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**EFFECT OF ALDOSTERONE ON MMP-9  
ACTIVITY IN MONOCYTE/MACROPHAGES**

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# ABSTRACT

**Background.** Atherosclerosis is a chronic, multifactorial pathology caused by long exposure to cardiovascular risk factors. Risk factors provoke oxidative stress and endothelial dysfunction, leading to expression of adhesion molecules, diapedesis of monocytes and transformation into macrophages. Macrophages internalize oxidized-LDL, become foam cells and release proinflammatory cytokines that increase atherosclerosis and metalloproteases that destabilize the plaque.

High plasma aldosterone level (PAC) was shown to predict short and long-term mortality and life-threatening arrhythmias in patients with myocardial infarction (AMI) and a prognostic role was attributed to PAC also in stable coronary artery disease (CAD) patients. Moreover, mineralocorticoid receptor antagonism (MRA) was demonstrated to reduce cardiovascular (CV) mortality in patients with heart failure (HF) or left ventricular dysfunction post-acute AMI. Recent clinical trials demonstrated a direct aldosterone pro-atherogenic or plaque-destabilizing effect, supported by the recent identification of pathways linking aldosterone to oxidative stress and metalloproteinase-9 (MMP-9) activation, which we postulate might occur in monocytic/macrophagic cells, recognized effectors of plaque instability and ensuing CV events.

**Aim.** The primary aim of our study was to investigate the effects of aldosterone and mineralocorticoid receptor antagonist on the MMP-9 production by monocytes/macrophages

**Material and Methods.** We employ a THP-1 monocytic cell-line and human monocytes from blood of healthy donors; to obtain macrophages, we used phorbol myristate acetate (PMA) for the cell line and GM-CSF for human cells.

We first confirmed the cell transition by FACS and gene expression of PPAR $\gamma$  gene.

Cells were investigated for the presence of the Mineralocorticoid Receptor (MR) at Western Blot and RT-PCR and to elucidate the effects of aldosterone on these cells we analyzed FKBP51 gene expression. The effect on MMP-9 expression and activity, was assessed by RT-PCR and zymography, respectively.

**Results.** We demonstrated that macrophages, both THP-1 and human, express an higher amount of PPAR $\gamma$  mRNA, confirming the successful phenotypic transition.

Macrophages express also an higher amount of MR protein and mRNA respect to monocytes, thus explaining the non-responsiveness of monocytes to aldosterone in MMP-9 increase. On the contrary, THP-1 macrophages (differentiated after 72 hours in PMA 100nM), showed in the dose and time-dependent curve, a significant MMP-9 activity in response to aldosterone in particular at lower concentrations ( $10^{-8}$  and  $10^{-9}$ M) between 16 and 48 hours. Responsiveness of macrophages to aldosterone was confirmed by FKBP51 gene expression.

We repeated the dose and time-response curve in human cells: MMP-9 gene expression was significantly higher after 2 hours of aldosterone at  $10^{-7}$ M and  $10^{-8}$ M ( $2,03 \pm 0,26$  and  $2,05 \pm 0,26$  fold change vs. control, respectively;  $p < 0.001$ ) and at 6 hours only aldosterone at  $10^{-7}$ M, while MMP-9 activity increases only at 24 hours in presence of aldosterone  $10^{-8}$  M aldosterone  $10^{-9}$  M ( $1,23 \pm 0,07$  and  $1,36 \pm 0,08$  respectively). In presence of canrenone as MR inhibitor, RT-PCR shows a clear trend in reduction both at 2 and 6 hours in presence of canrenone respect to aldosterone-induced MMP-9 gene expression, even if not significant ( $p = 0.08$  at 6 hours).

# INTRODUCTION

## Coronary artery disease

### Definition

Cardiovascular disease (CVD) is the most common heart disease and cause of death (17.3 million deaths per year) and represented the 30% of global deaths in 2008{{9008 Mozaffarian,D. 2015}}. Myocardial infarction is a common manifestation of ischemic heart disease, a condition in which interruption of blood supply to a region of the heart results in ischemia and subsequent cell death{{9042 He,B.J. 2013}}. The main cause of CVD is the coronary heart disease (CHD), also called coronary artery disease (CAD) that includes numerous problems, many of which are related to a process called atherosclerosis. Atherosclerosis is a type of arteriosclerosis, a general term for the thickening and hardening of arteries. This condition is rarely fatal, but thrombosis, superimposed on a ruptured or eroded atherosclerotic plaque, may cause a heart attack or stroke{{8966 Falk,E. 2006}}. Between the heart disease, stroke is the second global cause of death.

Other types of cardiovascular disease are hypertensive heart disease, arrhythmia, heart valves problems and heart failure.

CAD is a progressive disease that generally begins in childhood and manifests clinically during the mean and the elderly ages{{9009 Rosamond,W. 2008; 9010 Lloyd-Jones,D. 2010}}. Atherosclerosis is a chronic inflammatory

disease of the arterial wall in response to chronic multifactorial injuries that lead to the formations of fibrous plaques (atheromas) in the coronary arteries, that are composed of fibrous tissues, cells and lipids{{9012 Buja,L.M. 2012}}. It becomes clinically clear when the plaque limits the blood flow under increased requirement{{8967 Maiolino,G. 2015}}.

### **Risk Factors**

The American Heart Association periodically evaluated the cardiovascular health of the nation by tracking seven key factors and behaviours that increase risks for heart disease and stroke; the goal is to improve the cardiovascular health of all Americans by 20 percent and reduce deaths from cardiovascular diseases and stroke by 20 percent, by the year 2020.

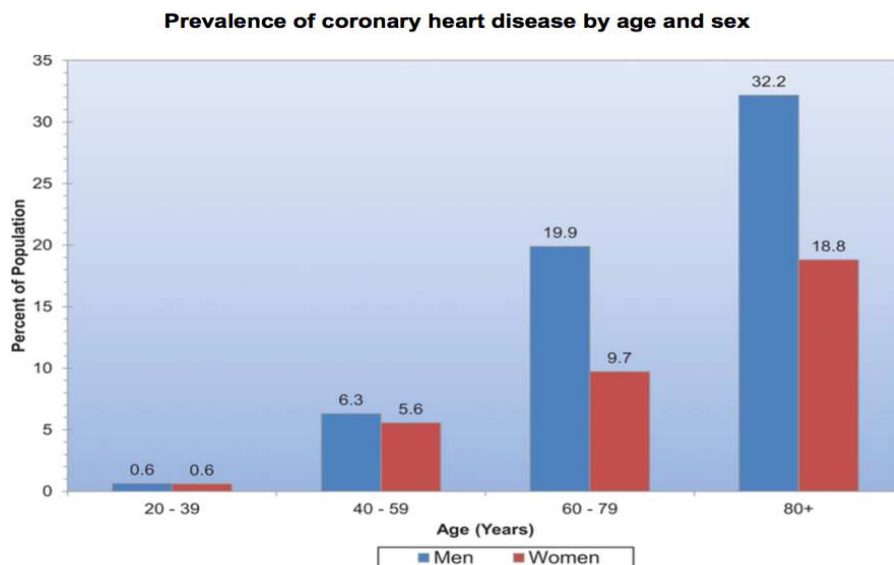
The risk factors are multiple and belong to lifestyle and biochemical and physiological area: behaviour as smoking, physical inactivity, excess of alcohol and fat diet stress leading to obesity, are considered as risk factors, alongside high plasma cholesterol, high blood pressure, low plasma HDL-cholesterol, high plasma triglyceride, diabetes mellitus, thrombogenic factors, age, gender and family history.

Life's Simple 7 are: not smoking, physical activity, healthy diet, body weight, and control of cholesterol, blood pressure and blood sugar.

CVD risk factors are commonly divided, from the operative standpoint, as non-modifiable and modifiable.

*Non-modifiable risks factors:*

Sex and age. There is an excess of cardiovascular risk for men than in women before menopause, and progressively increases in women, especially above 55 years; the protection of fertile women is due to the “umbrella-oestrogens”-effect that leads to a more favourable lipid profile.



**Figure 1.** Prevalence of coronary heart disease by age and sex{{9008 Mozaffarian,D. 2015}}.

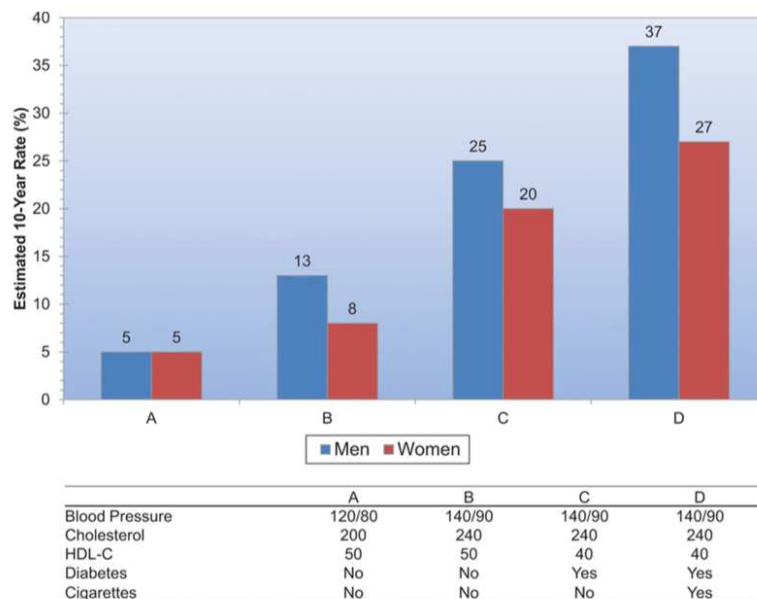
Family history of CVD is another known risk factor attributable to the transmission of other risk factors, such as hyperlipidaemia, diabetes, hyperhomocysteinemia, some of which are still to be discovered, as recently shown by the identification of the MEF2A mutation ([Topol, Wang; Colonna, Rossi JCV](#))

*Partially modifiable risk factors:*

Hypercholesterolemia is one of the most important risk factors and it is strictly associated with endothelial dysfunction because oxidized-LDLs are mediators

of endothelial damage. About 43 percent of Americans have total cholesterol higher of 200 mg/dL, but this factor alone is not sufficient to cause the disease, as shown by the fact that patients hospitalized for acute coronary syndrome, 50% have normal LDL values{{8968 Sachdeva,A. 2009}}.

**Estimated 10-year coronary heart disease risk in adults 55 years of age according to levels of various risk factors (Framingham Heart Study).**



**Figure 2.** Estimated 10-year coronary heart disease risk in adult 55 years of age according to levels of various risk factors (Framingham Heart study){9008 Mozaffarian,D. 2015}}.

Hypertension. About 33 percent U.S. adults have high blood pressure and about 77 percent of them are using antihypertensive medication. Notwithstanding this, only 54 of them have their condition controlled. This rate would be much lower if the target blood pressure matches for systolic blood pressure that were identified in the recently published SPRINT study,



e.g. 120 systolic, were adopted (1). Hypertension produce hemodynamic stress on the arterial wall that promotes oxidative processes.

Diabetes mellitus. Insulin normally lowers the free fatty acids plasma concentration and its lack of effect favours the increase, in diabetic patients. In the presence of high concentrations of glucose, lipoproteins may be modified by glycation (non-enzymatic glycosides), which alters the recognition and binding by the receptors. The prevalence of diabetes for adults worldwide was estimated to be 6.4 percent in 2010 but diabetes rates are growing: in fact, about 35 percent of Americans have pre-diabetes.

Obesity/Overweight. Most Americans older than 20, about 69 percent, are overweight or obese, and about 32 percent children are overweight or obese. Obesity enhances all the diseases associated with it, such as diabetes type II, dyslipidaemia and hypertension and low level of adiponectin.

*Modifiable risk factors:*

Smoking. In 2010 worldwide, tobacco smoking (including second hand smoke) was one of the top three leading risk factors for disease and contributed to an estimated 6.2 million deaths.

Smoking stresses the vasculature, decreases coronary blood flow and myocardial oxygen delivery, increases activation of leukocytes and platelets, blood pressure and insulin resistance.

The reactive oxygen species of the cigarettes directly causes endothelial damage and indirectly affect lipid peroxidation.

The endothelial dysfunction associated to smoke (Brunner, JH Guidelines...) is related with the decreasing activity of eNOS, enhanced expression of

adhesion molecules and deregulation of the local thrombotic balance. Studies demonstrated dose-related impairment of endothelium-dependent vasodilatation in smokers' circulation.

Unhealthy diet. Less than 1 percent of U.S. adults and no children meet the American Heart Association's definition for "Ideal Healthy Diet." Of the 5 components of a healthy diet, reducing sodium and increasing whole grains are the biggest challenges.

"Non traditional" risk factors are acquiring increasing importance as emerging risk predictors: C-reactive protein (CRP), lipoprotein associated phospholipase A2 (Lp-PLA2), fibrinogen, and homocysteine. These risk predictors were selected because substantial evidence is accumulating of their predictive abilities, there is a genetic basis for premature disease, modifying treatments are available, and/or these factors are the subject of ongoing or completed clinical trials{{8969 Hackam,D.G. 2003}}.

