

**ORIGINAL INVESTIGATION**

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# Angiotensin II type 1 and type 2 receptor expression in circulating monocytes of diabetic and hypercholesterolemic patients over 3-month rosuvastatin treatment

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## Abstract

**Background:** In diabetes, a variety of pro-inflammatory cellular changes has been found in various cell types, including monocytes which are known to be involved in all the phases of atherogenesis. Angiotensin II (Ang II) type 1 receptor (AT<sub>1</sub>R) mediates the pro-atherogenic effects of Ang II whereas the type 2 receptor (AT<sub>2</sub>R) seems associated with atheroprotection. We sought to investigate the potential changes of AT<sub>1</sub>R-AT<sub>2</sub>R expression in human monocytes of type 2 diabetic- hypercholesterolemic patients and in hypercholesterolemic subjects, upon clinical treatment with rosuvastatin.

**Methods:** The AT<sub>1</sub>R membrane protein and mRNA AT<sub>1</sub>R and AT<sub>2</sub>R expression in monocytes were investigated in 10 type 2 diabetic-hypercholesterolemic patients and in 10 hypercholesterolemic subjects, before and after 3-month rosuvastatin treatment. Moreover, the serum cytokine levels of interferon- $\gamma$  (IFN- $\gamma$ ) and interleukin-4 (IL-4) were detected.

**Results:** As expected, rosuvastatin was associated with a change in the lipid profile in the two groups. Both the membrane protein ( $P = 0.008$ ) and the AT<sub>1</sub>R mRNA expression ( $P = 0.038$ ) were significantly reduced during treatment in the absence of AT<sub>2</sub>R expression change in diabetic-hypercholesterolemic patients whereas no significant difference was observed in hypercholesterolemic subjects. The serum IL-4 levels were increased during treatment whereas no change was observed in IFN- $\gamma$  in diabetic-hypercholesterolemic patients. No cytokine change was observed in hypercholesterolemic subjects.

**Conclusions:** Our study on monocytes of diabetic-hypercholesterolemic patients, showing a reduced AT<sub>1</sub>R but not AT<sub>2</sub>R expression during rosuvastatin treatment, suggests that statin therapy may modulate favorably the AT<sub>1</sub>-AT<sub>2</sub> receptor balance in subjects with coexistent type 2 diabetes.

**Keywords:** Type 2 Diabetes, Angiotensin II type 1 receptor, Angiotensin II type 2 receptor, Monocytes, Statin

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## Background

Results from clinical trials show that systemic markers of inflammation correlate considerably with future cardiovascular events, both at baseline and during pharmacological treatments [1]. Atherosclerosis as an immuno-inflammatory disease has been associated with a prevalent T-helper (Th) 1 response, being the Interferon- $\gamma$  (IFN- $\gamma$ ) a key cytokine for pro-inflammatory response amplification; although controversial, data support an antiatherogenic effect of Th2 responses, and Interleukin (IL)-4, the prototypic Th2-related cytokine, is generally considered as an anti-inflammatory cytokine [2]. The renin-angiotensin-system has been strongly implicated in atherogenesis-related pro-inflammatory events. Angiotensin II (Ang II) exerts its effects through the binding of two major receptors: the Ang II type-1 receptor (AT<sub>1</sub>R) which mediates the majority of the pro-atherogenic well-known Ang II actions and type-2 receptor (AT<sub>2</sub>R) that is considered counteracting the AT<sub>1</sub>R-mediated effects [3]. Recently a direct role of AT<sub>1</sub>R activation in leukocyte and macrophages has been identified in the persistence and/or amplification of microinflammation in vessel walls [4,5]. Although little is known at present about the involvement of AT<sub>2</sub>R in atherosclerosis, these receptors appear to participate importantly in vascular biology with improvement in resistance artery remodeling and appear to be cardioprotective [6-8].

