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## **Antiatherogenic properties of high-density lipoproteins from arterial plasma are attenuated as compared to their counterparts of venous origin**

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## **Abstract**

Background and aims : High-density lipoprotein (HDL) particles play atheroprotective roles by their ability to efflux cholesterol from foam cells and to protect low-density lipoproteins (LDLs) from oxidative damage in the arterial intima. We hypothesized that antioxidative properties of HDLs can be attenuated in the oxygen-rich prooxidative arterial environment, contributing to the development of atherosclerosis. To evaluate this hypothesis, we compared antioxidative activity of HDLs from arterial and venous plasmas.

Methods and Results: Arterial and venous blood samples were simultaneously obtained from 16 patients (age  $68 \pm 10$  years; 75% males) presenting with ischemic or valvular heart disease. Major HDL subfractions and total HDLs were isolated by density gradient ultracentrifugation and their chemical composition and the capacity to protect LDLs from *in vitro* oxidation were evaluated. HDL-cholesterol, triglycerides and apolipoprotein (apo) B-100 levels were slightly but significantly reduced by -4 to -8% ( $p < 0.01$ ) in the arterial vs. venous samples. Total mass of HDL subpopulations was similar and HDL subpopulations did not reveal marked compositional differences between the arterial and venous circulation. Potent antioxidative activity of the small, dense HDL3c subpopulation was significantly reduced in the particles of arterial origin vs. their counterparts from venous plasma (increase of +21% in the propagation rate of LDL oxidation,  $p < 0.05$ ). Interestingly, antioxidative properties of venous HDLs were enhanced in statin-treated patients relative to untreated subjects.

Conclusion: Antioxidative properties of small, dense HDLs from arterial plasma are attenuated as compared to the particles of venous origin, consistent with the development of atherosclerosis in the arterial wall.

**Keywords**: antioxidative properties; arterial; venous; high-density lipoproteins; low-density lipoprotein; atherosclerosis

## Introduction

High-density lipoproteins (HDLs) play an important protective role towards the development of atherosclerosis, primarily by their ability to efflux cholesterol from foam cells and by protecting low-density lipoproteins (LDLs) from oxidative radical damage in the arterial intima [1]. By inhibiting the formation of lipid hydroperoxides and of short-chain oxidized phospholipids, HDLs exhibit antioxidative and anti-inflammatory properties [2,3]. HDL particles feature high structural and biological heterogeneity as well as display diverse biological functions. More specifically, the small, dense HDLs (HDL3c) provide a key contribution to the antioxidative activity of HDLs [4]. The HDL3c proteome contributes to LDL protection against peroxidation by virtue of proteins such as apolipoprotein (apo) A-I, apoA-II, apoJ, and others; a pro-inflammatory component, serum amyloid A, and a fraction of the lipoprotein-associated phospholipase A<sub>2</sub> (Lp-PLA<sub>2</sub>), are equally carried by HDLs [5]. In addition, the HDL3c lipidome is depleted in sphingomyelin and free cholesterol, displaying elevated fluidity of the HDL surface monolayer and facilitating incorporation of exogenous lipids [4,5].

Plasma lipoproteins are typically studied following their isolation from venous blood, whereas atherosclerosis develops at the arterial level. Arterial and venous media differ in partial oxygen pressure (higher in the arterial medium), parietal morphology, blood pressure and pH. These factors may impact antiatherogenic function of HDLs, including their antioxidative properties. Potential differences between venous and arterial blood regarding concentrations and properties of lipoprotein particles and inflammatory factors may impact the development of atherosclerotic lesions, as reported in the 1980s [6,7]. However, other studies found no difference between arterial and venous blood in the concentrations of lipid components [8,9]. Indeed, similar basal oxidative status and susceptibility of arterial and venous LDLs to copper-induced *in vitro* oxidation were reported [10]. In contrast, biological activities of HDLs have never been compared between particles isolated from venous and arterial blood.

