mouse anti-human PPARγ (E-8; Santa Cruz, USA; 1:1000 in TBS-T 5% milk) and monoclonal mouse anti-human β-actin (Sigma, USA; 1:5000 in TBS-T 3% BSA), as described [16,20]. Anti-mouse secondary antibody was coupled to horseradish peroxidase (Amersham Biosciences, UK). Proteins were visualized with an enzyme-linked chemiluminescence detection kit according to the manufacturer's (PerkinElmer) instructions. Chemiluminescence signals were analysed under non-saturating conditions with an image densitometer (Versadoc, Bio-Rad, USA). Semi-quantitative evaluation of PPARγ protein was performed as described [16,20], by calculating the ratio between its expression and the expression of the reference housekeeping protein, β-actin.

2.4. RNA isolation and Real-Time PCR

Total RNA was extracted from monocytes with the GenElute Mammalian Total RNA Miniprep kit (Sigma, USA), according to the manufacturer's instructions. The amount and purity of total RNA was 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 Biosystem; Carlsbad, CA) according to the manufacturer's instructions. Monocytes were challenged with NCX 6550, pravastatin, SNP, ISMN or the selective PPARγ ligands, PGJ and rosiglitazone, for 2 h. Real-Time PCR was carried out in a volume of 20 μl per well in a 96-well Optical Reaction Plates (Applied Biosystem) containing 1 μl TaqMan Expression Assay (PPARγ), 5 μl RNase free water, 10 μl TaqMan Universal PCR MasterMix (2×) (without AmpErase UNG) and 4 μl cDNA template. The plate was run on the Applied Biosystem 7000 ABI Prism System. To compensate for variations in cDNA concentrations and PCR efficiency between tubes, an endogenous gene control (beta-glucuronidase, GUSB) was included for each sample and used for normalization [22]. Results were analysed by the comparative cycle threshold method, according to Livak and Schmittgen [22].

2.5. NF-κB activation

The evaluation of NF-κB activation was performed by electrophoretic mobility shift assays (EMSA), as described [21]. Cells were pre-treated for 30 min with NCX 6550, pravastatin, SNP, ISMN, PGJ or vehicle, and stimulated by PMA 10^{-6} M for 1 h. According to previous experience, monocyte/macrophages challenged with PMA 10^{-6} M for 1 h presented a maximal NF-κB nuclear translocation [23]. Nuclear extracts were prepared by using "Nuclear Extraction kit" (Active Motifs, Rixensart, Belgium). Briefly, cells were resuspended in hypotonic buffer, lysed with 5% (v/v) detergent and centrifuged for 30 s at 14,000 × *g* in a microcentrifuge pre-cooled at 4 °C. Supernatant (cytoplasmic fraction) was recovered in a new tube; the pellet (nuclear fraction) was resuspended in lysis buffer and incubated for 30 min on ice on a rocking platform (150 rpm). Nuclear fraction was then centrifuged (10 min, 14,000 × *g*, 4 °C) and the supernatant (nuclear extract) transferred and stored at −80 °C until use.

Nuclear extracts (5 μg) were incubated with 2 μg poly (dI-dC) and the [γ-^{32}P]ATP-labelled oligonucleotide probe (100,000–150,000 cpm; Promega) in binding buffer (50% glycerol, 10 mM Tris–HCl, pH 7.6, 500 mM KCl, 10 mM EDTA, 1 mM dithiothreitol) in a final volume of 20 μl for 30 min at room temperature. The NF-κB consensus oligonucleotide (5′-AGTTGAGGGGAC TTTCCCAGGC-3′) was obtained from Promega. The nucleotide–protein complex was separated on a 5% polyacrylamide gel in 0.5 × TBE buffer (100 mM Tris–HCl, 100 μM boric acid, 2 mM EDTA) at 150 V on ice. The gel was dried and radioactive bands were detected by autoradiography [21]. Supershift assays were performed using a commercial antibody (anti-NF-κB p65(F6): sc-8008) from Santa Cruz Biotechnology, Inc., (CA, USA).

2.6. Cytokine release

Cytokine release was measured by enzyme-linked immunoassay kit (Pelikine Compact™ human ELISA kit; CBL, Netherlands), as previously reported [16,20,21]. TNF-α and IL-6 were evaluated as the most relevant pro-inflammatory cytokines, whereas IL-10 was selected as the reference anti-inflammatory cytokine. The amount of each cytokine was expressed in pg/ml, as indicated by the manufacturer's instructions. For these experiments, cells (1×10^6) were pre-treated for 1 h with NCX 6550 (1 nM–50 μM) and then stimulated by PMA 10^{-7} M for 24 h. According to previous experiments [20,21], the 24 h challenge period ensured the maximal cytokine release. NCX 6550 effects on cytokine release were compared with those elicited by PGJ (1 nM–50 μM), and the reference substances pravastatin, SNP and ISMN, all used at 50 μM.

2.7. Materials

FBS was from Gibco (Paisley, UK). PBS, Hystopaque, RPMI 1640 medium, glutamine, Hepes, streptomycin, penicillin, amphotericin B, protease inhibitors, SOD, cytochrome *C*, MTT, Poly (dI-dC) and monoclonal mouse anti-human β-actin antibodies were obtained from Sigma (St Louis, USA). The monoclonal mouse anti-human PPARγ (E-8) antibody was from Santa Cruz (CA, USA). Rosiglitazone and PGJ were from Cayman (Ann Arbor, USA). NCX 6550, pravastatin, SNP and ISMN were kindly provided by NicOx Research Institute, Bresso, Milan (Italy). They were dissolved in DMSO and then diluted in buffer and used at a maximal 50 μM concentration, so that DMSO final concentration did not exceed 0.5% and did not interfere with the assays reported here (not shown). Tissue-culture plates were from Nunc Ltd. (Denmark); all cell culture reagents, with the exception of FBS, were endotoxin-free according to details provided by the manufacturer.

2.8. Statistical analysis

All statistical analyses were performed using SPSS statistical software (version 15.0, SPSS Inc., Chicago, IL). Data are mean ± S.E.M. of '*n*' independent experiments. Concentration-effect curves for NCX 6550 and PGJ were constructed and their IC_{50} values (on PMA-induced cytokine release) were interpolated from curves of best fit. Statistical evaluation was performed by one-way analysis of variance between groups (ANOVA) followed by Bonferroni *post hoc* test or Student's *t*-test when appropriate. Differences were considered statistically significant when $P < 0.05$.