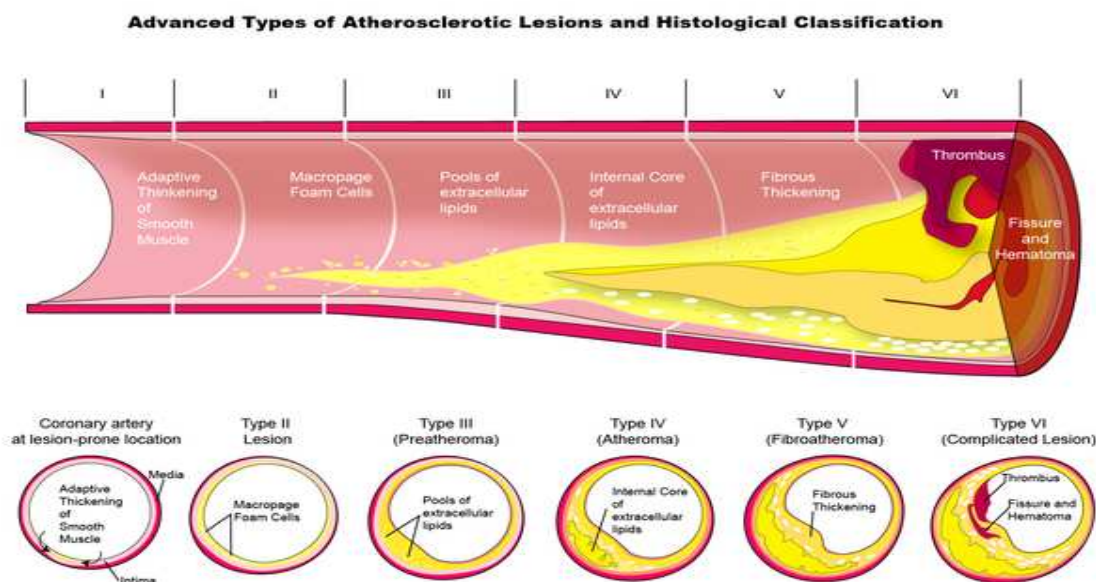
Between these new risk factors, LpPLA2 is acquiring increasing importance because of its role in the degradation of pro-inflammatory phospholipids (like ox-LDL) and in the generation of lysophosphatidilcholine (that in turn activates endothelial NADPH oxidase) and oxidized free-fatty acid, which are pro-atherogenic molecules. High levels of Lp-PLA2 predict CV mortality risk and acute myocardial infarction{{8967 Maiolino,G. 2015}}.

## **Pathogenesis**

Atherosclerosis affects mostly medium and large-sized arteries and is characterized by intramural thickening of the subintima that encroaches over the arterial lumen. Its typical lesions are plaques, which are composed of three major features:

- 1) Cellular component: endothelial cells, leukocytes and intimal smooth muscle cells
- 2) Connective tissue matrix and extracellular lipids.
- 3) Intracellular lipids that accumulate within macrophages, thereby converting them into foam cells.

The most affected sites are the aorta and its main branches, the vessels of the cerebral circulation, lower limb and the coronary arteries, where the disease affects epicardial arteries causing reduced blood flow reserve in the vessels.



**Figure 3:** Schematic representation of atherosclerotic plaque progression (<http://www.carlagoldenwellness.com>)

If plaques become unstable, because of denudation of the overlying endothelium or plaque rupture, a thrombotic occlusion of the overlying artery may result.

### Early atherosclerotic lesion

Endothelial cells undergo qualitative changes when subjected to irritative stimuli, i.e. hyperlipidaemia, hypertension, and pro-inflammatory cytokines, leading to an intact but leaky, activated and dysfunctional endothelium that promote the lesion initiation. Later, endothelial cells may vanish and denuded areas appear over advanced lesions, with or without platelets adhering to the exposed subendothelial tissue{{8966 Falk,E. 2006}}. Plasma molecules and lipoprotein extravasate through the weak endothelium in the subendothelial space; here, lipoproteins are oxidized and become cytotoxic, proinflammatory and pro-atherogenic.

Low-density lipoproteins (LDLs) have a shell of phospholipids, free cholesterol and apolipoprotein B100 (ApoB100) with a core of esterified cholesterol and triglycerides. These particles can accumulate in the subendothelial space, where the ApoB100 binds to proteoglycans of the extracellular matrix, trapping the LDLs. Under the influence of pro-inflammatory cytokines, these cells produce reactive nitrogen and oxygen species (ROS) that oxidize LDLs, which play a major role in the initiation and progression of the atherosclerotic process.

The oxidation step could be mediated by myeloperoxidase, 15-lipoxygenase, and/or nitric oxide synthase (NOS){{8966 Falk,E. 2006}}; nitric oxide could be produced by endothelium, where it exerts a vasodilator and protective role, or, in contrast it is produced by macrophages and it has a pro-atherogenic effect.

The activated endothelium produces and expresses adhesion molecules, primarily vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), P- and E-selectin which are involved in blood cells recruitment recruiting as monocytes and T-cells.

Once activated, endothelial cells also secrete chemokines, e.g., monocyte chemoattractant protein 1 (MCP-1), which attract monocytes and T-cells in the subendothelial space, acting on their CCR2 receptor. Monocytes roll on endothelial cells through the interaction of monocyte P-selectin glycoprotein ligand-1 (PSGL-1) with endothelial selectins and after firm adhesion to the dysfunctional endothelium, a process called *diapedesis* allows monocytes to enter in the subendothelial space. T-cells gain access to the intima with similar mechanisms involving chemokines and adhesion molecules.

Once within the intima, monocyte-derived macrophages may recruit themselves by secreting MCP-1; also cytokines as interleukin-8 (IL-8) are involved in monocyte-macrophages trafficking.

#### Monocyte differentiation into macrophages

Once in the intima monocytes are stimulated by macrophage colony-stimulating factor (M-CSF) produced by activated endothelial cells to differentiate into macrophage and/or dendritic cell-like features.

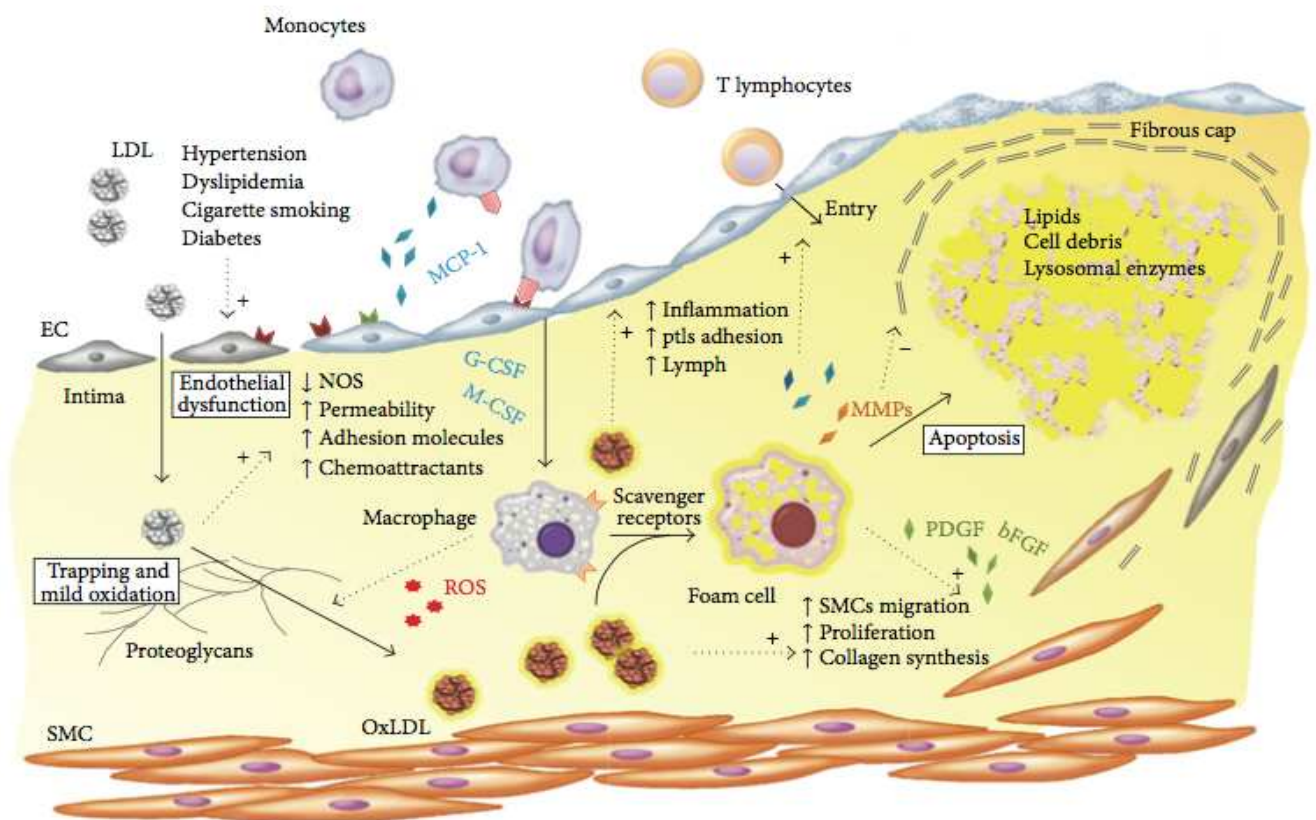
### Foam cells formation

Oxidation of LDL causes a loss of their affinity for the LDL receptor, which furthermore is downregulated by cholesterol intracellular overload, and determines a gain of affinity for the scavenger receptors (SR)A and CD36 which are, in contrast, up-regulated by cholesterol excess in macrophages. Thus, upon binding of oxidized LDL (oxLDL), these receptors are internalized with consequent foam cells formation.

Macrophages internalize ox-LDL until they die by (apoptosis and necrosis), thus contributing to formation of a soft lipid-rich core within the plaque. Then, they produce matrix proteolytic enzyme, mainly metalloproteinases, which are able to increase the thrombogenic and destabilizing properties of the plaque. Once internalized by macrophages cholesteryl esters of lipoproteins are hydrolyzed to free cholesterol and fatty acids. Free cholesterol is then transferred to other cell sites, like endoplasmic reticulum, and this lead to downregulation of LDL receptors and cholesterol synthesis through inhibition of the sterol-regulatory element binding pathway (SREBP). Free cholesterol could also migrate, probably through a Golgi vesicular transport to the plasma membrane, where it is removed from the cells by the ATP-binding cassette transporter (ABC)A1- and ABCG1-mediated transport to apolipoprotein A1 and HDL, respectively, or by "passive diffusion" to cholesterol-poor HDL.

### Other atheroma cell components

With progression of the disease, the immunoinflammatory response is joined by a fibro-proliferative response mediated by intimal smooth muscle cells, which proliferate under the stimulus of platelet derived growth factor (PDGF). These cells produce extracellular matrix molecules, like collagen and elastin, forming the fibrous cap that overlies the atherosclerotic plaque, to confer stability and to protect against rupture and destabilization. If the atherogenic stimuli persist over years, as they regularly do, the reparative response may become so voluminous that lumen is lost, blood flow is reduced, and ischemia sets in.



**Figure 4.** Putative pathway of oxidized low-density lipoprotein (oxLDL) in the atherosclerotic process according to the oxidative hypothesis of atherosclerosis{{8967 Maiolino,G. 2015}}.

## **Innate Immunity in atherogenesis**

The innate immunity is the first line of defense against pathogens and it is characterized by a fast response. During local inflammation, in the early stage of the disease, leukocytes enter a tissue in response to a perceived pathogen; in atherosclerotic lesions the nature of the inciting pathogen is not completely clear, probably there are many different candidate pathogens. The main candidates are the minimally oxidized LDLs and their late form, the oxidized LDLs{{8970 Binder,C.J. 2002}} which are themselves directly chemotactic for monocytes and T cells and stimulate both cellular and humoral response. Among other biological effects, ox-LDLs (and its various oxidized lipid components) are cytotoxic for endothelial cells, for macrophages and smooth muscle cells (SMCs) and stimulate the release of MCP-1 and of M-CSF from endothelial cells{{8971 Zeiher,A.M. 1991; 8972 Chatzizisis,Y.S. 2007}}.

Other candidate pathogens are molecules that trigger the inflammatory cascade, such as lysophospholipids or oxidized lipids, and metabolic events which lead to increased production of ROS, such as hypercholesterolemia or hypertension{{8970 Binder,C.J. 2002}}.

Monocytes constitute the main component of innate immunity; they are involved in exogenous and endogenous inflammation, counteracting pathogens and eliminating damaged cells and molecules. One of the most known extracellular inducer of macrophage activation is LPS, which binds toll-like receptor 4 (TLR-4) triggering different signalling cascades, including those mediated by transcription nuclear factor kB (NF-kB), Janus N-terminal



kinase (JNK) and p38 kinase{{8974 Barish,G.D. 2005}}. Recruitment of monocytes and their differentiation into macrophages are essential for lesion formation, as demonstrated in hypercholesterolemic mice deficient in MCP-1 or in its receptor that showed reduced atherosclerotic lesion{{8973 Gosling,J. 1999; 9013 Boring,L. 1998}}. Similar results were obtained in the mice lacking of M-CSF{{9014 Smith,J.D. 1995}}.

Oxidized LDLs and chemokines as MCP-1 increase the chemotaxis of monocytes and T cells, but not of neutrophils. In turn, the expression of MCP-1 in arterial cells is stimulated by oxidized phospholipids, cytokines, and activated complement{{8970 Binder,C.J. 2002}}.

Macrophages and dendritic-like cells have the capacity to ingest pathogens and to initiate adaptive immune responses presenting the antigen; they produce ROS, proteases and cytokines against the pathogens and they exert scavenging function mediated through pattern recognition receptors (PRR) as SR-A and CD36 and Toll-like receptors (TLR) involved in inflammation. CD14 (the non transmembrane receptor for lipopolysaccharide) initiates the inflammatory responses through interaction with TLRs.