The risk of atherosclerotic cardiovascular disease is increased 2- to 3-fold in type 2 diabetes mellitus and a recent meta-analysis in diabetic patients further strengthens the need of a clinical use of statins in this patient population, irrespectively from the baseline lipid characteristics of the patients [9]. Statins may interfere at various levels with the inflammatory processes leading to atherosclerosis and recent evidence, pointing to an interaction between statins and immune function, showed that isoprenoids could regulate T cell proliferation and Th1 differentiation [10,11]. Moreover, the effects of statins seem to influence pathways leading to Ang II-mediated atherosclerosis and we previously demonstrated, in circulating neutrophils of high-cardiovascular risk patients, interferences of treatment on both AT<sub>1</sub>R expression and the cell membrane translocation Rac 1, a guanosine triphosphate-binding protein playing a key role in Ang II-operated signaling pathways [12].

Among the circulating immune cells, monocytes are the most studied cell subset in relation to atherosclerosis and a key role of these cells in all the phases of atherogenesis has been demonstrated. Monocytes are the primary immune cells that appear early on the endothelial layer and adhere to the arterial endothelium and, after migrating into the intima and accumulating in fatty

streaks, contribute to their differentiation into macrophages which produce inflammatory cytokines, stimulate smooth muscle cell proliferation and migration and produce proteolytic enzymes that render the growing plaque more susceptible to rupture [13]. In the attempt to dissect the pathophysiological aspects and causalities of atherosclerosis and the potential effects of pharmacological treatments, it is worth focusing on cellular and subcellular pro-atherogenic changes associated with atherosclerosis and their modulation by drugs. However, most of the reported studies on the so-called pleiotropic effects of statins have been performed *in vitro* or in animal models of atherosclerosis [14,15] and only very few studies are available on cellular effects of clinically administered statin treatment in humans. Recently a word of caution has been raised on the basis of the notion that the various mouse models in use for studying atherosclerosis differ noticeably in their response to pharmacological modifiers of atherogenesis and differences in anatomy, lipid metabolism, and gene expression complicate translation of experimental results obtained in mice to humans [16].

Moreover, a challenge of the human cellular studies in atherosclerosis relies on the evidence that atherosclerosis is a highly heterogeneous condition. To minimize this limitation of human studies, we focused on diabetic patients who did not show clinical manifestation of atherosclerosis, such as healthy subjects with pre-clinical and early pro-atherogenic changes. We have previously demonstrated that polymorphonuclear cells and monocytes obtained from high-risk subjects express more AT<sub>1</sub>R than monocytes from healthy donors [17-19]. In the present study we sought to investigate whether rosuvastatin treatment in diabetic-hypercholesterolemic patients could be associated with a greater modulation of AT<sub>1</sub>R and AT<sub>2</sub>R expression in monocytes than in only hypercholesterolemic subjects treated with rosuvastatin. Moreover, we investigated whether the statin treatment could interfere with Th1/Th2 profile as expressed by the measure of serum IFN- $\gamma$  and IL-4 levels.

## Patients and methods

### Design of the study

In 10 subjects with type 2 diabetes and hypercholesterolemia (DM), and in 10 hypercholesterolemic (HC) subjects, monocytes were isolated from venous blood to investigate the AT<sub>1</sub>R and AT<sub>2</sub>R expression; moreover serum was also collected to measure cytokine production (IFN- $\gamma$ , IL-4) (see below). Patients were studied: 1] before any pharmacological treatment and 2] after 3-month treatment with rosuvastatin (10 mg/day; 10 PM). The subjects were enrolled consecutively at our Lipid Clinic (Research Center on Dyslipidemia, Clinical Medicine, University of Insubria, Varese, Italy) coming from