3. Results

3.1. Effects of NCX 6550 on nitrite accumulation and oxy-radical production in human monocytes

NCX 6550 was previously demonstrated to produce, in whole rat blood experiments, a linear time-dependent increase in nitrosylhemoglobin formation, which is consistent with a slow NO release kinetics [4]. In murine macrophage RAW 264.7 cells stimulated by IFNγ and LPS, it was also shown to significantly reduce iNOS protein expression, as well as nitrite accumulation [4]. Therefore, we have evaluated whether NO release could also be evidenced in human monocytes. In these experiments, human monocytes and RAW 264.7 cells (shown for comparison) were challenged with NCX 6550 at 50 μM for 30 min, and then stimulated by PMA or LPS for further 30 min or 24 h.

As reported in Fig. 1A, human monocytes spontaneously release low amounts of nitrite (one of the inactive oxidation products derived from NO), thus confirming data from the literature [5]. It is also worth noting that human monocytes challenged with NCX 6550 for 30 min displayed a small, although significant ($P<0.05$), increase in nitrite accumulation as compared to control monocytes (Fig. 1A). In RAW 264.7 cells, PMA or LPS did not affect nitrite accumulation after 30 min challenge, but significantly increased nitrite production after 24 h, as shown previously [4]. In keeping with previous data [4], NCX 6550 potently inhibited nitrite accumulation in activated RAW 264.7 cells challenged with stimuli for 24 h. Moreover, PMA-stimulated monocytes (but not LPS-treated cells) exhibited a significantly ($P<0.05$ *vs* control) increased nitrite accumulation after 30 min and 24 h challenge (Fig. 1A); this suggests that, in human monocytes, PMA is able to induce *de novo* synthesis of iNOS protein and, consequently, nitrite accumulation in the cell culture medium, as evidenced in other cell types [24]. Interestingly, unlike pravastatin (used at the same 50 μM concentration; not shown), NCX 6550 inhibited nitrite accumulation ($P<0.05$) in both RAW 264.7 cells and PMA-stimulated human monocytes (Fig. 1A).

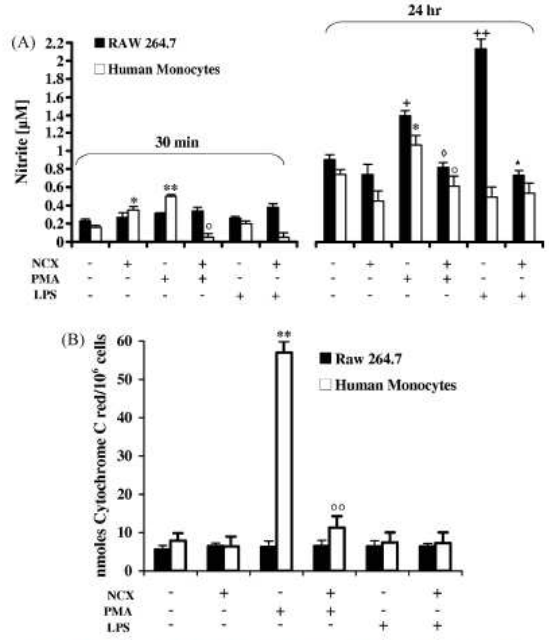

Fig. 1. Effects of NCX 6550 on nitrite accumulation (1A) and oxy-radical production (1B). In A: nitrite accumulation. RAW 264.7 cells (■) and human monocytes (□) were treated with or without NCX 6550 (NCX, 50 μM) and then stimulated by PMA or LPS for 30 min or 24 h. Values are mean ± S.E.M.; $n=3$. *$P<0.05$ *vs* control, un-stimulated monocytes; **$P<0.01$ *vs* control, un-stimulated monocytes; °$P<0.05$ *vs* PMA-stimulated monocytes; +$P<0.05$ *vs* control, un-stimulated RAW 264.7 cells; ++$P<0.01$ *vs* control, un-stimulated RAW 264.7 cells; ◊$P<0.05$ *vs* PMA-stimulated RAW 264.7 cells; ▲$P<0.05$ *vs* LPS-stimulated RAW 264.7 cells. In B: superoxide anion production. RAW 264.7 cells (■) and human monocytes (□) were treated with or without NCX and then stimulated by PMA or LPS for 30 min. Values are mean ± S.E.M.; $n=4$. **$P<0.001$ *vs* control, un-stimulated monocytes; °°$P<0.01$ *vs* PMA-stimulated monocytes.

Table 1
PMA-evoked superoxide anion production in human monocytes: effects of NCX 6550, pravastatin and SNP.

	% inhibition of PMA-evoked O_2^- production
NCX 50 μM	82 ± 4
NCX 10 μM	68 ± 4
NCX 1 μM	32 ± 2
PRAVA 50 μM	55 ± 6*
PRAVA 10 μM	30 ± 5*
PRAVA 1 μM	5 ± 3*
SNP 50 μM	32 ± 6*
SNP 10 μM	21 ± 5*
SNP 1 μM	6 ± 4*

Human monocytes were stimulated by PMA 10^{-8} M for 30 min, in the absence or presence of NCX 6550 (NCX), pravastatin (PRAVA) or sodium nitroprusside (SNP). PMA-evoked O_2^- production amounted to 59 ± 3 nmoles cytochrome *C* reduced/10^6 cells/30 min ($n=4$). Values are mean ± S.E.M.; $n=4$.
* $P<0.05$ *vs* corresponding NCX concentrations.