Components of oxLDL can activate macrophages and affect gene expression of genes such peroxisome-activated receptor-g, CD36 and ATP-binding cassette transporter A1, which influence macrophage inflammatory and atherogenic activity.

The complement factors are also involved in atherosclerosis, in fact deposition of C3,C4 and terminal C5b-C9 occurs in lesions, as well as IgM against specific epitopes of ox-LDL{{8970 Binder,C.J. 2002}}.

During atherogenesis, monocyte migration into the intima, and subsequent differentiation into macrophages, induce production and release of cytokines as tumor necrosis factor (TNF), IL-1 and IL-6 and several MMPs. Differentiated macrophages produce and release MMPs such as MMP-1, -2, -3, -8, -9 and -14.

NK cells in CAD patients are lower compared to healthy patients{{8976 Jonasson,L. 2005}}.

### **Macrophage phenotypes in atherosclerosis**

Macrophages exposed to specific micro-environments differentiate into heterogeneous range of macrophages, whose diversity can be assessed by assessing the expression of numerous surface markers as well as by their secretome.

#### *Classically activated M1 macrophages*

The classical M1 activation is driven by interferon- $\gamma$  (INF $\gamma$ ), inflammatory cytokines such as TNF, and microbial products as lipopolysaccharide (LPS). The M1 type is an inflammatory cell that increases and sustains inflammation secreting pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-1b, TNF, IL-12, IL-23 and they also produce ROS. They express chemokine receptors (CXCL9, CXCL10, CXCL5) that further promote the recruitment of Th-1 and NK cells, to counteract the acute infection provoked by pathogens. In case of chronic induction of M1 macrophages activation, tissue damage and impair wound healing occur.

## Alternatively activated M2 macrophages

M2 macrophages are considered as anti-inflammatory cells, and they counteract the inflammatory response sustained by the M1 type. They are mainly involved in tissue repair and show phagocytic, pro-angiogenic and pro-fibrotic properties. The M2 activation is first of all mediated by IL-4 and IL-13 produced by Th-2 cells.

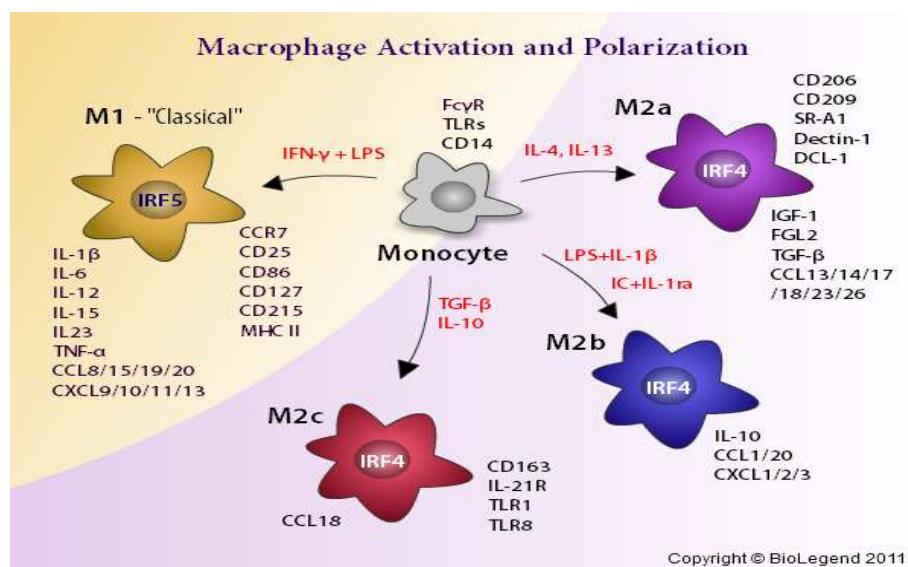
M2 macrophages can be subdivided into 4 subgroups:

M2a: activated in response to IL-4 and IL-13, which express high levels of mannose receptor (MR or CD206), secrete pro-fibrotic factors, fibronectin, insulin-like growth factor, and transforming-growth factor  $\beta$  (TGF $\beta$ ).

M2b: induced upon combined exposure to TLR ligands or IL-1 receptor agonists.

M2c: triggered by IL-10 and glucocorticoids, together with the M2b type, are

defined as "regulatory macrophages" M2d: induced by co-stimulation with TLR and adenosine A2A receptor agonists. They are characterized by high levels of IL-10 and vascular endothelial growth factor (VEGF) and low level of TNF and IL-12{{8977 Colin,S. 2014}}.



In the atherosclerotic plaque M1 and M2 macrophages represent respectively, the 40% and the 20% of total atherosclerotic lesion macrophages in mice{{8978 Kadl,A. 2010}}.

## **Metalloproteinases**

Metalloproteinase are enzymes that need metal for their catalytic mechanism; usually this metal is zinc ion, but some use cobalt. They divided into two groups based on their capacity to cleave or not cleave the terminal peptidic bond and consequently to generate monomers; exopeptidases have this characteristic, while endopeptidases cleave only the non-terminal peptide bond.

Matrix metalloproteases and ADAM proteins belong to the endopeptidases group. There are at least 23 structurally related matrix metalloproteinases that share a similar  $Zn^{2+}$  -based catalytic activity, and 24 genes (MMP-23 is duplicated). Most of them are secreted, except for six membrane-type, which are located on the cell surface{{8979 Newby,A.C. 2007}}.

MMPs have some common substrate to which they bind with different efficacy. These substrates are:

- ECM proteins (including collagen types I, II, III, IV, V, VI, VII, VIII, IX, X, XIV)
- Laminin
- Fibronectin
- Elastin
- Entactin
- Vitronectin
- Myelin basic protein

- Aggrecan
- Tenascin
- Gelatin I
- Fibrillin

MMPs are also able to cleave a number of non-matrix substrate, including plasminogen, fibrin and fibrinogen, E-cadherin, casein, MCP-3 and certain pro-cytokines such as membrane-bound TNF $\alpha$  and pro-TGF $\beta$  (Nagase, H. 2006)..

The degradation of extracellular matrix is an important step in development, morphogenesis, tissue repair and remodelling (Nagase, H. 2006), but uncontrolled ECM degradation is involved in multiple disease such as arthritis, nephritis, cancer, encephalomyelitis, chronic ulcers, fibrosis etc.

Their expression is transcriptionally controlled by inflammatory cytokines, growth factors, and hormones.

MMPs are synthesized as zymogens in the rough endoplasmic reticulum and secreted via endosomal pathway; they are then activated in the cytoplasm by the removal of a pro-peptide and inhibited by two major types of endogenous inhibitors:  $\alpha$ -macroglobin and Tissue Inhibitor of metalloproteinases (TIMPs).

$\alpha$ -macroglobin is a plasma glycoprotein that inhibits most proteinases entrapping them into the macroglobulin. TIMPs inhibit most of MMPs and some ADAMs. Selected MMPs are inhibited also by other proteins: MMP-2 for example is inhibited by the secreted form of  $\beta$ -amyloid precursor protein and by the C-terminal fragment of pro-collagen C-proteinase enhancer protein,

while RECK (a glycoprotein that suppressed angiogenesis) inhibits MMP-2, MMP-9 and MMP-14{{8981 Nagase,H. 2006}}.

A typical MMP consists of a pro-peptide of about 80 amino acids, a catalytic metalloproteinase domain of about 170 amino acids, a linker peptide of variable lengths (also called the 'hinge region') and a hemopexin (Hpx) domain of about 200 amino acids. Gelatinase A (MMP-2) and gelatinase B (MMP-9), have three repeats of a fibronectin type II motif in the metalloproteinase domain.

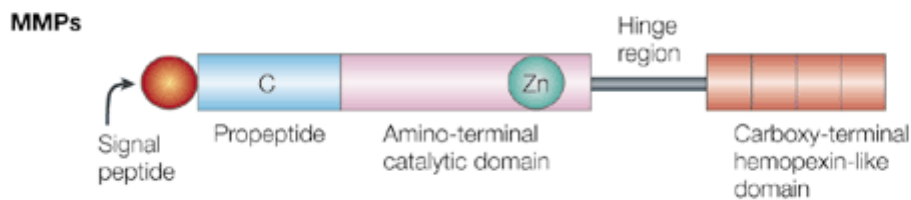


Figure 6. General structure of MMPs.

Uncontrolled ECM remodelling of the myocardium and vasculature are present in CVD, such as atherosclerosis, left ventricular hypertrophy, heart failure, aneurysm{{8981 Nagase,H. 2006}}.

As mentioned before, plaques rupture is the main cause of myocardial infarctions and strokes{{8979 Newby,A.C. 2007}}. MMPs have a dual effect on the plaque: on one side, they weaken the cap and promote rupture, on the other side, MMP-2, MMP-9 and MMP-14 facilitate migration and proliferation of smooth muscle cells, which promote fibrous cap stability.

### **Metalloproteinase-9 production in monocyte and macrophages**

Peripheral blood monocyte do not express high levels of MMPs, however attachment to extracellular matrix or to plastic leads to an up-regulation of MMP-9, -10, -14 and -19{{8979 Newby,A.C. 2007}}, which, for MMP-9 and -14, occurs also during monocytes to macrophages transformation, and promote cell invasion through the intima.

Exposure of human monocyte-derived macrophages to LPS, TNF- $\alpha$ , or ox-LDL up-regulates MMP-1 through NF-kB (Chase, 2002) and MMP-14 via serum-amyloid A activating factor 1 (Ray, 2004); MMP-3 is regulated via NF-kB but mediators remain unclear. These MMPs are involved in the early atherosclerosis and MMP-1 and -3 are selectively up-regulated in foam cells compared to normal macrophages.

CD14<sup>+</sup> monocytes expresses most of the 23 MMPs, with a preference for MMP-1, -3, -9, -10, -14, -19, -25, but also MMP-2 and -17 are represented.

MMP-9, also called gelatinase B or type IV collagenase, is a 92kDa in the latent form and after the removal of the propeptide became an 86kDa. It is able to degrade collagen IV, V, VII, X and XIV. It is not constitutively expressed and experimental conditions may influence its basal level, for example cellular adherence leads to higher level of MMP-9. It can be produced free or complexed to TIMP. The balance between MMP and TIMP decide whether it is activated. Together with MMP-8, it is stored in intracellular granules and, therefore, can be rapidly released independently of mRNA synthesis.

MMP-9 is down regulated in monocytes after stimulation with INF $\gamma$  and LPS, while it is up-regulated in the transition between monocytes and monocyte-

derived macrophages (MDM), and after 10-days culture there is an increase of 25-fold for MMP-9 and a 9-fold increase for MMP-2. M-CSF increases 5-fold in MMP-9 expression in freshly isolated monocytes.

MMP-9 is involved in monocyte migration for example in response to chemoattractants.

Positive inducers of MMP-9 in monocytes are:

- MCP-1
- HIV-1 Tat
- Lectins
- PMA
- Endotoxin
- IL-1 $\beta$
- Prostaglandin E2
- Chemokines (RANTES/CCL5, RANTES/CCR1)
- Cytokines (TNF $\alpha$  and GM-CSF)

Negative regulation is performed in macrophages by:

- IL-4
- IL-10
- INF $\beta$
- INF $\gamma$
- TGF $\beta$



MMP Designation	Other name(s)	Latent/active (kDa)	Collagen substrates	Other substrates	Pro-MM substrat
MMP-1	Collagenase-1, Fibroblast collagenase, interstitial collagenase, ColA, ColB	55/45	I, II, III, VI, VII, X	Agg, EN/NL, FN, Gel, LN, PG, VN, MBP, serpins, $\alpha_2$ M, Per, TNF precursor, $\alpha_1$ -ACT, $\alpha_1$ -PI, IGFBPs	-2 -9
MMP-2	Gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase	72/66	I, III, IV, V, VII, X, XI, XIV	Agg, EL, EN/NL, FN, Gel, LN, PG, VN, Dec, Fib, CCL-7, CXCL12, MBP, TNF precursor, plas, $\alpha_1$ -PI, IGFBPs, pro TGF $\beta$	-1 -9 -13
MMP-3	Stromelysin-1 Transin-1	57/45	III, IV, IX, X, XI	Agg, EL, EN/NL, FN, Gel, LN, PG, VN, Dec, Fib, E-cadherin, Latent TGF- $\beta$ 1, MBP, TNF precursor, plas, $\alpha_1$ -PI, IGFBPs, osteopontin	-1 -7 -8 -9 -13
MMP-7	Matrilysin, PUMP, Uterine metalloproteinase	28/19	IV, X	Agg, EL, EN/NL, FN, Gel, LN, casein, PG, VN, Dec, Fib, Pro- $\alpha$ -defensins, Fas ligand (CD95L), Latent TNF, Syndecan-1, E-cadherin, MBP, $\alpha_1$ -PI, plas, $\beta$ 4-integrins, $\alpha$ prodefensin, osteopontin	-1 -2 -9
MMP-8	Collagenase-2 Neutrophil collagenase	75/58	I, II, III, V, VII, VIII, X	Agg, EL, FN, Gel, LN, Mouse CXCL5, ser, $\alpha_2$ M	
MMP-9	Gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase	92/86	IV, V, VII, X, XIV	Agg, EL, Gel, LN, PG, VN, Dec, Fib, FBN, Agg, Zona occludens 1, Latent TGF- $\beta$ 1, Latent VEGF, NG2 proteoglycan, TNF precursor, $\alpha_1$ -PI, MBP, pro-IL-1 $\beta$ , IL-8, IL-2R $\alpha$ (CD25)	
MMP-10	Stromelysin-2 Transin-2	57/44	III, IV, V, IX, X	Agg, EL, FN, Gel, LN, PG, nidogen, carboxymethylated transferin	-1 -8 -7
MMP-11	Stromelysin-3	51/44		IGFBPs, $\alpha_1$ -PI	
MMP-12	Metalloelastase macrophage metalloelastase (MME), Human metalloelastase (MME)	54/45/22	I, IV	Agg, EL, EN/NL, FN, Gel, LN, caesin, PG especially heparin and chondritin sulphates, LN, Fib, latent TNF, plas plasminogen, $\alpha_1$ -PI	
MMP-13	Collagenase-3 Rat collagenase	60/48	I, II, III, IV, VII, IX, X, XIV	Agg, FN, Gel, Fib, Ser, PAI, $\alpha_1$ -ACT	-9
MMP-14	MT1-MMP Membrane-type MMP	66/56	I, II, III	Agg, EN/NL, FN, Gel, $\gamma$ 2-subunit of LN-5, FBN, syndecan-1, CD44 (Pgp-1), integrin $\alpha$ V, factor XII, $\alpha_2$ M, pro-TNF, latent TGF $\beta$ , $\alpha_1$ -PI	-2 -13
MMP-15	MT2-MMP	72/60		Agg, FN, Gel, LN, FBN	-2
MMP-16	MT3-MMP	64/52	III	FN, FBN, VN, LN, Gel, syndecan-1	-2
MMP-17	MT4-MMP	57/53		Fib, FBN, TNF $\alpha$ precursor	-2
MMP-18	Collagenase 4	70/53	I		
MMP-19	RASI 1	54/45	IV, I	FN, Gel, LN, COMP	
MMP-20	Enamelysin	54/22		Agg, amelogenin, COMP	
MMP-21	Xenopus MMP	70/53		ND	
MMP-22	Chick embryo MMP	51/42		Caesin, Gel	
MMP-23	CA-MMP			Gel	
MMP-24	MT5-MMP			Gel	-2
MMP-25	Leukolysin MT6-MMP		IV	Gel, Casein, FN, TACE substrate, $\alpha$ 1-PI	
MMP-26	Endometase Matrilysin-2	28/19	IV	FN, Gel	-9
MMP-27				ND	
MMP-28	Epilysin	56/45		Caesin	

MMPs designations and substrate{{8980 Webster,N.L. 2006}} .

As the production/inhibition of MMPs is strictly regulated by several molecules, the results obtained using a monocytic cell-line do not necessarily reflect data obtained in primary cells.

Monocyte stimulation with LPS and proinflammatory cytokines leads to induction of a number of MMPs, including MMP-1, -9, -2, -3; MMP-1 degrades fibrillar collagen I, II and III into gelatin which is further degraded by MMP-9, contributing to ECM degradation during inflammation.

MMPs have an important role in modulating immune responses via specific cleavage of pro-sequences in chemokines and cytokines.

Regulation of MMP expression is influenced by direct contact of monocytes and macrophages with other cell types in various tissues and with matrix components{{8980 Webster,N.L. 2006}}.

### **Metalloproteinases in atherosclerotic plaques**

High inflamed atheromatous plaques show increased global MMP activity (Choudhary 2006) and level of MMP-1, MMP-3, MMP-8, MMP-9 are higher in atheromatous than in fibrous plaques{{9016 Sukhova,G.K. 1999; 9017 Sluijter,J.P. 2006; 9019 Orbe,J. 2003}}: MMP-9 were found in macrophages rich of oxidized LDL{{9021 Hua,Y. 2009}}; moreover, MMP-9 knock-out mice develop a reduced atherosclerosis{{9020 Luttun,A. 2004}}.

In humans, the plaque fibrous cap prone to rupture also have increased MMP-8, -11, -14 and -16. Metalloproteinases-1, -2, -8, -13 and -14 can cleave straight-giving fibrillar type-I and type III collagens. Indeed MMP-1 and -13 co-localize with cleaved collagens in plaques.

MMP-9 and -12 fragment elastin, whereas MMP-3 and -7 degrade cleaved collagens and proteoglycan core proteins.

MMP-2 is increased in fibrous more than in atheromatous plaques, so not all MMPs are marker of plaques inflammation.

## **Mineralocorticoid receptor (MR)**

### **Structure**

The MR is a member of the steroid receptor family of hormone activated transcription factors, codified by the NR3C2 gene that is located on chromosome 4q31.1-31.2{{8982 Fan,Y.S. 1989}}. MR displays a modular structure comprising several separate domains with specific functions{{8983 Arriza,J.L. 1987}}.

The N-terminal domain of the MR contains an autonomous activation function (AF-1) that is considered to be constitutively active{{8984 Rupprecht,R. 1993}} ; Fuse et al., 2000) and plays a key role in the interaction with transcriptional coregulators{{8985 Tallec,L.P. 2003}}and with the ligand-binding domain (LBD){{8988 Rogerson,F.M. 2003}}. The central DNA-binding domain is composed of two zinc-finger structures that are involved in DNA binding and receptor homodimerization (Liu et al., 1996). By definition, the LBD is involved in ligand binding (Fagart et al., 1998), but also in the interaction with the heat shock protein 90 and transcriptional coactivators{{8990 Couette,B. 1998; 8998 Hellal-Levy,C. 2000}} and harbors a ligand-dependent activation function, AF-2{{8991 Nemoto,T. 1993}}.

In the absence of the ligand, MR is predominantly located in the cytoplasm{{8992 Fejes-Toth,G. 1998}}. It is associated with a multiprotein complex composed of heat shock proteins and immunophilins{{8993 Rafestin-Oblin,M.E. 1989}}. Heat shock protein 90 maintains the MR in an inactive state and in a ligand-binding-competent state{{8990 Couette,B. 1998}}. Binding of aldosterone to the MR induces changes in the receptor conformation{{8995 Trapp,T. 1995; 8996 Couette,B. 1996}} that trigger the translocation of the receptor into the nucleus{{8992 Fejes-Toth,G. 1998}}, the recruitment of transcriptional coactivators{{8998 Hellal-Levy,C. 2000}}, dimerization of the MR and interaction with DNA sequences located in regulatory region.

## **Function**

MR is a receptor with equal affinity for mineralocorticoids and glucocorticoids and is expressed in many tissues, such as the kidney, colon, heart, central nervous system (hippocampus), brown adipose tissue and sweat glands. In epithelial tissues, its activation leads to the expression of proteins regulating ionic and water transports (mainly the epithelial sodium channel or ENaC, Na<sup>+</sup>/K<sup>+</sup> pump, serum and glucocorticoid induced kinase or SGK1) resulting in the reabsorption of Na<sup>+</sup>, and as a consequence an increase in extracellular volume, increase in blood pressure, and an excretion of K<sup>+</sup> to maintain a normal salt concentration in the body.

The RAAS (Renin-Angiotensin-Aldosterone System) regulates the electrolyte balance and blood pressure. In response to decreasing blood pressure the

kidney release rennin, which cleaves angiotensinogen; thanks to the Angiotensin Converting Enzyme (ACE), the angiotensin II is active and can promote aldosterone release through its receptor AT1R in the adrenal. In the kidney, aldosterone binds the MR to enhance re-absorption of Na<sup>+</sup> and water, thus increasing blood pressure.

The MR can be activated by mineralocorticoids such as aldosterone (or deoxycorticosterone in rodents) as well as glucocorticoids like cortisol, with equal affinity. Since cortisol is 100-1000 fold more concentrated in the blood stream, the MR is mainly occupied by glucocorticoids.

In sodium-responding cells (kidney, colon), the MR is "protected" from glucocorticoids by 11-beta-dehydrogenase isozyme 2 (11-β-hydroxysteroid dehydrogenase 2; 11β-HSD2) an enzyme co-localized with the MR, which inactivates cortisol to cortisone.

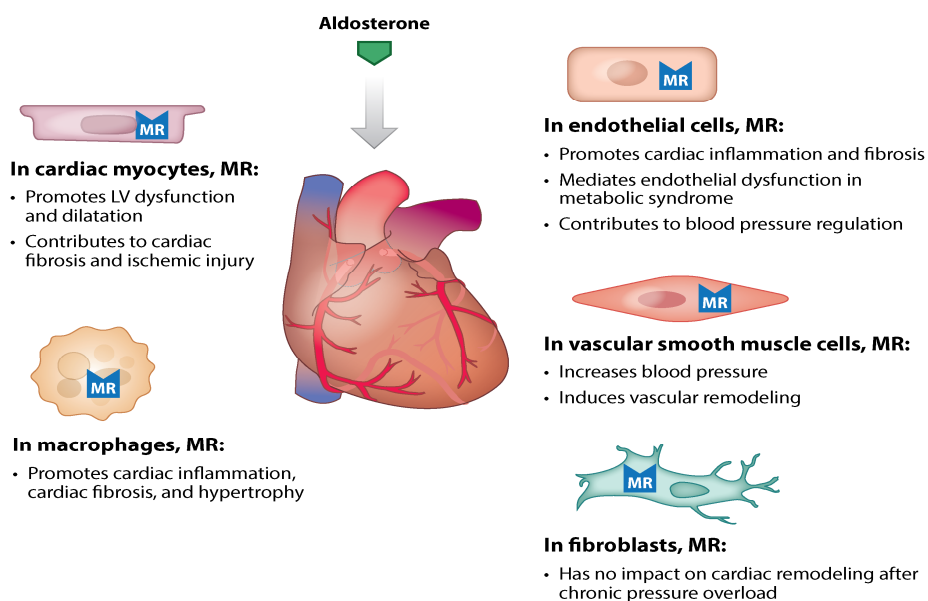
The enzyme is also expressed in non-epithelial cells, including vascular smooth muscle cells and endothelial cells, while cardiomyocytes do not.

Activation of the MR by binding of its ligand aldosterone, results in its translocation to the cell nucleus, homodimerization and binding to hormone response elements present in the promoter of some genes. This results in the complex recruitment of the transcriptional machinery and the transcription into mRNA of the DNA sequence of the activated genes.

### **Aldosterone, CVD and atherosclerosis**

Compelling evidence exists that aldosterone promote cardiac fibrosis, hypertrophy and cell death with a strong association with inflammation and oxidant signaling. (He, Anderson, Cell; Rocha; Weber and Brethe).

Primary aldosteronism (PA) is the most common form of secondary hypertension (PAPY, JACC, 2006); the two major causes are aldosterone-producing adenomas and bilateral adrenal hyperplasia(Rossi Nat Rev Endocrinol). Patients with PA have a significant higher risk of nonfatal myocardial infarction, atrial fibrillation and stroke compared with age, sex and blood-pressure matched essential hypertensives (Zennaro MC, hypertension, 2012)-(Rossi, Funder TEM).



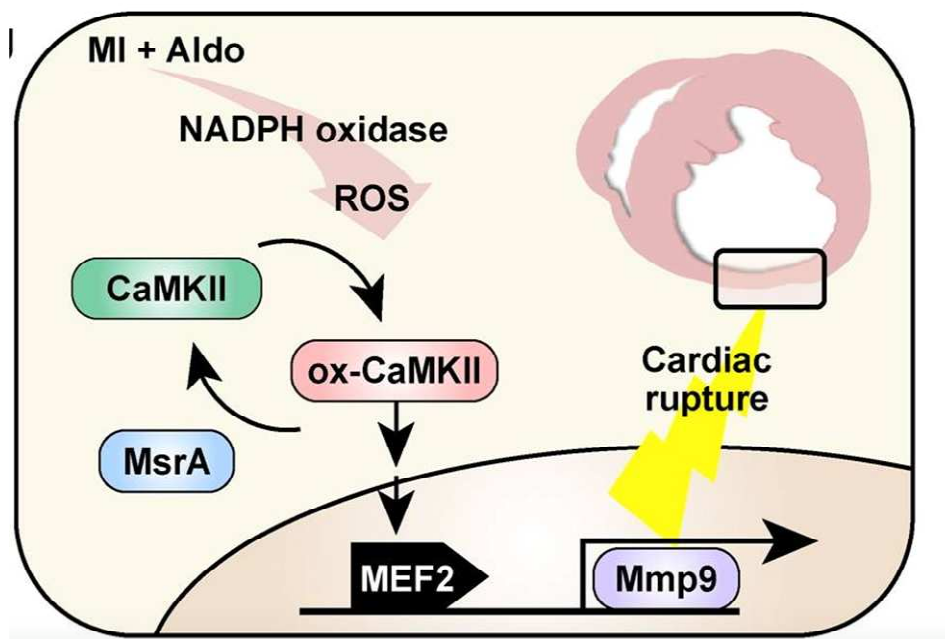
Lothar A, et al. 2015. Annu. Rev. Pharmacol. Toxicol. 55:289–312

**Figure 6.** Role of MR and aldosterone in different cell types involved in atherosclerosis.

The heart is exposed to several sources of ROS (NADPH oxidase, mitochondrial enzymes, xanthine oxidase and NOS), and aldosterone is able

to generate oxidative stress through mechanisms not completely understood yet. Recent studies by He{{8965 He,B.J. 2011}} identified the *Ca<sup>2+</sup>/calmodulin dependent protein kinase II* (CaMKII) as a direct downstream target of oxidation by NADPH-derived ROS mediated by aldosterone. Activated CaMKII in turn stimulates an increase in MMP-9 action to promote cardiac rupture driving myocardial infarction.