The environment may influence functionality of lipoprotein particles primarily in the arterial blood where oxidative stress induced by the relative local hyperoxia can decrease antioxidative properties of HDLs. Consequently, it can be hypothesized that the ability of HDLs to be more protective against oxidation in the venous relative to the arterial circulation might contribute to the absence of atherosclerosis in the venous wall.

Our study therefore aimed at (i) comparing biological serum parameters of the routine lipid profile between venous and arterial blood in a well-defined population; (ii) comparing lipid and protein composition of HDL subpopulations isolated from arterial and venous plasma; (iii) studying antioxidative properties of HDLs isolated from arterial plasma towards *in vitro* LDL oxidation, in comparison with HDLs isolated from venous plasma.

## **Materials and Methods**

### Study population

Twenty-six consecutive subjects were recruited from January 2, 2012 to February 15, 2012 in the Department of Thoracic and Cardiovascular Surgery of the Pitié-Salpêtrière-Charles Foix hospital in Paris. Eight healthy normolipidemic subjects were recruited at the same hospital as a control group. The blood samples were drawn after informed procedures, issue of a newsletter and obtaining consent from the patients before surgery. All patients presented with ischemic or valvular heart disease requiring cardiac surgery under general anesthesia. Their diagnoses included ischemic cardiomyopathy, atrial fibrillation, coronary artery bypass and mitral stenosis.

The blood samples from the patients were taken after a 12-hour fasting period in the pre-anesthesia room during the usual conditioning of the patient by antebrachial peripheral venipuncture during the placement of a peripheral venous route. Obtaining arterial samples needs a medical procedure that is invasive by its potential hemorrhagic risk and that imposes septic conditions. Unlike venous samples, arterial samples are not obtained in routine except for the conditioning of patients in cardiac surgery, which explains our choice in the selection of subjects. Arterial punctures were

performed on the radial artery after arterial catheter placement prior to sedation and oro-tracheal intubation. Eight tubes of 7 mL blood are collected, i.e., 2 tubes without anticoagulant to obtain serum and 2 tubes with EDTA on each venous and arterial sites. The tubes were immediately sent to the Department of Metabolic Biochemistry of the Pitié-Salpêtrière Hospital after conditioning at 4°C. The exclusion criteria for the choice of subjects were as follows: non-menopausal women or women on hormone replacement therapy; BMI > 40 kg/m<sup>2</sup>; smoking; assisted ventilation; secondary cause of dyslipidemia including renal insufficiency (defined by creatinine > 135 µmol/L), hypothyroidism (defined as TSH > 5 mIU/L), hepatopathy or hepatocellular insufficiency (defined by transaminases > 2N); evolutionary infection.

The studies of antioxidative properties of HDLs according to their arterial or venous origin were performed in a smaller number of subjects given the necessity of a laborious and time-consuming HDL isolation by ultracentrifugation. In order to avoid any potential modification of lipoproteins related to oxygenation, only 16 patients whose samples were taken before oro-tracheal intubation for assisted ventilation were included in the study. In healthy controls, only venous blood samples were obtained after overnight fast and HDLs of a venous origin were studied.

#### Analytical methods

After blood centrifugation at 4,500 rpm at 4°C for 10 minutes, plasmas and sera were stored at -80°C until analysis. A complete serum lipid profile [total cholesterol, triglycerides (TG), HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C)] on the both venous and arterial types of sampling was performed by conventional automated enzymatic methods. Concentrations of apoB-100 and of two major apolipoproteins of HDLs, apoA-I and apoA-II, were determined by immunonephelometry [11,12].

Determination of Lp-PLA<sub>2</sub> concentration was performed on serum by ELISA (diaDexus PLAC kit, Eurobio, Courtaboeuf, France) using two monoclonal antibodies 2C10 and 4B4, with a linearity between 0 and 1000 ng/mL.