We have also checked the ability of NCX 6550 to affect superoxide anion (O_2^-) production, since human monocyte/macrophages are major phagocytes and release relevant amounts of oxy-radicals upon challenge with appropriate stimuli. We confirm that the murine macrophage cell line does not undergo a significant respiratory burst, in contrast to human monocytes (Fig. 1B). As expected, a robust O_2^- production (59 ± 3 nmoles cytochrome *C* reduced/10^6 cells/30 min; $n=4$) was documented in PMA-stimulated human monocytes, but not in LPS-challenged cells (Fig. 1B). Interestingly, NCX 6550 at 50 μM potently reduced (about 80% inhibition) PMA-induced O_2^- production (Fig. 1B; Table 1). When used at 50 μM, pravastatin also inhibited (by about 55%) PMA-induced respiratory burst in human monocytes, whereas SNP, at the same concentration, showed less effects on PMA-induced O_2^- generation (Table 1). Interestingly, at 1 μM concentration, NCX (but not the two other compounds) still inhibited PMA-induced O_2^- generation (Table 1).

3.2. NCX 6550 inhibits the release of pro-inflammatory cytokines in human monocyte/macrophages

According to previous experiments [20,21], a 24 h challenge with PMA (10^{-7} M) induces the maximal release of pro-inflammatory cytokines from human monocytes and MDM. PMA-evoked TNF-α release was higher in monocytes (1323 ± 58 pg/ml) than in MDM (574 ± 113 pg/ml; $n=8$; $P<0.05$ *vs* monocytes).

As depicted in Fig. 2, NCX 6550 and the PPARγ ligand PGJ inhibited, in a concentration-dependent manner (1 nM–50 μM), PMA-induced TNF-α release in both monocytes (Fig. 2A) and MDM (Fig. 2B). NCX 6550 was less potent than PGJ either in monocytes (Fig. 2A) with IC_{50} values of 240 and 92 nM, respectively, or in MDM (Fig. 2B), with IC_{50} values of 52 and 48 nM, respectively. Interestingly, the maximal inhibition (about 90%) afforded by NCX 6550 at 50 μM was significantly higher ($P<0.001$) compared to that of pravastatin and the two NO donors, SNP and ISMN, used at the same concentration (Fig. 2).

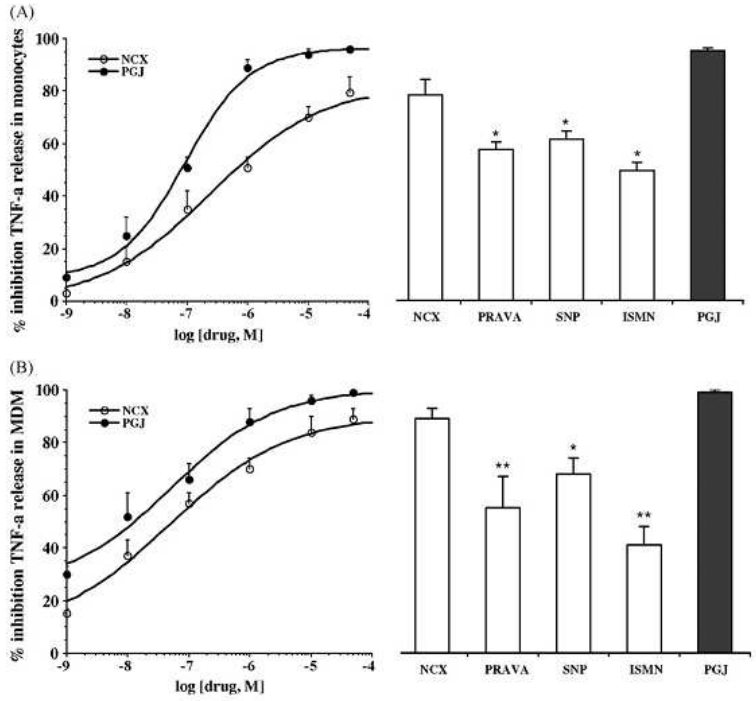

Fig. 2. NCX 6550 dose-dependently inhibits PMA-induced TNF-α release in human monocytes (A) and MDM (B). On the left: cells were pre-treated for 1 h with PGJ (1 nM–50 μM: ●) or NCX (1 nM–50 μM: ○) and then stimulated by PMA 10^{-7} M for 24 h. On the right: inhibition afforded at the maximal 50 μM concentration by NCX, pravastatin (PRAVA), sodium nitroprusside (SNP), isosorbide mononitrate (ISMN) or PGJ. Values are mean ± S.E.M.; $n = 8$. *$P < 0.05$ *vs* NCX; **$P < 0.01$ *vs* NCX.

Similar results were obtained with IL-6. PMA-induced IL-6 release was 468 ± 70 pg/ml ($n = 8$) in monocytes and 309 ± 28 pg/ml in MDM ($n = 8$; $P < 0.05$ *vs* monocytes). As shown in Fig. 3, NCX 6550 inhibited, in a concentration-dependent manner, PMA-induced IL-6 release in both human monocytes (Fig. 3A) and MDM (Fig. 3B), with IC_{50} values of 122 nM (monocytes) and 185 nM (MDM). Also for this marker, PGJ was more potent than NCX 6550 (IC_{50} values: 72 nM in monocytes and 89 nM in MDM). At the maximal 50 μM concentration, NCX 6550 was significantly more effective than pravastatin, SNP and ISMN (Fig. 3).

NCX 6550, as well as the other drugs, had no significant effect on PMA-induced IL-10 release (data not shown). In these experiments, PMA 10^{-7} M released small amounts of IL-10 (50 ± 7 pg/ml in monocytes and 45 ± 8 pg/ml in MDM; $n = 8$), significantly lower than the levels of pro-inflammatory cytokines.

3.3. NCX 6550 inhibits PMA-induced NF-κB translocation in human monocyte/macrophages

In these experiments, cells were pre-treated with drugs for 30 min and then stimulated by PMA (10^{-6} M) for 1 h, that ensures maximal effects [23]. In human monocytes, PMA induced a marked NF-κB nuclear translocation, with a major involvement of the p65 subunit, as demonstrated by supershift assays (Fig. 4A). NCX 6550, at 50 μM, potently inhibited PMA-induced NF-κB activation, whereas pravastatin was almost ineffective; SNP also reduced PMA-induced NF-κB activation (Fig. 4A). Moreover, NCX 6550 (10 nM–50 μM) produced a concentration-dependent inhibition of PMA-triggered NF-κB translocation in human monocytes (Fig. 4B). The endogenous PPARγ agonist PGJ, known to inhibit NF-κB activation [25], was also tested for comparison (Fig. 4B). Likewise, in human MDM, NCX 6550 and NO donors, but not pravastatin, inhibited PMA-induced NF-κB translocation (Fig. 4C).