The same authors showed that CaMKII is also activated in myocytes by aldosterone through the MR pathway and is involved in the early pathological effects of aldosterone after myocardial infarction, when both CaMKII and ROS are elevated.



**Figure 7.** Proposed model for MI + Aldo-induced CaMKII activation leading to myocardial rupture. In the acute post-MI setting, ox-CaMKII leads to MMP-9 up-regulation to accelerate matrix breakdown, leading to cardiac rupture and premature death. MsrA reduces ox-CaMKII to prevent Mmp9 expression and protect against post-MI cardiac rupture{{8965 He,B.J. 2011}}.

Animal studies demonstrated a clear role of aldosterone as inducer of atherosclerosis in mice{{9022 Gamliel-Lazarovich,A. 2010; 9006 Keidar,S. 2004; 9023 Keidar,S. 2003; 9024 Suzuki,J. 2006}}, rabbits{{9025 Van Belle,E. 1995; 9027 Imanishi,T. 2008}} and monkeys{{9028 Takai,S. 2005}}.

In humans, studies{{9029 Ivanes,F. 2012; 9030 Hillaert,M.A. 2013; 9031 Beygui,F. 2006}} evidenced that high plasma aldosterone levels are associated with a worse prognosis for patients with stable coronary atherosclerosis or acute coronary syndrome. Therapy with antagonist of MR (MRA) have a positive effect in patients with coronary atherosclerosis with or without previous myocardial infarction{{9032 Rossignol,P. 2011}}.

### **Galectin -3 and atherosclerosis**

Galectin-3 (Gal-3) belongs to a family of soluble  $\beta$ -galactoside binding lectins<sup>1</sup> that reside in the nucleus and cytoplasm of several cell types, as well as in the extracellular space. It has multiple actions that can be relevant for promoting vascular damage and cardiovascular fibrosis.

Accordingly, in patients with heart failure plasma Gal-3 level has been shown to be the best short-term predictor of events, thus leading to incorporation of this measurement in the current American Heart Association heart failure guidelines for risk stratification purposes of such patients.<sup>12</sup>

Galectin-3 enhances monocyte interleukin-10 production to a TLR2/1 ligand; furthermore, galectin-3 diminished monocyte to dendritic-like cell differentiation and T-cell antigen presentation{{9033 Chung,A.W. 2013}}.

Studies have shown that of Gal-3 is implicated in a variety of processes



associated with heart failure, including myofibroblast proliferation, fibrogenesis, tissue repair, inflammation, and ventricular remodelling.

Some actions of Gal-3, as monocytes chemoattraction, enhancement of phagocytosis, and induction of vascular smooth muscle cells proliferation,<sup>7</sup> can play an important role in atherogenesis{{9034 Maiolino,G. 2015}}. In the Genetic and ENvironmental factors In Coronary Artery disease(GENICA) study, which enrolled high cardiovascular risk patients referred for coronary angiography for suspected coronary artery disease (CAD), plasma Gal-3 levels predicted cardiovascular deaths and events during long-term follow-up after adjustment for many other cardiovascular risk factors and potential confounders.

### **MR in immune cells**

The extra-renal effects of MR are clearly involved in CVD, and the involvement of inflammation in its initiation and progression is emerging. A direct role of MR in immune cells is emerging, albeit not fully understood.

Barish et al. In 2005 showed that 28 of 49 known nuclear receptor are expressed in mice macrophages; 9 of 12 known endocrine receptors are expressed and they included GR, MR, oestrogen receptor  $\alpha$  (ER $\alpha$ ), progesterone receptor, vitamin D receptor, thyroid receptors  $\alpha$  and  $\beta$ , and retinoic acid receptor  $\alpha$  and  $\gamma$ {{8974 Barish,G.D. 2005}}. The localization of glucocorticoid receptor in the immune system is distinct, reflecting their specific and opposing role: thymus for example expresses only GR (at one of the highest level in the body), while spleen expresses both GR and MR.

The classical and well characterized macrophages activators LPS or INF- $\gamma$  induced a distinct temporal gene expression profile for each of the nuclear receptor in macrophages; LPS stimulation increased GR expression fivefold over baseline after just 4 hours, whereas MR gene expression was completely suppressed, supporting the well described and potent anti-inflammatory action of GR signalling{{8974 Barish,G.D. 2005}}. Furthermore, the reduced MR expression is consistent with studies demonstrating the pro-inflammatory role of MR signalling in non-epithelial tissues ([Rocha 2002](#), [Young 2003](#)). In contrast, INF $\gamma$  induced a sustained fourfold increase of both receptors in macrophages{{9002 Rickard,A.J. 2009}}. Rickard et Young in 2009{{9002 Rickard,A.J. 2009}} concluded that the immunostimulatory effects produced by low-corticosterone concentrations are mediated via MR signalling whereas the immunosuppressive effects of high corticosterone are produced through GR.

Administration of MRA, such as eplerenone or spironolactone, reduces macrophages accumulation in a number of disease models including peritoneal fibrosis ([Nishimua et al 2008](#)), myocardial infarction ([Fraccarollo 2008](#)) and vascular inflammation induced by angiotensin II ([Neves 2005](#)) and aldosterone ([Rocha 2002](#)). As demonstrated by Calò et al. ([2004](#)), aldosterone enhances inflammatory and oxidative stress markers such as p22phox and PAI-1 in human monocytes; moreover it was reported to increase oxidative stress (in terms of enhanced ability to oxidize LDL and superoxide anion production) in macrophages derived from Apo-E deficient mices{{9006 Keidar,S. 2004}}.

Aldosterone activation of MR in endothelial cells specifically modulates ICAM-1 expression, thus promoting leukocytes adhesion. Mineralocorticoid/salt treatment is also associated with increased monocyte/macrophages infiltration and expression, of inflammatory markers such as osteopontin, MCP-1, IL-6, and IL-1 $\beta$  in the kidney{{9004 Blasi,E.R. 2003}}. In macrophages, due to the lack of 11 $\beta$ HSD2, glucocorticoids may be the predominant MR ligand. However, the interplay between MR- and GR-mediated effects in these cells, as well as the role of MR in T and B lymphocytes remains to be explored{{9005 Bene,N.C. 2014}}.

The role of MR in hypertension is well known, in fact MRA are active drugs against essential hypertension. In experimental models with high blood pressure it was demonstrated that anti-hypertensive drugs reduce both recruitment of macrophage in heart, brain and kidney and blood pressure.

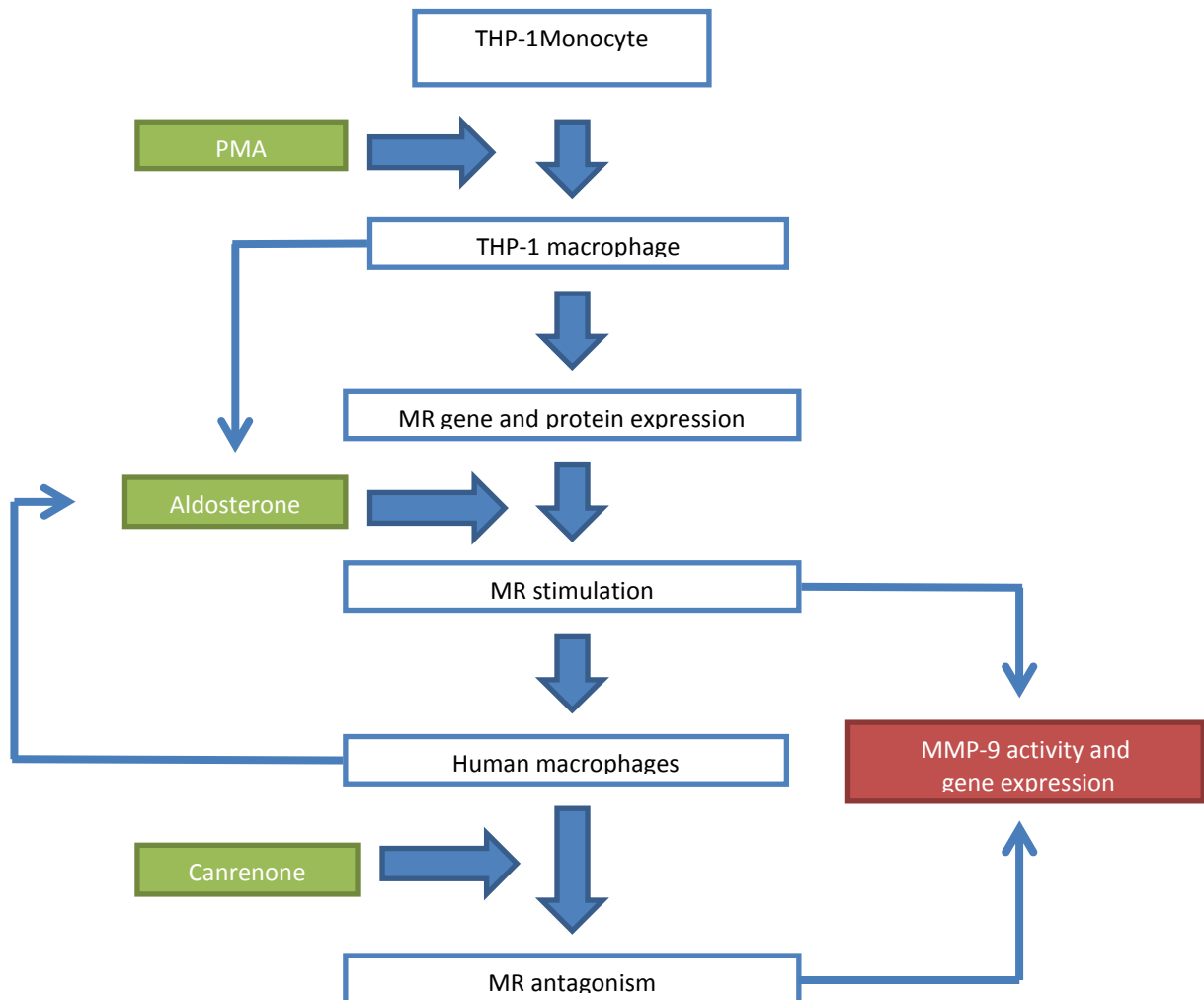
MR seems to have a role in macrophages polarization: as reported by Usher{{9007 Usher,M.G. 2010}}, in mouse peritoneal macrophages treated with aldosterone, the MR activation resulted in increased expression of the M1 classical activation markers TNF $\alpha$ , RANTES, MCP1, IL-12, and spironolactone prevented the induction of these markers by LPS; furthermore, MR-deficient macrophages showed reduced expression of M1 markers. Similarly, an immortalized mouse microglial cell line, which are macrophage-like cells of the central nervous system, MR activation with aldosterone potentiated LPS induction of the pro-inflammatory cytokines TNF $\alpha$  and IL-6 in a MR but not GR dependent manner ([Chantong, j neuroinflammation 2002](#));

these cytokines are regulated by the transcription factor NF- $\kappa$ B, that is in turn activated in macrophages in a MR-dependent manner.

# AIM

The primary aim of our study was to investigate the effects of aldosterone and mineralocorticoid receptor antagonist on the MMP-9 production by monocytes/macrophages.

The following flow chart show the experimental procedure we adopted:



# MATERIALS AND METHODS

## Cell cultures

Human monocytes were isolated from buffy coat by double gradient separation with Ficoll 1077 and Percoll (2 separate centrifugation 400x g 30' without brake and accelerator); cells were rinsed in RPMI 1640 supplemented with L-glutamine (1%), antibiotic-antimycotic solution (1%), and 2% of heat-inactivated foetal bovine serum (FBS), counted by trypan blue exclusion method, and are allowed to adhere to the plate for 1 hour at 37°C. All reagents were purchased from *Sigma Aldrich, Italy*.

Cells were seeded at the concentration of  $2 \times 10^6$  cells in a 24-well plate for monocyte, and at  $5 \times 10^5$  cells/well to obtain macrophages, then washed 3 times with adhesion and stimulation medium, in order to eliminate all non adherent cells (lymphocytes).

Monocytes are stimulated immediately after separation, while GM-CSF was used to obtain macrophages (*Miltheniy Biothech*) in RPMI 20% FBS for 1 week, with a partial change of medium at day 4.

THP-1 is a human monocytic cell line derived from an acute monocytic leukemia of 1-year-old male patient ([Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K \(August 1980\). "Establishment and](#)

characterization of a human acute monocytic leukemia cell line (THP-1)". *Int. J. Cancer* 26 (2): 171–6. doi:10.1002/ijc.2910260208)

THP-1 cell line were grown in suspension in RPMI 1640 medium supplemented with 10% FBS, L-glutamine (1%), antibiotic-antimycotic solution (1%).

To obtain THP-1 macrophages, we added 100nM PMA (phorbol 12-myristate 13-acetate, *Sigma Aldrich*) for 72 hours; after this period, the majority of cells are attached to the well.

Medium was discarded and replaced with new fresh medium without PMA for 3-6 days (resting time).

To stimulate cells for zymography, we added stimulus in RPMI medium without FBS or in presence of Nutridoma (*Roche/Sigma Aldrich*), a chemical supplement that can completely replace serum in cell culture media.

### **Dose-response curve of Aldosterone**

THP-1 macrophages and human macrophages were obtained as described before. The aldosterone stimulus is placed in RPMI without FBS at decreasing concentrations ( $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$ ,  $10^{-9}$ M) and samples were collected after 6-16-24-48 hours.

After this time, the supernatant was centrifuged ( $4,7 \times g \times 5'$ ) to eliminate cells in suspension, and supernatant were collected at  $-80^{\circ}\text{C}$  until use.