the Diabetic Unit or General Practitioner for evaluation of dyslipidemia. As for inclusion criteria to be enrolled in this study, no disease was found after a clinical examination and routine laboratory tests (apart from diabetes), and the patients did not assume any pharmacological treatment except for anti-diabetic treatment. Anti-diabetic medications were allowed if the patients were satisfactorily treated and no changes in treatment were scheduled for the duration of the study. Eight patients were on diet treatment, 1 assumed metformin and 1 patient was on treatment with the association glybenclamide/metformin. Ongoing clinical infection and/or the presence of infections in the previous three months as well as a smoking *habitus* and competitive sporting activities were exclusion criteria. All the patients had previously been instructed about the lifestyle modification required for the treatment of their disease, including dietary treatment (qualitative counseling) and mild physical activity. The patients were then asked to maintain the same level of physical activity and a similar diet throughout the study. All the patients were evaluated with a clinical visit including familial and personal history. Among the diabetic-hypercholesterolemic patients studied, 8 were males, mean age was  $68 \pm 8$  years whereas among hypercholesterolemic subjects mean age was  $65 \pm 10$  years. The body-mass-index ( $28.4 \pm 2.7$  and  $27.4 \pm 2.8$  Kg/m<sup>2</sup>) and waist circumference ( $102.6 \pm 10.4$  and  $99 \pm 10$ cm) were similar respectively in diabetic and hypercholesterolemic patients and the values did not change throughout the study. Blood samples were obtained to perform routine laboratory exams and to isolate circulating monocytes for subsequent studies and to measure serum cytokines by using heparinized tubes between 8.00 and 9.00 A.M., after a night of fasting. Glomerular filtration rate was measured according to MDRD formula [20]. All the subjects were previously asked not to take coffees, teas, chocolates, or cola-containing substances for the 24 hours preceding the evaluations. Our study complies with the Declaration of Helsinki; the local Ethics Committee has approved the research protocol and informed consent has been obtained from the subjects.

#### Cell isolation

Whole blood was allowed to sediment on dextran at 37°C for 30 min. Supernatant was recovered and peripheral blood mononuclear cells (PBMCs) were separated by Ficoll-Paque Plus density-gradient centrifugation. A typical PBMC preparation contained about 80% lymphocytes and 16% monocytes, and cell viability was always >99% as assessed by flow cytometric analysis.

Monocytes (for real-time evaluation) were further separated from PBMCs by immunomagnetic cell sorting. To this end, antibodies targeted to CD14 (monocytes)

were obtained from Dynal A.S. (Oslo, Norway) and added to separate aliquots of the cell suspension using a target-to-bead ratio of 1:4 as previously described [17].

#### Flow cytometric analysis of AT<sub>1</sub>R expression

We have evaluated the expression of AT<sub>1</sub>Rs on the cell membrane of monocytes. To this end, 1 ml of whole blood was used and the analysis was performed by using conventional immunofluorescence techniques together with a multiparametric flow cytometric analysis as previously described [17]. A minimum of 50,000 cells were analyzed from each sample, and AT<sub>1</sub>R density on positive cells [mean fluorescence intensity (MFI)] was obtained.

#### RNA isolation and real-time polymerase chain reaction (PCR) analysis of AT<sub>1</sub>R and AT<sub>2</sub>R mRNA

Total mRNA was extracted from  $1 \times 10^6$  monocytic cells by Perfect RNA Eukaryotic Mini kit (Eppendorf, Hamburg, Germany) and the amount of extracted RNA was estimated by spectrophotometry at 260 nm. Total RNA was reverse transcribed using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's instructions.

Real-time PCR was performed by means of an ABI prism 7000 apparatus (Applied Biosystem, Foster City, CA) using the assay-on-demand kits (Table 1) as previously described [17,21].

Threshold cycle values (Ct<sub>1</sub>) for the genes of interest were calculated, normalized to 18s RNA (Ct<sub>2</sub>) (house-keeping) content and finally expressed as  $2^{-\Delta Ct}$ , where  $\Delta Ct = Ct_2 - Ct_1$ .

#### Measurement of serum cytokines

Serum samples collected were analysed for cytokine content. To this end, IFN- $\gamma$ , IL-4 levels in serum were quantified using a sandwich-type enzyme-linked immunosorbent assay (ELISA kit; Amersham Biosciences, UK). The detection limit of the assay was 1 pg/ml. The control values for serum IL-4 levels were: not detectable; and the serum control IFN- $\gamma$  levels were: 0–1.5 pg/ml, as reported for the kit used.

#### Statistical analysis

Data are presented as mean  $\pm$  standard deviation (SD). A paired *t* test was used to compare variables before and during pharmacological statin treatment. Calculations were performed using a commercial software (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com) and a two-sided *P* < 0.05 was retained for statistical significance.