3.4. NCX 6550 enhances PPARγ expression in human monocyte/macrophages

Since PPARγ is expressed in human monocyte/macrophages [16,17,20] and NO has been reported to increase PPARγ activity [26], we evaluated whether NCX 6550 affected PPARγ protein expression in human monocyte/macrophages.

In keeping with previous results [16,17,20], a lower constitutive expression of PPARγ protein was detected in human monocytes (PPARγ/β-actin ratio: 0.21 ± 0.031; $n = 4$; Fig. 5A) compared to MDM (PPARγ/β-actin ratio: 1.78 ± 0.38; $n = 4$; $P < 0.001$ *vs* monocytes; Fig. 5B). NXC 6550 and SNP, in the range of 1–50 μM, significantly enhanced PPARγ expression in monocytes, whereas pravastatin and ISMN were ineffective (Fig. 5A). On the contrary, the effects obtained in MDM were not statistical significant, possibly due to the higher constitutive PPARγ expression in these cells (Fig. 5B). In fact, even the PPARγ agonist PGJ (10 μM), tested as positive control, was less effective in MDM than in monocytes (Fig. 5).

By the Real-Time PCR, we also evaluated the effects of NCX 6550 on PPARγ gene expression in monocytes, in comparison with pravastatin, SNP, ISMN and two different PPARγ agonists, the endogenous ligand PGJ and the oral anti-diabetic drug rosiglitazone (Table 2). All drugs enhanced PPARγ mRNA levels of about twofold ($P < 0.05$ *vs* control monocytes; Table 2), NCX 6550 resulting particularly effective.

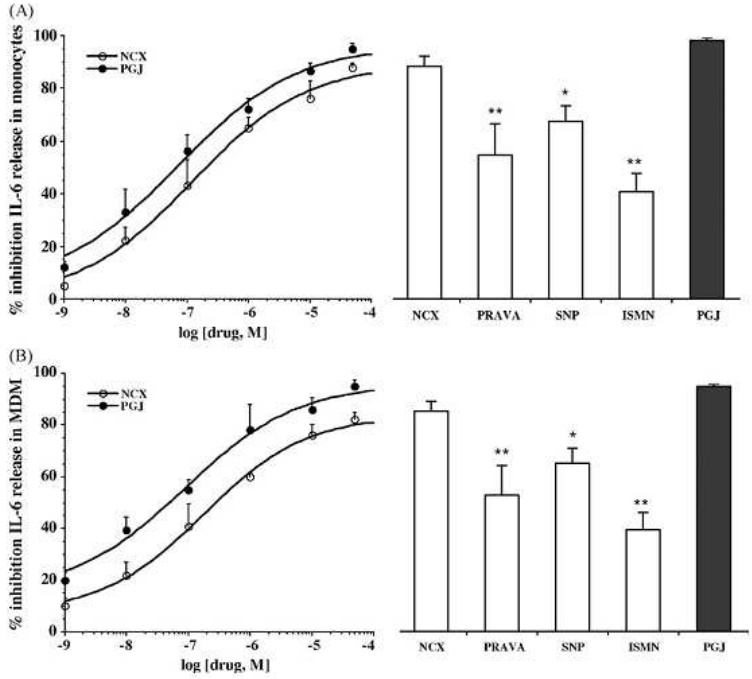

Fig. 3. NCX 6550 dose-dependently inhibits PMA-induced IL-6 release in human monocytes (A) and MDM (B). On the left: cells were pre-treated for 1 h with PGJ (1 nM–50 μM; ●) or NCX (1 nM–50 μM; ○) and then stimulated by PMA 10^{-7} M for 24 h. On the right: inhibition afforded at the maximal 50 μM concentration by NCX, pravastatin (PRAVA), sodium nitroprusside (SNP), isosorbide mononitrate (ISMN) or PGJ. Values are mean ± S.E.M.; $n = 8$. *$P < 0.05$ *vs* NCX; **$P < 0.01$ *vs* NCX.

Table 2
PPARγ gene expression in monocytes.

	Fold increase *vs* control (control = 1)	P^* value
Control	1	
PGJ	1.61 ± 0.05	0.05
ROSI	1.91 ± 0.04	0.05
NCX	2.01 ± 0.12	0.05
ISMN	1.68 ± 0.03	0.05
SNP	1.91 ± 0.06	0.05
PRAVA	1.97 ± 0.02	0.05

Monocytes were challenged for 2 h with PGJ (10 μM), rosiglitazone (ROSI; 1 μM), NCX (50 μM), pravastatin (PRAVA; 50 μM), SNP (50 μM) or ISMN (50 μM). mRNA from un-stimulated (control) monocytes ($n = 4$) represents the 'calibrator' (as called by the 7000 System JDS Software, Applied Biosystem) value = 1. Values are mean ± S.E.M.; $n = 4$.

* $P = 0.05$ *vs* control, un-stimulated monocytes. No significant differences among the different group treatments.

4. Discussion

The main finding of this study is that the NO-donating pravastatin, NCX 6550, inhibits PMA-induced cytokine release and NF-κB activation in human monocyte/macrophages and, at the highest concentration used (50 μM), enhances PPARγ expression in monocytes. Conversely, under the same experimental conditions, pravastatin (the native compound) and two reference NO donors, SNP and ISMN, do not display similar level of activity. It is worth noting that NCX 6550, a compound with a dual mode of action, i.e., inhibition of HMG CoA reductase and NO donation, maintains HMG CoA reductase inhibition properties, since, in rat aortic smooth muscle cells, it inhibits cholesterol biosynthesis with an IC_{50} value (9.4 μM: NicOx, internal data) in the same low micromolar range as reported with other NO-donating statins [27].