Five major subpopulations of HDLs were isolated by density gradient ultracentrifugation, using a SW41 rotor, at 15°C, for 48 hours at 40,000 rpm in a Beckman ultracentrifuge [13,14]. The densities of the subpopulations are well defined and were as follows: HDL2b (d 1.063-1.087 g/mL), HDL2a (d 1.088-1.110 g/mL), HDL3a (d 1.110-1.129 g/ml), HDL3b (d 1.129-1.154 g/mL) and HDL3c (d 1.154-1.170 g/mL) [12]. Total HDLs were reconstituted at equivalent plasma concentrations of all the subfractions. LDLs were isolated by density gradient ultracentrifugation at a density between 1.018 and 1.065 g/mL. Lipoproteins were isolated in the presence of EDTA to avoid oxidation; no oxidation of methionine residues of apoA-I was observed during isolation (data not shown).

HDL composition was analyzed by determination of all major constituents, including free and esterified cholesterol, phospholipids, triglycerides and total proteins [11,12].

Antioxidative activity of HDL subpopulations and total HDLs was evaluated towards reference LDL isolated from blood samples obtained from a healthy normolipidemic control subject [11,12]. LDL (10 mg total cholesterol/dL) was oxidized at 37°C in phosphate-buffered saline (PBS) in the presence of 1 mM 2,2-azobis (2-amidinopropane) hydrochloride (AAPH); HDLs were added to LDLs directly before oxidation. Specific antioxidative activity of HDLs was measured at the same final HDL concentration for each subfraction of 10 mg HDL mass/dL, or at 40 mg HDL mass/dL for total HDL, at a HDL/LDL ratio reflecting physiological conditions (2-10 mol/mol). Conjugated dienes were determined as products of lipid peroxidation, by recording the absorbance at 234 nm at 37°C every 5 min for 16 h (Uvikon 933, Kontron AG Corp, Zurich, Switzerland).

#### Statistical analysis.

Distributions of all variables were analysed for normality using the Kolmogorov Smirnov test. Normally distributed variables are expressed as means  $\pm$  SD. Between-group differences in normally distributed variables were analysed using Student's t-test. Differences in dichotomous variables were analyzed by Fisher's exact test. Spearman's correlation coefficients were calculated to evaluate relationships between variables. Differences were considered statistically significant at  $p < 0.05$ . The statistical software employed was Statistica (StatSoft, Tulsa, OK, USA).

## Results

All patients included in the study suffered from ischemic or valvular cardiopathy that required surgical intervention. The patients were over 60 years old and predominantly (75%) male (Table 1). Although sex-matched healthy normolipidemic controls were significantly younger than patients, the groups did not differ in their BMI (Table 1). Expectedly, cardiovascular risk factors, including dyslipidemia, hypertension and type 2 diabetes were highly prevalent in the patient population, resulting in high proportions of hypotensive, cholesterol-lowering and antiplatelet treatments.

Mean concentrations of total cholesterol and LDL-C in venous blood serum were below 4 and 2.1 mmol/L, respectively (Table 2), reflecting frequent statin treatment (37%). Consistent with this result, venous lipoprotein and apolipoprotein concentrations were similar in the patient and control groups, with the only between-group difference involving reduced triglyceride levels in controls (-34%). Triglyceride and HDL-C levels were within usual values for such population. Circulating levels of triglycerides (-8%), HDL-C (-7%) and apoB (-4%) were slightly but significantly reduced in the arterial vs. venous serum (Table 2). Levels of total cholesterol, apoA-I and apoA-II tended to be similarly reduced in the arterial circulation but these differences did not reach significance. Concentrations of Lp-PLA<sub>2</sub> were high and did not differ between arterial and venous serum.

When the population was stratified according to the presence or absence of cardiovascular risk factors, decreases in the concentrations of serum lipids in the arterial vs. venous samples were not specifically associated with any of such factors (data not shown). Similarly, the differences between the arterial and venous lipid concentrations were similar in patients without and with hypotensive, cholesterol-lowering and antiplatelet treatment (data not shown).