### **Other cell stimulations**

Canrenone (*Doppel Farmaceutici*) was added at a concentration 100-fold higher than that of aldosterone and put 30 minutes before, to allow the occupancy of the MRs.

Lipopolysaccharide from *Escherichia coli* (*Sigma Aldrich*) was used at concentration of 100 ng/ml.

Modified Citrus Pectin (*PectaSol-C, ecoNugenics*) was dissolved in PBS at final concentration of 0.5 mg/ml.

## **RT-PCR**

Total RNA was extracted from cells using the High Pure RNA Isolation Kit (*Roche*). One  $\mu\text{g}$  of total RNA were reverse transcribed with iScript™ Reverse Transcription Supermix for RT-qPCR (*BioRad; Milan, Italy*) in a final volume of 20  $\mu\text{l}$ . The mRNA was measured with a real time qRT-PCR with universal ProbeLibrary probes in the LightCycler 480 Instrument (*Roche; Milan Italy*). The expression was calculated applying DDCt method relative to actin or 18S used as an internal control.

## **Zimography**

To investigate the gelatinolytic activity of MMP-9 in response to aldosterone, we used zymography, a semi-quantitative electroforetic running, performed in a 8% gel containing gelatin as substrate, under non-reducing conditions. Equal volumes of surnatant proteins were used for each sample.



After the running, the gels were rinsed in 2.5% Triton X-100 (*Sigma Aldrich*) 2x30' to remove SDS, followed by incubation at 37°C overnight in developing buffer (Tris 100 mM, NaCl 400 mM, CaCl<sub>2</sub> 20 mM).

Gels were then stained (0.5% Coomassie Brilliant Blue, *Sigma Aldrich*) for 30' and destained with a destaining solution (methanol 5%, acetic acid 7%); areas of enzymatic activity appeared as clear bands over the blue background.

Band intensity were measured with VersaDoc Imaging System (*Biorad*) and the MMP9 activity was calculated as ratio between the treated and non-treated samples; the quantification was performed with ImageJ (*NIH, U.S.*).

### **Western Blot**

MR protein expression in THP-1 cells was evaluated by Western Blot analysis; cells were homogenized in lysis buffer (*Thermo Scientific, Italy*) and protein concentration was determined in the soluble supernatant with BCA (*Thermo Scientific, Italy*).

Lysate fraction (50 µg) was separated in a polyacrylamide gel and then electroblotted onto nitrocellulose membrane (*Hybond ECL-Amersham Biosciences Europe, Germany*). The membranes were blocked for 1h at room temperature in 5% non-fat dry Blocking Milk (*Roth*) and thereafter incubated overnight at 4°C with a primary mouse monoclonal antibody against human MR (1:100 dilution) (a kind gift from Prof. Celso Gomez Sanchez){9038 Gomez-Sanchez,C.E. 2011}}. Blots were analyzed with the QuantityOne software.

# RESULTS

## **THP-1 monocytes and differentiation into macrophages**

Phorbol 12-myristate 13-acetate (PMA) was used to differentiate THP-1 monocytes into macrophages through the activation of Protein Kinase C (PKC). The different cells phenotype was detected by their different features, entailing a large, round cell morphology and growth in suspension of THP-1 monocytes and a “star-like” shape with adherence to the well plastic of THP-1 macrophages.

THP-1 monocytes differentiation was induced through exposure different PMA concentrations, i.e. 50 nM, 75 nM, 100 nM, 200 nM, for 24 hours.

To verify the cells transformation they were analyzed by means of flow cytometry (Fluorescence Activated Cell Sorting, FACS); due to the cost of this technique, we decided to test THP-1 monocytes and THP-1 + PMA 100 nM. Flow cytometry allows to scatter the cells by dimension and granularity; after PMA treatment for 24 hours the cells began to show the phenotypic transition toward macrophages (Figure 1A), although a relevant amount of them remained in suspension.

Furthermore, the sorting based on HLA-DR antibody discrimination evidenced a difference between untreated THP-1 and THP-1 PMA $10^{-7}$ M-treated monocytes: after 24 hours this marker was expressed by 57% of monocytes and by only the 27% of PMA $10^{-7}$ M-treated cells (Figure 1B).

Based on these preliminary results, the time of PMA with different PMA concentrations (50 nM, 75 nM, 100 nM, 200 nM) stimulation was extended to different periods (24, 48 and 72 hours). The optimal PMA concentration and stimulation interval selected were 100 nM for 72 hours, which guaranteed adherent and healthy cells, whereas at lower PMA concentration the adherence was not complete, and at higher concentration the cells displayed unhealthy features, evidenced by the presence of cell debris and shape dissimilarities.

The gene expression of PPAR $\gamma$  was evaluated at RT-PCR to confirm the differentiation of monocytes into macrophages according to the method reported by Maeß ([Selection of reliable reference genes during THP-1 monocyte differentiation into macrophages, Stefanie Sendelbach and Stefan Lorkowski](#)). This is based on the premise that PPAR $\gamma$  is the least stable gene in the transition of THP-1 monocytes toward macrophages; hence its gene expression was found to be very different between THP-1 macrophage and THP-1 monocytes. For these reasons the gene expression of PPAR $\gamma$  was determined to confirm the shift from monocytes to macrophages evidenced at morphological examination. As shown in figure 2, PPAR $\gamma$  gene expression increases in a concentration-dependent manner, confirming that the 100 nM concentration of PMA leads to an increased transition of the cells into macrophages.

### **THP-1 monocytes/macrophages expression of MR**

Preliminary to the investigation of the effects of aldosterone on monocyte/macrophages, it was initially necessary to seek for the expression of MR in these cells.

Gene expression studies (RT-PCR) data evidenced that THP-1 monocytes show lower expression of MR compared to THP-1-derived macrophages treated with 100 nM PMA for 72 hours: MR gene expression was  $1.63 \pm 0.26$  ( $p = 0.008$ ) fold more expressed compared to THP-1 monocytes (Figure 3).

The western blot (Figure 4) data confirmed that THP-1 monocytes have lower expression of MR compared to THP-1-derived macrophages: the stimulation performed included three different concentrations of PMA for 72 hours; after this time of PMA stimulation the MR protein expression was significantly higher at PMA concentration of 100 nM compared to THP-1 monocytes ( $4.11 \pm 0.35$   $p = 0.001$ ). Thus, we choose this experimental setting to induce THP-1 differentiation in all further experiments.

### **Demonstration of aldosterone activity through the MR on THP-1 macrophages**

To demonstrate that THP-1-derived macrophages are responsive to aldosterone MR-mediated effects, FKBP51, a 51KDa FK506 binding protein whose expression is known to be induced by aldosterone, was determined.

THP-1-derived macrophages showed a significant increase of FKBP51 gene expression ( $p = 0.002$ ) with increasing concentrations of aldosterone, as assessed by RT-PCR (Figure 5).

### **Metalloproteinase-9 activity in THP-1 monocyte**

After demonstrating that THP-1 monocytes express the MR, these cells were stimulated with aldosterone for 24 hours and supernatants were collected to test MMP-9 activity. As shown in Figure 6, at zymography THP-1 monocytes did not show any increase in MMP-9 activity in response to aldosterone. In particular there were no differences between the band density of control THP-1 monocytes (treated with vehicle) and aldosterone-treated cells.

### **MMP-9 activity in THP-1 macrophages**

To test the effects of aldosterone on MMP-9 activity induced by THP-1 macrophages a dose- (aldosterone concentration  $10^{-5}$  M,  $10^{-6}$  M,  $10^{-7}$  M,  $10^{-8}$  M,  $10^{-9}$  M) and time-response (5, 16, 24, and 48 hours) curve were completed. As shown in Figure 7, at higher aldosterone concentrations, i.e.  $10^{-5}$ M and  $10^{-6}$ M, the stimulation effect on MMP-9 activity occurs early in the time-course analysis, between 5 and 24 hours. At variance, lower aldosterone concentrations, i.e.  $10^{-8}$ M and  $10^{-9}$ M, the increase in MMP-9 activity occurs later, with a significant peak at 16 and 48 hours (aldosterone  $10^{-8}$  M for 16 hours,  $p = 0.003$  vs baseline, and aldosterone  $10^{-9}$ M at 16 and 48 hours,  $p \leq 0.001$ ). Stimulations of cells for zymography must be performed in serum-free medium, which is a stressful condition and when too protracted, i.e. 48 hours, induced THP-1 cells detachment from the plate. Thus, 48 hours stimulations were excluded for further experiments.

### **MMP-9 activity on human macrophages**

The same experiments performed on THP-1 cell line were repeated on human monocytes differentiated into M1 subtype macrophages, using GM-CSF.

FKBP51 gene expression was tested before starting the dose-response curve experiments to verify that the human cells are responsive to aldosterone. Compared to macrophages vehicle-treated, the gene expression of FKBP51 raised in a concentration dependent manner, as expected, with a  $5.25 \pm 0.30$  ( $p < 0.0001$ ) fold increase for cells stimulated with aldosterone  $10^{-5}$ M (Figure 8).

Considering the aforementioned data obtained with the THP-1 cell line, we tested MMP-9 activity on human macrophages, in a simplified dose- and time-response curve, focusing on lower concentrations of aldosterone ( $10^{-7}$  M,  $10^{-8}$  M,  $10^{-9}$  M) for 2, 6 and 24 hours.

Gene expression data (Figure 9, panel A) showed a significant increase of MMP-9 expression after 2 hours of aldosterone at  $10^{-7}$ M and  $10^{-8}$ M concentrations ( $2.03 \pm 0.26$  and  $2.05 \pm 0.26$  fold change vs. control, respectively;  $p < 0.001$ ). Only at 6 hours  $10^{-7}$ M aldosterone showed higher MMP-9 expression compared to control ( $1.71 \pm 0.25$ ,  $p = 0.01$ ). Treatment of human macrophages with aldosterone for 24 hours did not result in an MMP-9 gene expression increase compared to control.

After stimulation of macrophages with decreasing concentrations of aldosterone for 2 hours there was no increase in MMP-9 activity at zymography, except for a slightly increased activity at lower aldosterone

concentration ( $10^{-9}\text{M}$ ). After stimulation for 6 hours MMP-9 activity showed high variability at higher aldosterone concentrations, which was borderline significant at  $10^{-7}\text{M}$  ( $1.26 \pm 0.12$   $p = 0.05$ ). Finally, after 24 hours of stimulation MMP-9 activity was significantly higher at lower aldosterone concentrations, i.e.  $1.23 \pm 0.07$  fold increase compared to vehicle-treated cells ( $p = 0.006$ ) at aldosterone  $10^{-8}\text{M}$  and  $1.36 \pm 0.08$  fold increase at aldosterone  $10^{-9}\text{M}$  ( $p < 0.001$ ) (Figure 9, panel A).

Based on these data, we choose to investigate the effect of aldosterone  $10^{-7}$  exposure for 2 and 6 hours. Figure 10 shows the gene expression (panel A) and zymography (panel B) of MMP-9 in presence of the MRA canrenone.

RT-PCR shows a borderline significance in reduction, both at 2 and 6 hours, in presence of canrenone respect to aldosterone-induced MMP-9 gene expression ( $p = 0.18$  and  $p = 0.08$  at 2 and 6 hours, respectively).

At zymography, canrenone appear to have a small inhibitor effect on MMP-9 activity both at 2 and 6 hours (Figure 10, panel B).

## DISCUSSION

Atherosclerosis is initiated by stimuli, which lead to endothelial dysfunction with consequent expression of adhesion molecules and increased permeability to macromolecules. In these conditions LDLs enter the endothelium and are oxidized by resident vascular cells, promoting monocytes recruitment and transformation into macrophages, which can further promote oxidation of LDLs. Completely oxidized LDLs are recognized by macrophage scavenger receptors and internalized with consequent foam cells formation and accumulation inside the plaque together with other inflammatory cells, which promote plaque rupture through the release of matrix metalloproteinases and other collagenolytic enzymes ([Maiolino, Rossitto, Caielli, Bisogni The Role of Oxidized Low-Density Lipoproteins in Atherosclerosis: The Myths and the Facts, 2013](#)).

There is currently growing attention on the aldosterone role in atherosclerosis development, which was postulated based on animal and clinical data. In fact, aldosterone promotes endothelium dysfunction, mainly through generation of vascular ROS ([Spironolactone Improves Angiotensin-Induced Vascular Changes and Oxidative Stress, Agostino Virdis, Mario Fritsch Neves, Farhad Amiri, Emilie Viel, Rhian M. Touyz, Ernesto L. Schiffrin, J Hypert 2002; Calo; Keidar](#)), vascular inflammation ([Am J Physiol Heart Circ Physiol. 2002 Nov;283\(5\):H1802-10. Aldosterone induces a vascular inflammatory phenotype in the rat heart. Rocha R1, Rudolph AE, Friedrich GE, Nachowiak](#)



DA, Kekec BK, Blomme EA, McMahon EG, Delyani JA), and macrophages polarization toward the inflammatory phenotype (M1) (Usher, *J Clin Invest* 2010v120p3350). Furthermore, animal studies demonstrated that aldosterone plays an important role in atherosclerosis in mice (gamliel-lazarovich *J Hypertens* 2010v28p1900; keidar *circ*; keidar *J Cardiovasc Pharmacol* 2003v41p955, suzuki *Arterioscler Thromb Vasc Biol* 2006v26p917), rabbits (Van Belle *Cardiovasc Res.* 1995 Jan;29(1):27, Imanishi *Hypertension* 2008v51p734) and monkeys (takai *Hypertension* 2005v46p1135). These findings were corroborated by studies on humans showing that patients with stable coronary atherosclerosis{{9029 Ivanes,F. 2012}} or with acute coronary syndrome{{9031 Beygui,F. 2006}} had a worse prognosis with raising plasmatic levels of aldosterone. Clinical non-randomized studies evidenced the positive effect of mineralocorticoid receptor antagonists (MRA) in patients with coronary atherosclerosis with or without previous myocardial infarction{{9032 Rossignol,P. 2011}}. These data were confirmed by recently published randomized clinical trials demonstrated in patients with ST elevation myocardial infarction MRA decreased events (montalescot *Eur Heart J* 2014v35p2295) and mortality (ALBATROSS study *Esc* 2015). In conclusion, despite compelling evidences of the relevant role played by aldosterone in atherosclerosis development the mechanisms responsible for these effects have been elusive.