Overall, NCX 6550 shows its anti-inflammatory properties in a cell model, as human monocytes, that is relevant to atherosclerosis and coronary artery disease. In fact, the earliest noticeable lesions of atherosclerosis, fatty streaks, contain large numbers of foam cells, derived from circulating monocytes. After recruitment, monocytes adhere to the activated endothelium and migrate into the artery wall, where they differentiate into macrophages and accumulate lipids to form foam cells, which largely affect the progression of atherosclerosis. Indeed, not only they mediate the uptake of oxidized low-density lipoproteins (LDL), but also promote LDL oxidation, undergo a respiratory burst, release pro-inflammatory cytokines and secrete matrix metalloproteinases [28].

Moreover, human monocyte/macrophages are intriguing cell types to evaluate the NO involvement in inflammatory responses. Different experimental data of the early 1990s clearly point out that, at variance from rodent macrophages and murine cell lines, human monocyte/macrophages release little or no NO after *in vitro* challenge with standard stimuli, yet they express the iNOS gene and respective protein [5,6]. The lack of biopterin [6] and/or a different arginine transport-specific response [29] in human monocyte/macrophages could explain this discrepancy. Moreover, Carreras et al. [30] clearly showed the different kinetics of NO, oxy-radical and peroxynitrite production during the respiratory burst of human neutrophils.

Our results are consistent with these previous data, as we demonstrate a low but significant nitrite accumulation and a high superoxide anion production in PMA-stimulated monocytes. Interestingly, a 30-min challenge of human monocytes with NCX 6550 results in a low, although significant, increase in nitrite

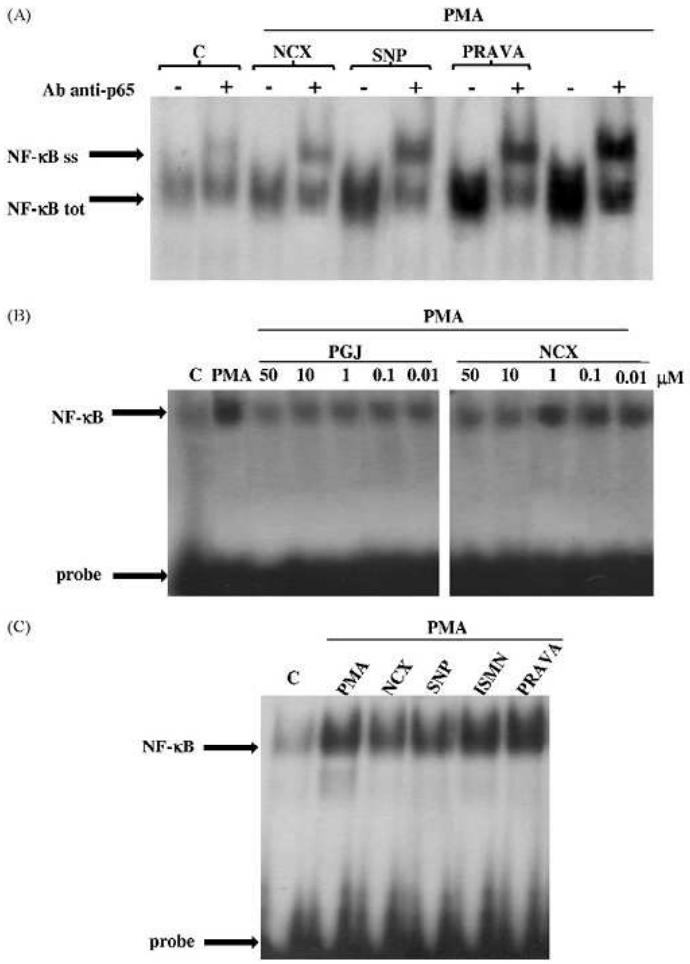

Fig. 4. NCX 6550 inhibits PMA-induced NF-κB translocation in human monocytes and MDM. In A: Effects of NCX 6550 and other compounds in human monocytes. Cells were pre-treated for 30 min with NCX, sodium nitroprusside (SNP), or pravastatin (PRAVA), all at 50 μM, and then stimulated by PMA 10^{-8} M for 1 h. Supershift (NF-κB ss) for p65 subunit is demonstrated. In B: NCX 6550 and PGJ inhibit PMA-induced NF-κB translocation in human monocytes in a concentration-dependent manner. Cells were pre-treated for 30 min with PGJ (10 nM–50 μM) or NCX (10 nM–50 μM), and then stimulated by PMA 10^{-8} M for 1 h. In C: effects of NCX 6550 and other compounds in human MDM. Cells were pre-treated for 30 min with NCX, pravastatin (PRAVA), sodium nitroprusside (SNP) or isosorbide mononitrate (ISMN), all at 50 μM, and then stimulated by PMA 10^{-8} M for 1 h. Each blot is representative of three other independent experiments.

accumulation, that further validates its NO-donating properties. Even more relevant is the NCX 6550 ability to inhibit, in a concentration-dependent manner, superoxide anion production in PMA-stimulated monocytes, thus confirming previous *ex vivo* data in splenocytes from both C57BL/6 and ApoE$^{-/-}$ mice [9]. NCX 6550 is significantly more effective than the native pravastatin and SNP: as reported here, at the highest 50 μM concentration, it inhibited PMA-induced O_2^- production by more than 80% and still displayed a 30% inhibition at 1 μM. On the contrary, neither pravastatin nor SNP exerted significant inhibition at 1 μM concentration.

Over the past years, several studies demonstrated that the pleiotropic effects of statins contribute to their therapeutic efficacy, beyond lipid lowering [13]. In particular, statins have been shown to inhibit NF-κB binding activity in LPS-stimulated human monocytes [14] and in TNF-α-challenged endothelial cells [31], as well as to activate PPARγ in murine macrophage cell lines [15]. Even more relevant, in patients with coronary artery disease, statins exert vascular protection and significantly improve endothelial function [32], possibly through mechanisms involving the NO pathway.