Five major HDL subpopulations isolated from the patients revealed no marked compositional differences between arterial and venous plasmas, except slight decreases in the content of cholesteryl esters (-9%,  $p < 0.05$ ; Supplemental Table 1) and apoA-I (-25%,  $p < 0.01$ ; Supplemental Table



2) in HDL3c and HDL2a particles, respectively, in the arterial samples. Total mass of HDL subpopulations was also similar in the arterial and venous plasmas.

When small, dense HDL subfractions or total HDL were added to reference LDL at a physiological HDL/LDL ratio of about 2-10 mol/mol, LDL oxidation was markedly delayed as reported earlier [11,12,15]. Such antioxidative activity of HDL particles was reduced in arterial HDLs relative to their counterparts from venous plasma (Figure 1). Indeed, under conditions used to measure such specific antioxidative activity (HDL mass, 10 mg/dL; LDL-C, 10 mg/dL), potent inhibitory effects of the small dense HDL3c subpopulation towards LDL oxidation were significantly lower in the arterial than in venous HDL, with respect to the decrease in LDL oxidation rate in the propagation phase (-21%,  $p < 0.05$ ; Figure 1, A). Consistent with this result, the duration of the propagation phase tended to be shorter in the presence of the arterial vs. venous HDL3c, although this difference did not reach significance (Figure 1, B). By contrast, no difference in the maximal production of conjugated dienes was observed between LDLs incubated with arterial and venous HDL3c (Figure 1, C). Similarly, no difference in antioxidative properties of HDL3b and total HDLs was observed between arterial and venous samples (Figure 1). Interestingly, the capacity of venous HDLs to delay LDL oxidation was similar in patients and healthy controls. Indeed, HDL3b, HDL3c and total HDLs obtained from controls decreased LDL oxidation rate in the propagation phase by  $-48 \pm 12$ ,  $-28 \pm 15$  and  $-67 \pm 7\%$ , respectively, and their effects did not significantly differ from those of venous HDLs from the patient group (Figure 1). The only significant between-group difference in the antioxidative activity of venous HDLs involved enhanced prolongation of the propagation phase by total HDLs in the controls (by  $+58 \pm 30\%$  relative to LDLs alone,  $p < 0.001$  vs. patients).

When antioxidative activity of small, dense HDL3c was analysed according to the subgroups involving the presence or absence of cardiovascular risk factors and treatment, this functional metric was significantly higher in venous particles obtained from patients treated with statins as compared to untreated subjects. Notably, both the inhibition of LDL oxidation rate in the propagation phase and the prolongation of the duration of this phase induced by venous HDL3c were significantly higher in

statin-treated vs. untreated patients (Figure 2). By contrast, no such difference was observed when arterial HDL3c was studied.

Expectedly, LDL-C levels were significantly lower in statin-treated vs. untreated subjects (Figure 3), whereas differences in HDL-C, TG and Lp-PLA<sub>2</sub> did not reach significance.

## **Discussion**

In the present study we found that antioxidative activity of small, dense HDL particles derived from arterial circulation was significantly impaired relative to their counterparts from venous plasma.

### Antioxidative properties of HDLs isolated from arterial or venous plasma towards *in vitro* LDL oxidation.

Antioxidative activity of arterial HDLs was intermediate between those observed in healthy subjects and in subjects with chronic diseases associated with elevated cardiovascular risk, such as type 2 diabetes or metabolic syndrome [12]. These data suggest that *in vitro* LDL oxidation in the presence of HDLs isolated from venous blood cannot generally be applied to the conditions of arterial circulation. Local conditions of arterial blood differ from the venous environment, with increased partial pressure of oxygen and a less acidic medium than in the venous blood. It can be hypothesized that oxidative stress is increased reflecting the presence of oxygen and its reactive species which cause a greater oxidation of lipoproteins in the arterial environment. Indeed, LDL oxidation occurs more rapidly at higher oxygen pressure [16]. Moreover, in the arterial circulation, the hemodynamic conditions are not comparable to those of the venous circulation. Pressures, shear rates, and turbulences are increased at the origin of focal endothelial dysfunction in certain arterial segments [17].