Therefore, to investigate the effects of aldosterone a cellular model entailing monocytes/macrophages was selected, based on their crucial role in

atherosclerosis. To increase the robustness of the results, we conducted the experiments both on THP-1 cell line and human cells.

After confirming the expression of the MR in THP-1-derived and in human macrophages, which was higher compared to monocytes, the responsiveness to aldosterone stimulation was demonstrated showing the concentration-dependent increased gene expression of FKBP51, a downstream effector of MR.

After demonstration that aldosterone increases MMP-9 expression in cardiomyocytes{{8965 He,B.J. 2011}} we postulated a similar effect in monocytes and macrophages, where this metalloproteinase might promote atherosclerosis development and plaque destabilization.

In fact, MMP-9 was found in atherosclerotic plaques{{9019 Orbe,J. 2003}} and in macrophages rich of oxidized low-density lipoprotein{{9021 Hua,Y. 2009}}. Moreover, in an animal model of atherosclerosis, ApoE-MMP-9 knock-out mice showed a decreased aortic atherosclerotic burden compared to wild type animals{{9020 Luttun,A. 2004}}. Finally, in humans this metalloproteinase was associated to carotid plaque destabilization ([Tan Atherosclerosis 232 \(2014\) 199](#)) and acute coronary syndrome [Tretjakovs Clin. Chim. Acta 413 (2012) 25].

I first tested the MMP-9 production in response to aldosterone in the THP-1 monocytes, demonstrating a minimal metalloproteinase production under aldosterone stimulation (figure 7). Being the macrophage the effector of inflammatory responses in the atherosclerotic plaque I completed the

experiments after PMA-induced differentiation of THP-1 monocytes into macrophages.

The morphological transition of the THP-1 monocytes toward macrophages was confirmed at FACS; among the available antibodies, we select HLA-DR as a marker to test the differentiation of the cells, because it was demonstrated as highly expressed in THP-1 monocytes compared to THP-1-derived macrophages ([flow cytometry and high-content imaging to identify markers of monocyte-macrophage differentiation, 2011, Dev Mittar, Rosanto Paramban, and Catherine McIntyre BD Biosciences](#)). Accordingly, the majority of THP-1 monocytes displayed a HLA-DR<sup>+</sup> profile, while 72% of THP-1+PMA for 24 hours were HLA-DR<sup>-</sup>.

We performed several experiments to clarify if aldosterone has a role in MMP-9 expression and activity in macrophages. Despite the difficulties in quantifying zymography and the variability of PCR data, we found that MMP-9 significantly increases in presence of aldosterone both in THP-1 cell line and human macrophages even if this effect is not macroscopic; aldosterone in fact acts on a cell-system which is already stimulated during the transition between monocyte to macrophage.

NF- $\kappa$ B is involved in MMP-9 production in response to LPS: it was demonstrated that the translocation of NF- $\kappa$ B into the nucleus (where it acts as transcriptional factor) occurs within 2 hours after LPS challenge. Therefore we decided to analyse more in detail what happens with shorter time of incubation in dose-response and time-response experiments with human

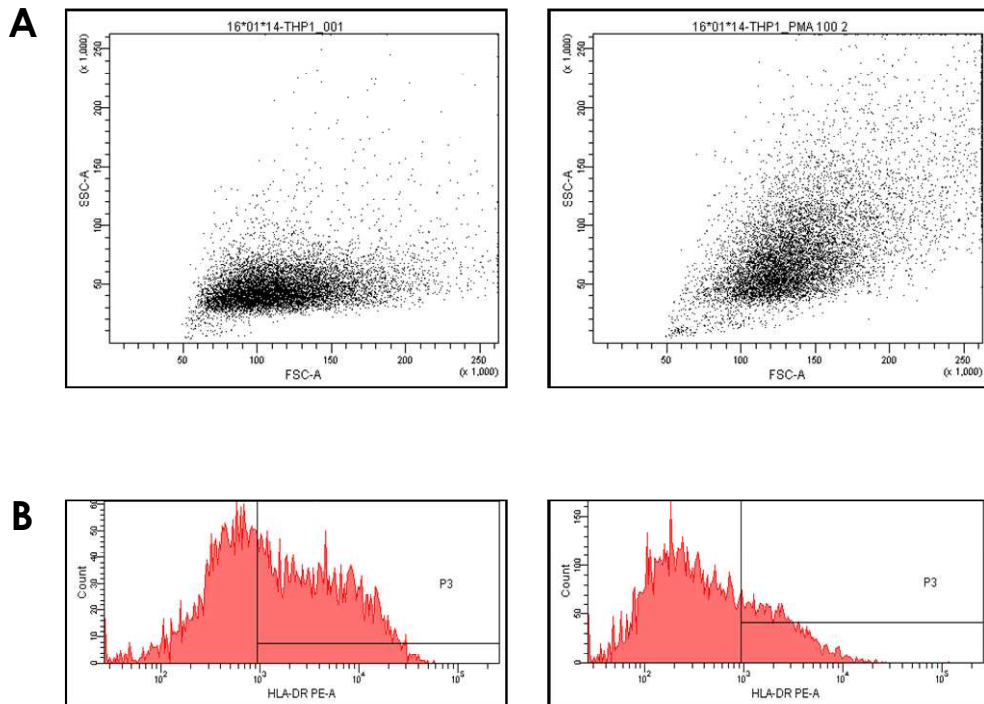
macrophages. Under these conditions, we found a clear effect on gene expression, hence we would like to contend that:

- NF- $\kappa$ B is a downstream effector of MR;
- Canrenone decreases aldosterone effect on MMP-9, confirming its antagonist action on MR and consequently demonstrating that the aldosterone-mediated MMP-9 increase is MR-dependent.

MMP-9 activity assessed by zymography and MMP-9 gene expression appear to have a different timing: MMP-9 activity seems to increase at 24 hours while gene expression is higher between 2 and 6 hours. This point could be explained with the storage in granules of MMP-9, which is released in response to a stimulus, hence not necessarily there is a relation between the gene/protein expression and the activity of the enzyme.

# CONCLUSIONS

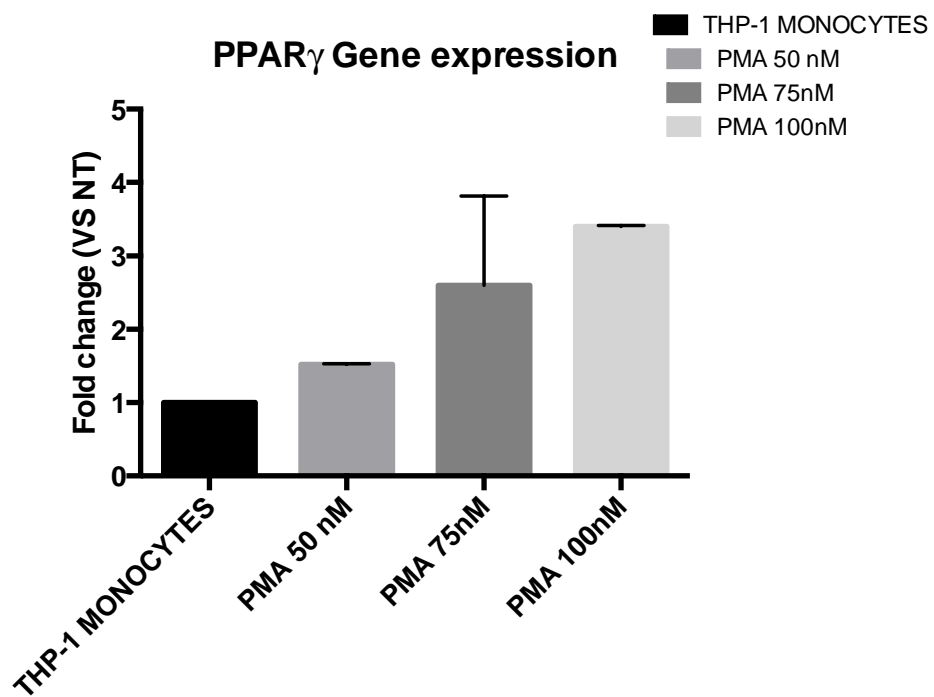
In my PhD thesis I demonstrated for the first time that aldosterone induces matrix metalloproteinase-9 expression in macrophages, a crucial player of atherosclerotic plaque destabilization, and that this effect is mediated by the mineralocorticoid receptor. These results might explain the association between aldosterone and cardiovascular events in patients with stable coronary artery disease and the beneficial effects of mineralocorticoid receptor antagonists in acute ST-elevation myocardial infarction ([montalescot Eur Heart J 2014v35p2295](#); [ALBATROSS study Esc 2015](#)). Further studies are required to determine if a more liberal use of mineralocorticoid receptor antagonists might benefit patients with atherosclerosis.



**Figure 1.**

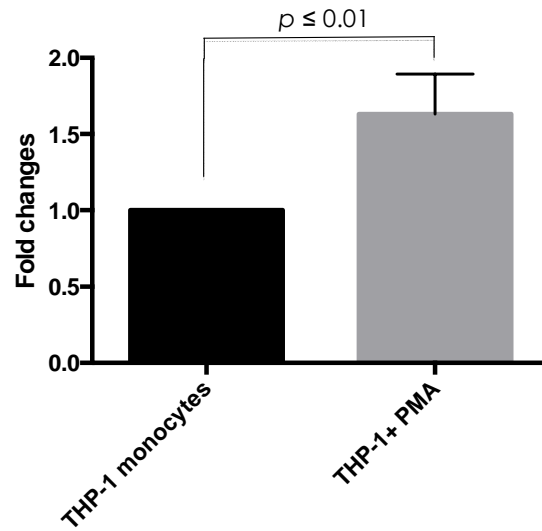
**A)** Scatterplot of THP-1 monocytes in PBS (left panel) and of THP-1+ PMA100 nM after 24 hours of stimulation (right panel).

**B)** Sorting of THP-1 monocytes (left panel) and THP-1 + PMA 100 nM 24 hours (right panel) for HLA-DR antibody.



**Figure 2:** PPAR $\gamma$  gene expression on THP-1 macrophages in presence of different concentration of PMA for 72 hours.

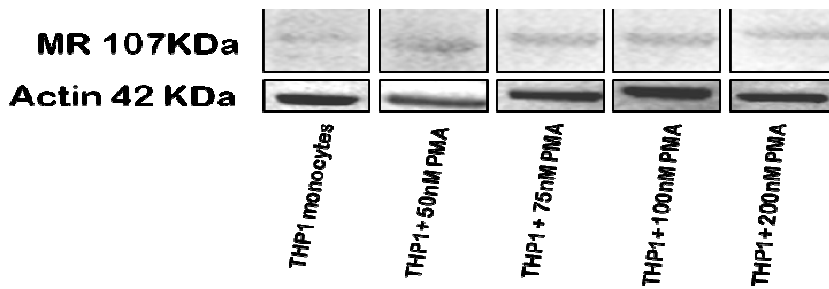
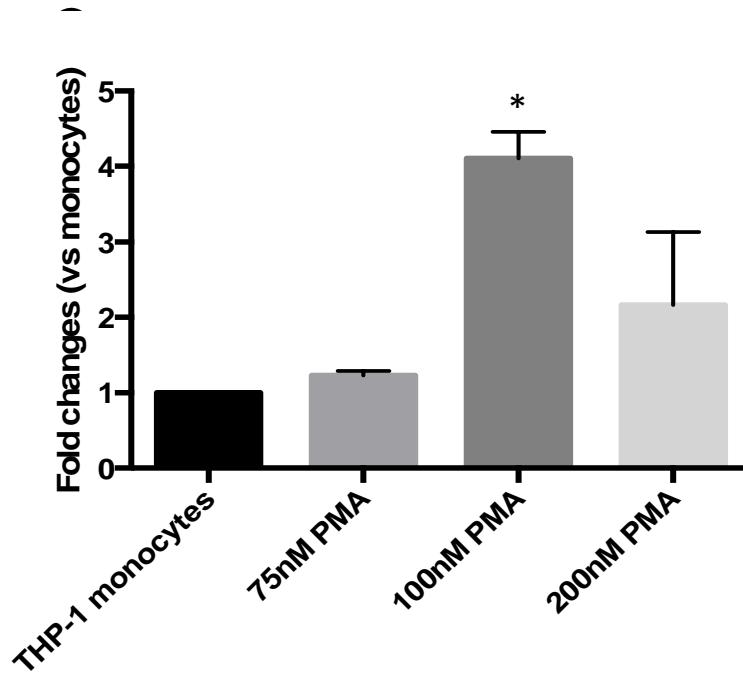
### MR gene expression



**Figure 3.** MR gene expression in THP-1 vehicle-treated and PMA $10^{-7}$ M monocytes on THP-1 + for 72 hours (macrophages).