NO is a key mediator of endothelial function and statins are shown to enhance endothelial NO bioavailability by promoting NO production [33] and/or preventing its inactivation by free radicals [34]. NO has been shown to have a dual action on iNOS expression, that largely depends on the concentration that is released in a specific tissue, low physiological concentrations of NO inhibiting iNOS expression through a negative feedback mechanism [35]. Therefore, the incorporation of a NO-releasing moiety into a statin structure, and its slow release, as for NCX 6550, has the potential to confer further anti-inflammatory properties to the native molecule, besides increasing its liposolubility as previously demonstrated [4]. In fact, in other cell types, Ongini et al. [4] showed an increase of the theoretical partition coefficients (Log *P*, as a mea-

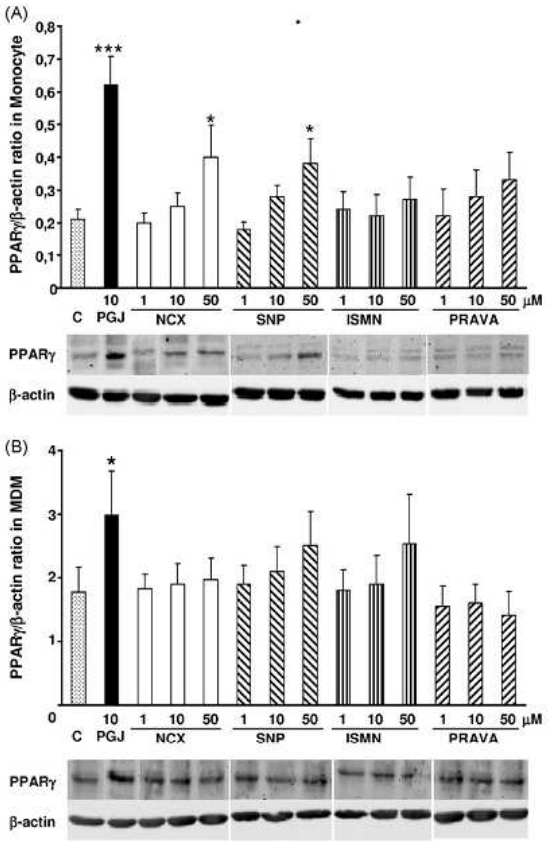

Fig. 5. PPARγ protein expression in human monocytes (A) and MDM (B). Semi-quantitative evaluation of PPARγ protein and representative Western blots for PPARγ and β-actin are shown. Cells were challenged for 6 h with the PPARγ ligand PGJ (10 μM), NCX (1–50 μM), sodium nitroprusside (SNP; 1–50 μM), isosorbide mononitrate (ISMN; 1–50 μM) or pravastatin (PRAVA; 1–50 μM). C denotes control, un-stimulated cells. Values are mean ± S.E.M.; $n = 4$. *$P < 0.05$ *vs* control cells; ***$P < 0.001$ *vs* control cells.

sure of lipophilicity), pravastatin and NCX 6550 being 2.5 and 4, respectively.

The results here reported corroborate the above mentioned notions, since, in human monocyte/macrophages, NCX 6550 is more effective than pravastatin in inhibiting PMA-evoked release of TNF-α and IL-6, major biomarkers of cardiovascular risk [36]. As shown, NCX 6550 produced a concentration-dependent inhibition of cytokine release, being more potent in macrophages (IC_{50} value for TNF-α inhibition: 51 nM) than in monocytes (IC_{50} value: 240 nM), possibly due to a lower PMA-stimulated cytokine release in MDM. Interestingly, NCX 6550 appears to be more effective in human monocytes/MDM as compared to murine cells: the IC_{50} values found here are lower than those reported in RAW 264.7 cells (27.7 μM) and PC12 cells (2.3 μM) [4].

Release of pro-inflammatory cytokines is generally the result of gene transcription, which is controlled by the activation of various transcription factors. Among the different signal transduction pathways involved in cytokine secretion, we focused our attention on NF-κB and PPARγ, functionally active in human monocyte/macrophages [16,17,21]. Regulation of TNF-α production is largely NF-κB-dependent, although evidence exists that TNF-α and other cytokines can also be induced through NF-κB-independent pathways [21]; on its own, TNF-α is a potent inducer of NF-κB activation [21,31], as other inflammatory cytokines and oxy-radicals. So, an auto-regulatory cross-talk can occur, that is further tightly modulated by several mediators. As an example, NO can inhibit NF-κB through the induction and stabilization of its inhibitor, IκBα [37]. In addition, endogenous and exogenous PPARγ ligands have been repeatedly demonstrated to inhibit the release of pro-inflammatory cytokines [16–18] and NF-κB signalling pathway [25,38]. Our results are in keeping with the framework reported above. In fact, in both human monocytes and MDM, NCX 6550 inhibits, in a concentration-dependent manner, PMA-induced NF-κB translocation, with maximal inhibition at 50 μM. As expected from previous reports [25], PGJ, the endogenous agonist for PPARγ, is highly effective in inhibiting PMA-induced NF-κB translocation and more potent than NCX 6550; the maximal inhibition is achieved at 50 μM for both compounds and is significantly superior to that shown by the reference NO donors. Moreover, in these experiments, pravastatin is ineffective, at variance from previous data in human monocytes [39]. This discrepancy can be, at least in part, related to the different experimental procedures. In fact, Zelvyte et al. [39] used either un-stimulated monocytes or monocytes stimulated by oxidized low-density lipoproteins, and a longer incubation time (24 h) with pravastatin, compared to the present experiments (30 min).

Another interesting finding of the present study is that NCX 6550 significantly enhances PPARγ protein expression (about twofold increase) and mRNA levels in human monocytes, with a profile similar to that of PGJ. On the contrary, while effective at the mRNA level, pravastatin, SNP and ISMN do not induce a significant increase in PPARγ protein. Even if the effect of NCX 6550 is observed only at the highest (50 μM) concentration, it could contribute to its anti-inflammatory and anti-atherosclerotic potential, also in keeping with the suggested cross-talk between NO and PPARγ.

In fact, Ptasinska et al. [26] demonstrated that NO activates PPARγ signalling in endothelial cells, supporting, at least in part, the shared vascular protective properties of NO and PPARγ. Interestingly, Gonon et al. [40] reported that the cardio-protective effects of the PPARγ agonist rosiglitazone are mediated by NO. Moreover, in patients with type 2 diabetes, 2-week treatment with rosiglitazone reduced superoxide anion production, restored NO bioavailability and improved the *in vivo* re-endothelialization capacity of endothelial progenitor cells [41].