**Table 1 Real-time-Protein Chain Reaction primers**

Gene name	Interrogated sequence	Translated protein	Exon boundary	Assay location	IMAGE clone ID	
AT <sub>1</sub> R	RefSeq	NM_031850.1	NP_114038.1	1-2	248	-
		NM_000685.3	NP_000676.1	1-2	248	-
	GeneBank mRNA	S77410.1	-	1-2	201	-
		BC068494.1	-	1-2	224	30337806
AT <sub>2</sub> R	RefSeq	NM_000686.3	NP_000677.2	2-3	129	-
	GeneBank mRNA	U16957.1	-	2-3	107	-

AT<sub>1</sub>R = Angiotensin II type 1 receptor; AT<sub>2</sub>R = Angiotensin II type 2 receptor.

## Results

Lipid pattern and other laboratory characteristics were not different at baseline in the two groups (Additional file 1). As expected and shown in Table 2, rosuvastatin treatment significantly affected the lipid profile of the patients after the 3-month treatment. No change was observed in liver and renal function. High-sensitive serum C-reactive protein was also reduced after statin treatment.

### Flow cytometric analysis of AT<sub>1</sub>R expression

Figure 1 shows data of a representative flow cytometric analysis of AT<sub>1</sub>R expression on monocytes of a diabetic subject obtained before and after statin treatment (panel A). The data shown are obtained from one representative subject. A 3-month rosuvastatin treatment significantly reduced AT<sub>1</sub>R membrane expression in monocytes of diabetic patients. Values (measured as

MFI) observed before the institution of therapy were  $1.84 \pm 0.55$  and reached the values of  $1.17 \pm 0.45$  after the clinical statin treatment ( $P = 0.008$ ). Moreover in hypercholesterolemic subjects we didn't see any difference (panel B).

### AT<sub>1</sub>R and AT<sub>2</sub>R mRNA expression in monocytes from patients before and after rosuvastatin treatment

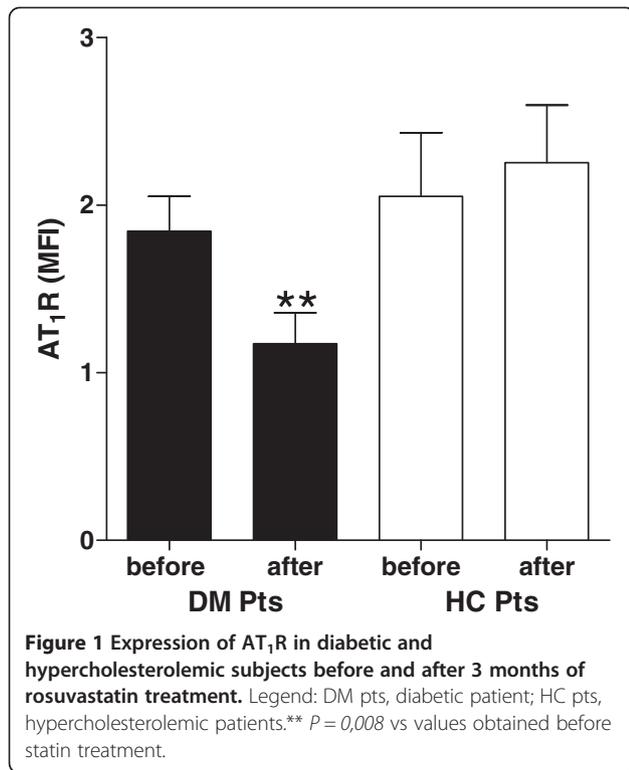
As observed for the protein expression, the mRNA expression of AT<sub>1</sub>R was significantly reduced during treatment in monocytes of diabetic patients (Figure 2, panel A) whereas no difference was observed for the AT<sub>2</sub>R expression measured before and during statin therapy (Figure 3, panel A).

No differences was observed for AT<sub>1</sub>R and AT<sub>2</sub>R expression measured before and during therapy in hypercholesterolemic subjects (Figures 2 and 3, panel B).