Attenuated antioxidative properties of arterial HDLs can reflect their altered composition. Decreased antioxidative activity of arterial small, dense HDLs can therefore be hypothetically explained by diminished acquirement and/or inactivation of lipid hydroperoxides due to a deficiency in apoA-I and its methionine residues, as suggested by Zerrad-Saadi et al. [18]. No difference in apo-A-I content

was however observed between small, dense HDLs isolated from the arterial blood relative to their venous counterparts. By contrast, depletion of cholesteryl esters in arterial vs. venous HDL3c might reflect reduced capacity of arterial HDLs to acquire lipids.

#### Parameters of lipoprotein metabolism in arterial and venous media

Our study showed a significant decrease in circulating levels of triglycerides, HDL-C and apoB-100 in the arterial compared to the venous blood. Aviram et al. [6] had already reported, in 10 healthy subjects, significantly lower HDL contents in cholesterol, TG and protein in the arterial medium, as well as lower plasma concentrations of apoA-I, without finding differences in plasma levels of total cholesterol, TG, apoB, or even LDL-C. In 1989, Keidar et al. found in healthy coronary subjects, increased circulating levels of LDL-C and arterial HDL protein content [7]. In contrast, a larger study conducted on 42 subjects treated for peripheral vascular disease found no difference in the concentration of total cholesterol, HDL-C and LDL-C between arterial and venous media [8]. A more recent study conducted in subjects to be explored by right or left catheterization similarly found no difference in lipid profile parameters between the two media [9].

The results of available studies are thus controversial and difficult to compare due to differences in the analytical and preparative methods used. The selection of the subjects could also be an issue since the first study only involved healthy donors.

Determination of Lp-PLA<sub>2</sub> seems justified in our population that was constituted of more than 50% of subjects suffering from valvular heart disease, such as aortic stenosis. Lp-PLA<sub>2</sub> levels are high in our population, whatever the arterial or venous medium, even higher than those observed in a previous study of subjects with stable ischemic heart disease or aortic valvular cardiopathy [19], in agreement with its elevation in cardiovascular diseases.

#### Effects of lipid-lowering treatment with statins on the lipid profile parameters, Lp-PLA<sub>2</sub> levels and the antioxidative properties of HDLs.

Patients treated with statins displayed a better lipid profile than untreated patients, both in venous and arterial blood. Statins exert a cholesterol-lowering effect through competitive inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase [20], a rate-limiting enzyme involved in hepatic synthesis of cholesterol, which acts on the activity of LDL receptors by increasing their number on the surface of peripheral and liver cells [21]. The effectiveness of statin treatments is evaluated by the decrease in LDL-C. Depending on the molecule and posology, expected reductions in LDL-C range from 10 to 60% [22,23]. Protective effects of the statins were also evidenced by enhanced antioxidative capacities of small, dense HDLs from treated patients in our study.

Our study presents a major limitation related to its small sample size. Moreover, HDLs do not account for the total antioxidative activity of plasma, since albumin and uric acid largely contribute to this activity [24]; however, such water-soluble antioxidants can be depleted in microenvironments within arterial intima, enhancing the role of HDL-mediated protection [3]. Another limitation involves clinical heterogeneity of the patient group which presented with different diagnoses. We still believe that our study design was valid as every patient acted as his/her own control. In addition, this design reflected a real-world practice, rendering our study clinically relevant. In addition, we cannot rule out the effect of other, than statins, drugs on the HDL function. It still appears reasonable to assume that these effects if any should not differ between arterial and venous circulation.