Therefore, the major ability of NCX 6550 to induce PPARγ protein expression, as well as its capability to inhibit PMA-induced cytokine release, oxy-radical production and NF-κB translocation, compared to the native pravastatin and reference NO donors, clearly indicate that this NO-donating statin might have relevant beneficial effects for the treatment of cardiovascular inflammatory disorders, such as atherosclerosis.

5. Conclusions

Altogether, these findings indicate that NCX 6550 is significantly more effective than pravastatin, SNP and ISMN and suggest this compound as a potent regulator of human monocyte/macrophage activity. NCX 6550 anti-inflammatory effects result in a significant reduction of PMA-evoked respiratory burst and cytokine release, as well as inhibition of NF-κB translocation and stimulation of PPARγ expression. We suggest that, in human monocyte/macrophages, the anti-inflammatory potential of this compound is determined, at least in part, by its NO-donating properties, since the effects exerted by NCX 6550 are significantly higher than those evoked by the native pravastatin. These data further indicate that the incorporation of a NO-donating moiety into a statin structure confers

pharmacological properties which may translate into useful therapeutic benefits.

Conflict of interest

Ennio Ongini is employed by the NicOx Research Institute (Via Ariosto 21, 20091 Bresso, Milan, Italy), the company that develops NCX 6550, and holds stock in the NicOx company. All the other authors state no conflict of interest.

Acknowledgements

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DISCUSSION

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

My first paper was on the expression of PPARγ in monocytes and MDM isolated from CAD patients. The role of these cells in the initiation of atherosclerosis is well established (Osterud and Bjorklid, 2003)**,** as well as the influence of PPARγ in the inflammatory milieu**.** Therefore**,** we investigated the possible different expression of the receptor in cells isolated from CAD patients compared the healthy volunteer, and we checked for a possible gender difference**.** We demonstrated that monocytes from CAD patients express significantly higher levels of both PPARγ protein (approximately 10-fold) and mRNA (approximately 60-fold) compared with healthy donors. These results are in keeping with previous papers demonstrating that PPARγ is 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 Nagy 2008, for review). Interestingly, analysis by gender indicates that cells from female CAD patients have a higher PPARγ protein expression than male CAD patients. The existence of a gender difference was suggested previously (Vidal-Puig et al., 1997) in subcutaneous adipose tissue from lean and obese individuals, with women presenting higher levels of PPARγ1 and γ2 mRNA expression than men and lowest basal release of TNF-α.

Moreover**,** relevant observations concern the release and the expression of inflammatory mediators in CAD. Our results demonstrate that monocyte/macrophages of CAD patients release more TNF-α and IL-6 than healthy donors and, inside the CAD group, the expression of PPARγ protein in monocytes and MDM is inversely related to the spontaneous release of pro-inflammatory cytokines: in fact, CAD females, who present the highest level of PPARγ expression, have the lowest spontaneous release of inflammatory cytokines.

When considering clinical parameters, such as body mass index, diabetes mellitus, unstable angina, multivessel disease, and hypertension, this study documents a significant GD in PPARγ protein expression only in the hypertension subgroup. Because approximately two thirds of these CAD patients are treated with ACE-inhibitors and AT1 blockers that, at high in vitro concentrations, enhance PPARγ expression in differentiated adipocytes (Schupp et al., 2004, 2006; Storka et al., 2008), this observation warrants further evaluation. Currently, we can exclude that the pharmacological therapy accounts for the GD; in fact, a similar number (10 females and 12 males) of patients is treated with an ACE-inhibitor, and only two patients in each group also receive losartan. We can also evidence a significant inverse correlation (but no clear GD) between

PPARγ/β-actin ratio and LDL-C levels: the higher the PPARγ expression, the lower the LDL-C level. It has to be noted that all CAD patients were on current medical therapy (more than 50% receiving statins) and that their lipid profile was near normal, with no major differences between males and females (Table 1).
Therefore, the enhanced PPARγ expression we detect in monocytes and MDM from CAD patients (and especially in females) could represent a beneficial condition.

Moreover, we also evaluated PPARγ expression in established RA, a disease which, as reported in the Introduction, has a higher incidence in females.
This study indicates that monocytes and macrophages isolated from RA patients under pharmacological treatment (MTX and corticosteroids, mainly) present an increased PPARγ expression (both protein and mRNA) as compared to cells of healthy donors, in good agreement with previous findings in experimental arthritis and patients (Koufany et al., 2008; Jiang et al., 2008). In fact, an increase in PPARγ (but not adiponectin) mRNA levels was observed in a model of adjuvant-induced arthritis (Koufany et al., 2008). Moreover, Jiang et al., by evaluating PPARγ 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 PPARγ mRNA levels than fractured subjects (Jiang et al., 2008). Increased PPARγ 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) and we previously reported that monocyte/macrophages from patients with CAD present a constitutively enhanced PPARγ expression as compared to healthy subjects (Amoruso et al., 2009).
Analysis by gender indicates that MDM from the 10 male RA patients have a higher PPARγ protein expression than female RA patients. However, as shown in Table 1, the male RA patients enrolled in this pilot study are in the remission phase (DAS28 < 3.2) and, as discussed below, the disease severity largely affects PPARγ protein expression.
The fact that PPARγ protein and/or mRNA might be increased in inflammatory/immune diseases with a relevant involvement of monocyte/macrophages is not surprising, since PPARγ is a key modulator of macrophage differentiation (Amoruso et al., 2007; Moore et al., 2001; Tontonoz et al., 1988) and participates in different inflammatory and autoimmune disorders (Szeles et al., 2007; Szanto et al., 2008 for review). On this regard, a recent paper demonstrates that synovial tissues obtained from arthritis-susceptible and highly erosion-prone (DA rats) or arthritis-resistant (DA.ACI.Cia25 rats) animals, 21 days after induction of arthritis, present different levels of PPARγ expression, cytokine release and disease severity (Brenner et al., 2011). Indeed, a reduced expression of inflammatory cytokines and proteases is detected in arthritis-resistant rats as

compared to DA rats that are matched by a 5-fold increased expression of PPARγ in resistant animals (Brenner et al., 2011). Analysis of gene expression also indicates reduced numbers of infiltrating macrophages in resistant animals, both Cd163 and Cd68 genes (that are relevant for M2 and M1 macrophages, respectively) being more expressed in arthritis-susceptible rats (Brenner et al., 2011). 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 et al., 2003). Therefore, the local environment created by the activation of PPARγ might induce a switch from M1 toward M2 activated macrophages, thus contributing to the anti-inflammatory effect. Interestingly, PPARγ 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). In addition, glucocorticoids represents another stimulus that favours the M2 phenotype (Manovani et al., 2002) and this switching could be relevant for their anti-inflammatory action at the macrophage level (Hamilton et al., 2009).