**Table 2 Laboratory characteristics of patients at baseline and after three-month rosuvastatin therapy**

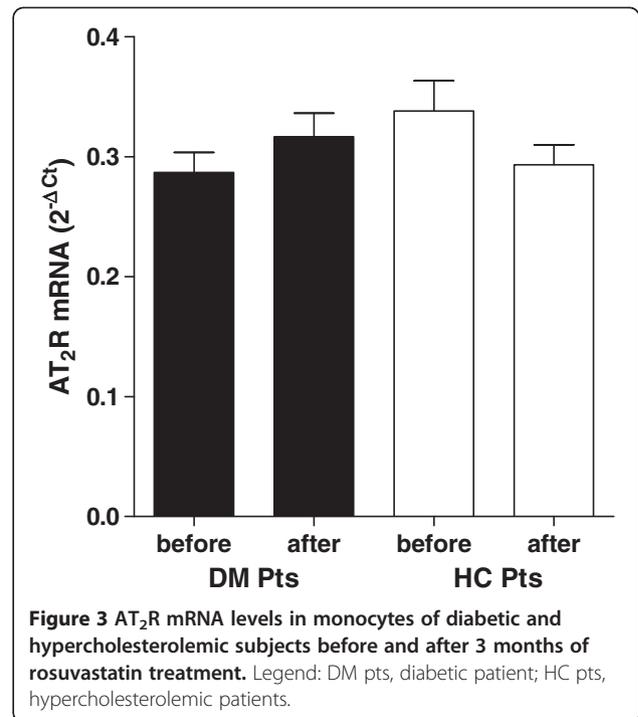
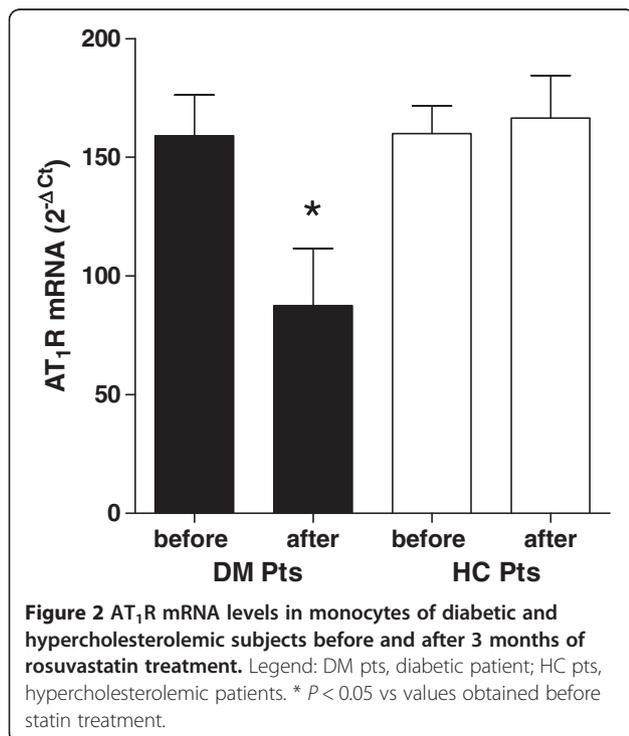
	Diabetic patients (n = 10)			Hypercholesterolemic patients (n = 10)		
	baseline	treatment	#P	baseline	treatment	#P
TC (mg/dl)	223.7 ± 34.0	168.5 ± 45.9	0.003	288.2 ± 41.0	165.5 ± 24.4	<0.001
TG (mg/dl)	165.3 ± 73.0	118.5 ± 31.5	0.012	180.9 ± 61.4	133.5 ± 58.9	0.004
HDL-c (mg/dl)	50.0 ± 2.8	50.2 ± 6.5	0.94	55.1 ± 9.9	50.7 ± 12.1	0.164
LDL-c (mg/dl)	135.7 ± 20.6	85.0 ± 25.6	<0.001	197.0 ± 34.1	88.1 ± 25.1	<0.001
ApoA (mg/dl)	128.0 ± 53.0	163.0 ± 14.4	0.69	144.5 ± 28.5	160.1 ± 55.4	0.34
ApoB (mg/dl)	117.2 ± 29.1	54.0 ± 14.9	<0.001	147.1 ± 38.8	75.1 ± 24.6	<0.001
Glucose (mg/dl)	137.7 ± 19.4	135.5 ± 31.8	0.79	98.6 ± 5.7	97.0 ± 12.9	0.77
Insulinemia (ng/ml)	14.2 ± 3.4	16.3 ± 3.5	0.63	18.8 ± 14.7	19.7 ± 17.0	0.63
AST	24.2 ± 4.3	24.0 ± 4.1	0.94	18.3 ± 5.6	17.1 ± 3.5	0.658
ALT	37.7 ± 19.2	39.2 ± 27.1	0.78	27.6 ± 12.9	21.3 ± 4.4	0.122
GGT	43.3 ± 27.2	46.5 ± 39.6	0.65	21.7 ± 5.9	20.1 ± 5.6	0.131
CK	226.8 ± 113.7	212.7 ± 103.5	0.73	135.7 ± 64.9	134.4 ± 56.6	0.994
s-creatinine	0.98 ± 0.11	0.97 ± 0.10	0.89	0.98 ± 0.17	0.99 ± 0.18	0.597
GFR	76.33 ± 12.18	77.5 ± 3.56	0.79	73.22 ± 33.31	74.00 ± 12.09	0.667
hsCRP	2.94 ± 2.02	1.76 ± 0.51	0.03	1.70 ± 1.69	1.15 ± 0.79	0.350