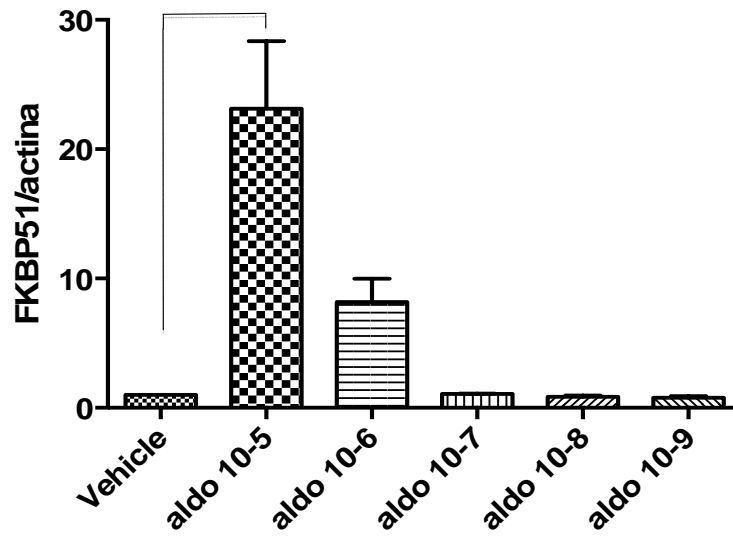
### MR Western Blot



**Figure 4.** Representative immunoblot of MR protein expression in THP-1 monocytes and THP-1 with different concentration of PMA, for 72 hours.

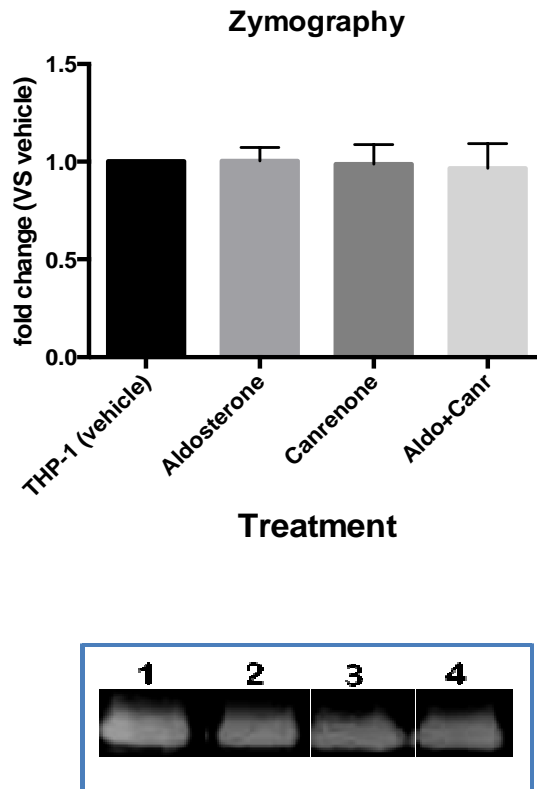
\*  $P \leq 0.001$  vs THP-1 monocytes.

## FKBP51 gene expression

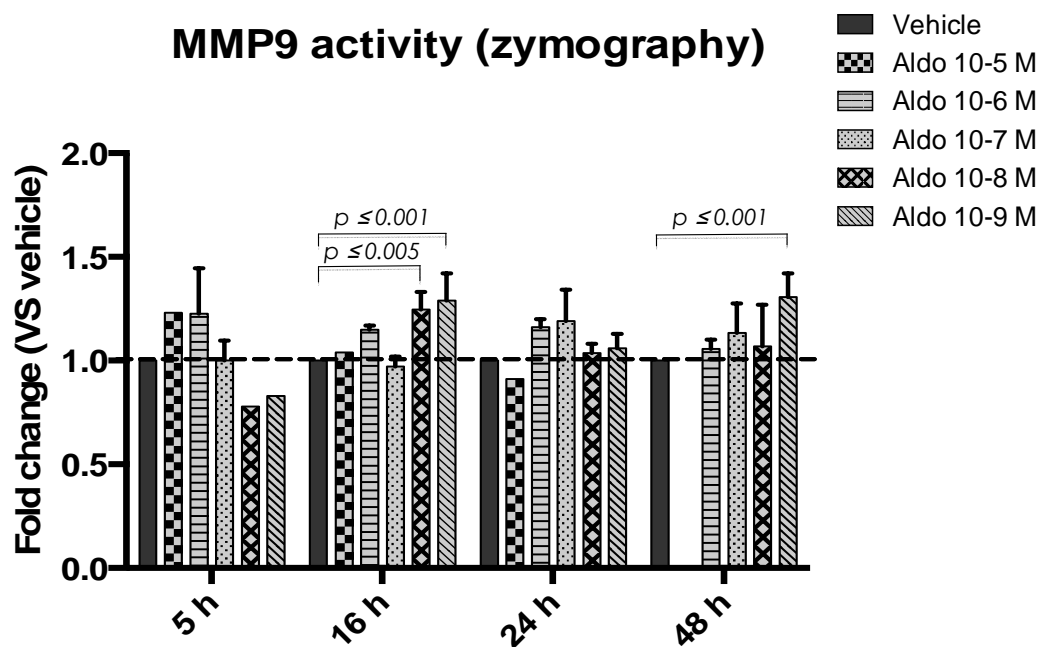


**Figure 5.** FKBP51 gene expression in macrophages stimulated with different concentration of aldosterone.

\*  $P \leq 0.005$  vs Vehicle



**Figure 6.** Representative zymography on THP-1 monocytes supernatants and the corresponding bar graph with the plot of the ratio between band density of treated cells on band density of non-treated cells (vehicle); THP-1 supernatants at baseline (lane1), in presence of aldosterone  $10^{-8}$ M (lane 2), Canrenone  $10^{-6}$  M (lane 3) and in presence of both (lane 4) for 24h.



**Figure 7.** MMP-9 activity at different time points (5-16-24-48 hours), in response to decreasing concentrations of aldosterone, assessed by zymography.

Fold change in MMP-9 activity of THP-1 macrophages treated with aldosterone was measured versus THP-1 macrophages treated with vehicle.

#  $p \leq 0.005$ , \*  $p \leq 0.001$  vs Vehicle

### FKBP51 gene expression

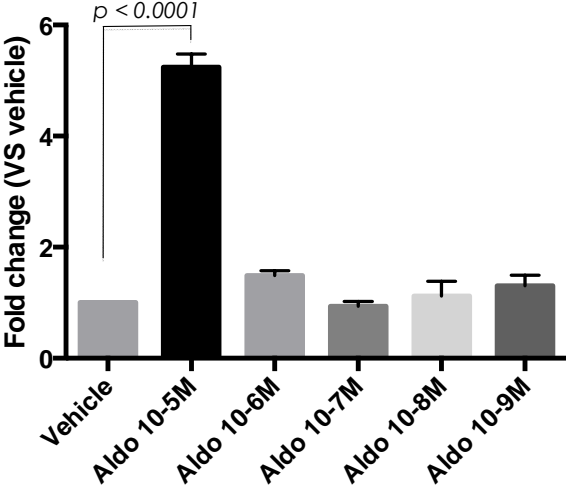
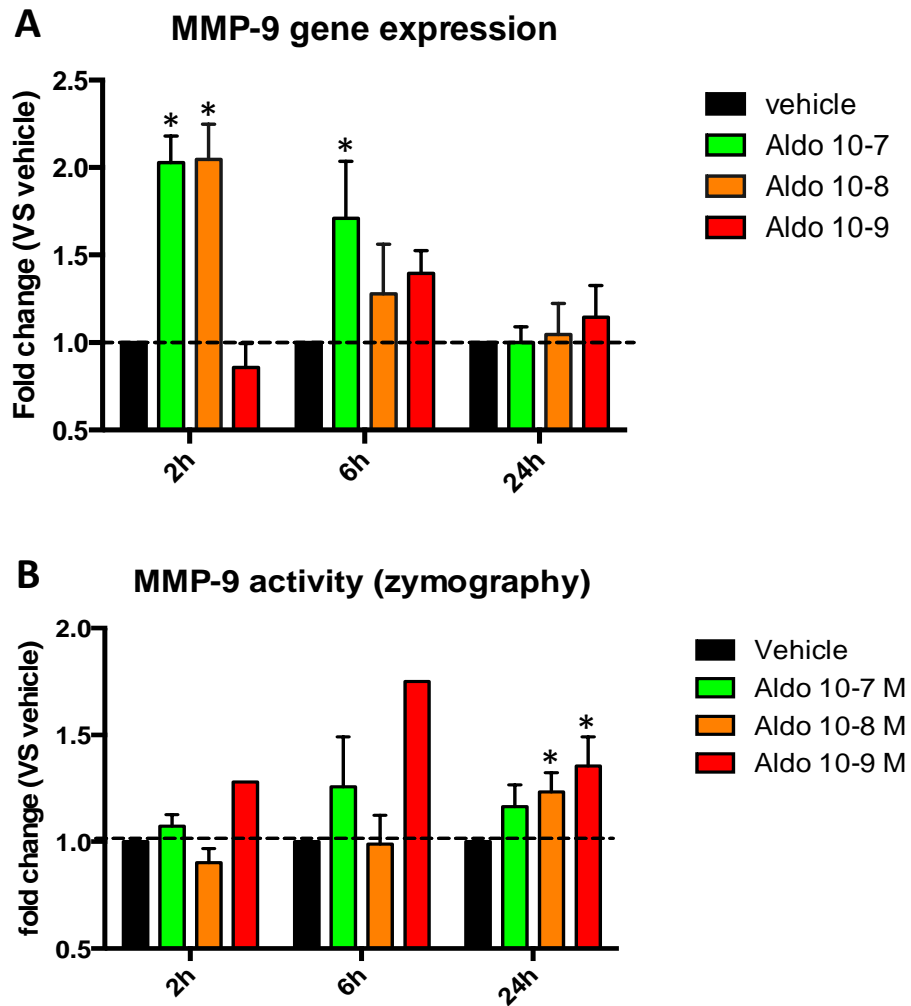


Figure 8. FKBP51 gene expression on human macrophages.

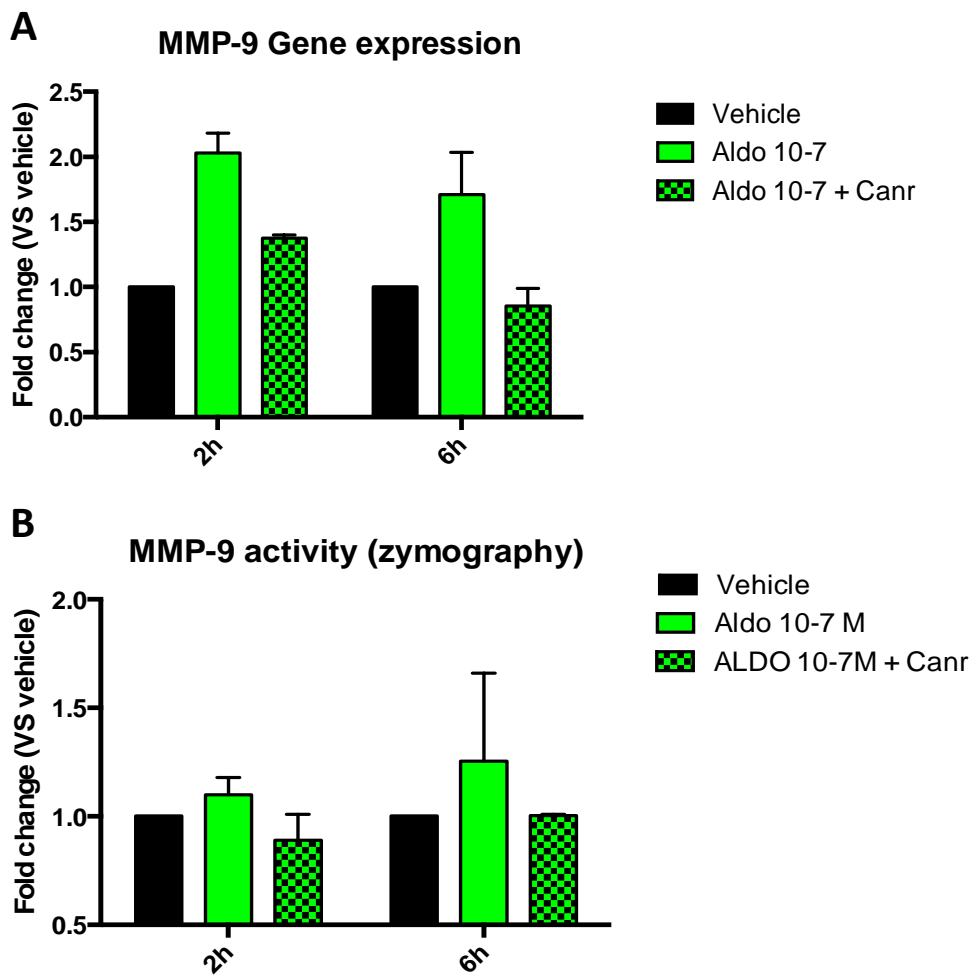


**Figure 9.**

A)MMP-9 gene expression on human monocyte-derived macrophages in presence of different concentration of aldosterone.

B)Corresponding MMP-9 activity assessed by zymography.

\*  $p \leq 0.01$  vs Vehicle



**Figura 10.**

**A)** MMP-9 gene expression in presence of Canrenone (as MR inhibitor)

**B)** Corresponding MMP-9 activity