As largely demonstrated (see Mantovani et al., 2002, 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 MTX (Dolhain et al., 1998) or corticosteroids (Gerlag et al., 2004). Although most of our RA patients were treated with oral prednisone, as a prototype of corticosteroids for *in vitro* studies**,** we used methylprednisolone (MP), since its characteristics - bioactivity per *se* and higher liposolubility - make it more suitable for a 6hr-challenge, as for the evaluation of PPARγ protein expression and MMP-9 activity.

In this study, we show that MTX and MP, besides reducing MMP-9 activity (please, see later), enhance about 2-fold PPARγ protein expression in monocytes and MDM, as the endogenous ligand 15d-PGJ does. The fact that different drugs may affect PPARγ expression is not surprising, since indomethacin and other selected non-steroidal anti-inflammatory drugs, telmisartan and some statins have been described to act as PPARγ agonists at relatively high concentrations, this property contributing to their overall therapeutic activity (Lehmann et al., 1997; Jaradat et al., 2001; Xue et al., 2010; Amoruso et al., 2010; Matsumura et al., 2011). Given that MTX and MP increase PPARγ expression at therapeutic concentrations in our *in vitro* experiments, it is tempting to speculate that this activity may contribute to their clinical efficacy in RA.

On this regard, it is worth noting that we measured a different PPARγ 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 (please, see also Table 1) express significantly higher PPARγ protein levels than patients with DAS28 score

>3.2, that could be assimilated to “inadequate-responding” patients. Interestingly, the highest PPARγ protein expression (PPARγ/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 PPARγ mRNA levels, even if significantly enhanced as compared to healthy donors, are similar in both DAS28 cohorts. Although we have no definite explanation, this is in accordance with our previous results in patients with CAD (Amoruso et al., 2009).

Therefore, it seems conceivable to suggest PPARγ expression in human monocyte/macrophages as an indicator of RA disease activity and/or therapy efficacy. However, further prospective studies with a larger cohort of patients are needed to confirm our preliminary results.

Since proteinase activity of synovium is stronger in RA than in osteoarthritis and PPARγ ligands inhibit MMP-9 secretion (Shu et al., 2000; Marx et al., 2003), we also measured MMP-9 activity in monocyte/macrophages. Our results confirm that MMP-9 activity is potently increased in RA patients and that 15d-PGJ, the endogenous PPARγ agonist, inhibits by 60-70% LPS-induced MMP-9 activity in cells from healthy donors. MTX and MP, evaluated *in vitro* at conceivable therapeutic concentrations (1 μM 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.
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.

Taken together, the key findings of enhanced PPARγ expression and concomitant reduction in MMP-9 activity in cells from RA patients with a less severe disease suggest PPARγ 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 and MTX 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; Bodmeret al., 2009), possibly due to PPARγ 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).

Based on the above considerations and the experimental findings of this pilot study, we suggest that the evaluation of PPARγ expression in monocyte/macrophages could be a useful biomarker to monitor response to RA therapy.

Over the past years, several studies demonstrated that the pleiotropic effects of statins contribute to their therapeutic efficacy, beyond lipid lowering (Wang et al., 2008). In particular, statins have been shown to inhibit NF-κB binding activity in LPS-stimulated human monocytes (Habib et al., 2007) and in TNF-α-challenged endothelial cells (Jarvisalo et al., 1999), as well as to activate PPARγ in murine macrophage cell line (Yano et al., 2007). Even more relevant, in patients with CAD, statins exert vascular protection and significantly improve endothelial function (Jarvisalo et al., 1999), possibly through mechanisms involving the NO pathway. Therefore, the incorporation of a NO-releasing moiety into a statin structure, and its slow release**,** has the potential to confer further anti-inflammatory properties to the native molecule, besides increasing its liposolubility**,** as previously demonstrated (Ongini et al., 2004). During my PhD**,** I had the possibility to test NCX 6550 (pravastatin linked to a NO moiety).
We demonstrated that NCX6550 inhibits, in a concentration-dependent manner, superoxide anion production in PMA-stimulated monocytes, being significantly more effective than the native pravastatin and SNP. Moreover, in human monocyte/macrophages, NCX 6550 is more effective than pravastatin in inhibiting PMA-evoked release of TNF-α and IL-6, major biomarkers of cardiovascular risk. As shown, NCX 6550 produced a concentration-dependent inhibition of cytokine release and was more potent in macrophages than in monocytes **(**possibly due to a lower PMA-stimulated cytokine release in MDM**)**. In both cell types, NCX6550 inhibited, in a concentration-dependent manner, PMA-induced NF-κB translocation and significantly enhanced PPARγ protein expression (about twofold increase) and mRNA levels in human monocytes.
Therefore, the major ability of NCX 6550 to induce PPARγ protein expression, as well as its capability to inhibit PMA-induced cytokine release, oxy-radical production and NF-κB translocation, compared to the native pravastatin and reference NO donors, clearly indicate that this NO-donating statin might have relevant beneficial effects for the treatment of cardiovascular inflammatory disorders, such as atherosclerosis.

Moreover, statins have been used in patients with RA for their favourable effects on inflammation and endothelial dysfunction. Two studies have shown that statins may improve endothelial dysfunction, reduce arterial stiffness, lipid oxidation and inflammatory markers (Maki-Petaja et al., 2007; Charles-Schoeman et al., 2007). The TARA (Trial of Atorvastatin in Rheumatoid Arthritis) study revealed a significant reduction in CRP, ESR and other useful effects on RA; as expected, the anti-inflammatory effect was paralleled with the reduction in total- and LDL-cholesterol levels (McCarey et al., 2004). Based on the current data, it is possible to suggest that statins should be considered in patients with severe RA and an unfavourable lipid profile.

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