TC = total cholesterol; TG = triglycerides; HDL-c = high density lipoprotein-cholesterol; LDL-c = low density lipoprotein-cholesterol; ApoA = Apolipoprotein A; ApoB = Apolipoprotein B; AST = aspartate aminotransferase; ALT = alanine aminotransferase; GGT = γ-glutamyl transpeptidase; CK = creatine kinase; GFR = glomerular filtration rate; hsCRP = high sensitivity C reactive protein. # Paired t test.



#### Serum cytokine levels from patients before and after rosuvastatin treatment

Rosuvastatin treatment did not affect serum levels of IFN- $\gamma$  ( $7.97 \pm 2.41$  pg/ml vs  $6.45 \pm 3.35$  pg/ml;  $P = 0.912$ ). A significant increase of IL-4 was observed after three



months of statin treatment ( $22.96 \pm 16.53$  pg/ml vs  $35.10 \pm 16.93$  pg/ml;  $P = 0.020$ ). No cytokine change was observed in hypercholesterolemic subjects.

#### Discussion

The main finding of the present study is the observation that medium-term rosuvastatin clinical treatment is associated with reduced AT<sub>1</sub>R expression in monocytes of type 2 diabetic patients in the absence of AT<sub>2</sub>R expression changes.

Various factors act in diabetes to induce atherosclerotic processes and vascular changes. Both hyperinsulinemia and the lipid pattern called atherogenic dyslipidemia are well known pro-atherogenic factors [22]. Recently, the central role of inflammation in diabetes-related atherosclerosis has been underlined by the finding that pro-inflammatory preconditioning is required for the development of high-glucose-induced inflammation in human aortic smooth muscle cells [23]. Moreover, altered cellular functions of various cell types have been related to diabetes and these abnormalities may contribute to systemic inflammation. In particular, in experimental models of diabetes monocytes/macrophages show a decreased phagocytosis and an increased expression of inflammatory mediators, and cells from diabetic subjects exhibit enhanced adherence to the endothelium [24]. As regards the Ang II receptor expression, the functional relevance of up-regulated AT<sub>1</sub>R expression in diabetes has been underlined by a recent study showing that upregulation of the ligand-Receptor for Advanced

Glycation End products pathway via AT<sub>1</sub>R is an essential mechanism in diabetic atherosclerosis [25]. In diabetic myocardium a significant increase in protein expression and median mRNA expression of the AT<sub>1</sub>R was reported [26]. In carotid human specimen the mRNA expression of both angiotensinogen and angiotensin-converting enzyme were higher in type 2 diabetic patients than in non-diabetic subjects whereas AT<sub>1</sub>R mRNA did not differ [27]. Previously we reported that subjects with increased cardiovascular risk according to the Adults Treatment Panel III guidelines had an increased leukocyte AT<sub>1</sub>R expression at mRNA level and that this expression was reduced during simvastatin treatment but no study has previously focused on cellular changes upon statin treatment in a specific diabetic population [12,17]. In this study we did not enrolled a control group since the comparison of the receptor expression of high risk subjects has been already investigated [17]. Moreover, we show here for the first time that not only mRNA, but also protein membrane expression of AT<sub>1</sub>R was significantly reduced during rosuvastatin treatment.

AT<sub>2</sub>Rs are present in the vasculature and may induce vasorelaxation in vivo [6-8]. Very few studies are at present published on the AT<sub>2</sub>R expression and functioning in humans, and it is now generally accepted that the AT<sub>1</sub>R blockade is associated with AT<sub>2</sub>R overexpression and that AT<sub>2</sub>R stimulation by Ang II induces counterregulatory vasodilatation that opposes AT<sub>1</sub>R-mediated vasoconstriction [28]. Indeed, in a study on resistance arteries dissected from gluteal subcutaneous tissues of hypertensive diabetic patients treated with valsartan, Ang II evoked a significant vasodilatory response which was blocked by an AT<sub>2</sub>R inhibitor [6]. However, studies performed on human internal mammary arteries, AT<sub>2</sub>R receptor stimulation did not mediate vasodilation and AT<sub>2</sub>R-mediated vasodilation in the human heart was shown to be limited to coronary microarteries [29,30].

To our knowledge, no study is available on both AT<sub>1</sub>R and AT<sub>2</sub>R modulation by rosuvastatin. In this study, we show for the first time that, after treatment with rosuvastatin, no changes are observed in AT<sub>2</sub>R mRNA expression in monocytes of type 2 diabetic patients. This finding strengthens the results of reduced AT<sub>1</sub>R observed during treatment, suggesting a possible favorable modulation of the AT<sub>1</sub>-AT<sub>2</sub> receptor expression during statin treatment. Moreover, according with the already reported finding of a relatively lower AT<sub>2</sub>R tissue expression in the adult when compared with AT<sub>1</sub>R expression [28], we show in human monocytes of diabetic subjects that the AT<sub>2</sub>R is expressed at a lower level than AT<sub>1</sub>R. However we have to acknowledge that the results reported here, obtained in a population mainly constituted by male subjects, apply only to type 2 diabetic patients. Even if we have not recruited a control group,

the reduction of AT<sub>1</sub> receptor expression after rosuvastatin treatment could be explained by a specific effect of diabetes, given that the two groups didn't differ in their lipid profile. Moreover, the relatively small number of the patients enrolled in this study or the short-term could have influenced the results in hypercholesterolemic patients.

It has been recently reported that rosuvastatin was able to affect the Th1/Th2 response in humans with acute coronary syndrome [31]. In our study, in subjects prone to atherosclerosis but in a stable phase of the process as the type 2 diabetic patients studied, a significant increase of the Th2-profile-related IL-4 was observed whereas we did not evidence a reduction in circulating IFN- $\gamma$  during treatment. Previously, a reduction in pro-inflammatory cytokine production by circulating monocytes has been associated with statin treatment in humans [32,33]. As regards statin effects on cytokines directly involved in immunomodulation, although simvastatin-treated dendritic cells showed a Th2 transcription profile which was accompanied by increased Th2 (IL-4, IL-5, and IL-13) and decreased Th1 (IFN- $\gamma$ ) cytokine secretion from the T cells [10], no longitudinal study in humans has previously shown increased IL-4 levels during clinical statin treatment. Other authors showed no immunomodulatory effect by atorvastatin on the Th1/Th2 balance in human T cells in vitro [34].

## Conclusions

In conclusion, this longitudinal study in humans show that rosuvastatin treatment is associated with reduced monocyte AT<sub>1</sub>R but not AT<sub>2</sub>R expression, suggesting that the clinically administered statin therapy may modulate favorably the AT<sub>1</sub>-AT<sub>2</sub> receptor balance in diabetic patients.

## Additional file

**Additional file 1: Laboratory characteristics of patients at baseline.**

### Abbreviations

Ang II: Angiotensin II; AT<sub>1</sub>R: Angiotensin II type 1 receptor; AT<sub>2</sub>R: Angiotensin II type 2 receptor; IFN- $\gamma$ : Interferon- $\gamma$ ; IL: Interleukin; Th: T-helper; PBMCs: Peripheral blood mononuclear cells; MFI: Mean fluorescence intensity; SD: Standard deviation.

### Competing interests

The authors declare that they have no competing interests

### Authors' contributions

FM, AMM and LG: design, data collection, drawing the manuscript, data analysis and statistics. All authors: design, critical revision of article and approval of article.

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