In conclusion, our study allowed us to evidence arteriovenous differences in lipid profile, suggesting that cardiovascular risk factors measured in the venous blood may not always accurately reflect lipid metabolism in the arterial environment. Moreover, small, dense HDLs isolated from arterial blood appeared less efficient in protecting LDLs from lipid peroxidation than their counterparts isolated from venous circulation. This finding is consistent with the development of atherosclerosis in the arterial wall and can challenge our current diagnostic approach, enriching current knowledge of atheromatous disease.

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Table 1. Characteristics of the study population (n=16).

Clinical characteristics	Patients (n=16)	Controls (n=8)
Age (years)	68 ± 10	43 ± 10***
Gender		
Male (%)	12 (75%)	6 (75%)
Female (%)	4 (25%)	2 (25%)
BMI (kg/m <sup>2</sup> )	27.7 ± 5.6	24.3 ± 2.5
Cardiovascular risk factors (%)		
Dyslipidemia	8 (50%)	0
Arterial hypertension	8 (50%)	0
Type 2 diabetes	4 (25%)	0
Treatments (%)		
Beta-blockers	9 (56%)	0
Antiplatelet therapy	6 (37%)	0
Statins	6 (37%)	0
ACE inhibitors/ARA II	6 (37%)	0
Hospitalization for (%):		
Coronary artery bypass	7 (44%)	0
Valvular surgery	7 (44%)	0
Both	2 (12%)	0

ACE : Angiotensin Converting Enzyme ; ARAII : Angiotensin II Receptor Antagonist ; BMI : body mass index.

Dyslipidemia was defined according to AFSSAPS 2005 recommendations; arterial hypertension was defined as > 140 mm Hg for systolic blood pressure and >90 mm Hg for diastolic blood pressure. Diabetes was defined according to the criteria of the World Health Organization (fasting plasma glucose level ≥ 7.0 mmol/L (126 mg/dL), or plasma glucose ≥ 11.1 mmol/L (200 mg/dL) 2 h after a 75-g oral glucose load in a glucose tolerance test (OGTT), or symptoms of high blood sugar and casual plasma glucose ≥ 11.1 mmol/L (200 mg/dL)). Among six patients who received statins, three were on atorvastatin (from 10 to 40 mg/d), two were on rosuvastatin (10 mg/d) and one was on pravastatin (20 mg/d); all the patients were treated for 1 to 5 years.

Data are shown as mean ± SD or number (percentage of total).

Table 2. Plasma lipids in venous and arterial blood sera (n=16).

Lipid and lipoprotein profile	Venous serum	Arterial serum	Controls
Total cholesterol (mmol/L)	3.74 ± 0.72	3.35 ± 0.93	4.45 ± 0.79
Triglycerides (mmol/L)	<b>1.31 ± 0.44</b>	<b>1.21 ± 0.40**</b>	<b>0.87 ± 0.28*</b>
LDL-cholesterol (mmol/L)	2.06 ± 0.64	2.03 ± 0.67	2.62 ± 0.53
HDL-cholesterol (mmol/L)	<b>1.11 ± 0.39</b>	<b>1.03 ± 0.36**</b>	1.40 ± 0.27
ApoB-100 (g/L)	<b>0.71 ± 0.19</b>	<b>0.68 ± 0.19**</b>	0.70 ± 0.07
ApoA-I (g/L)	1.21 ± 0.25	1.16 ± 0.27	1.47 ± 1.14
ApoA-II (g/L)	0.267 ± 0.091	0.256 ± 0.083	n.d.
Lp-PLA <sub>2</sub> (µg/L)	395 ± 183	397 ± 175	n.d.

Parameters significantly differing between arterial vs. venous samples (Student's t test for paired samples) are shown in bold; \* p<0.05; \*\*p<0.01 vs. venous plasma; n.d.: not determined.

## Figure legends

Figure 1. Antioxidative activity of HDL3c, HDL3b and total HDL subfractions isolated from arterial and venous blood plasmas during *in vitro* LDL oxidation (n = 16). **A.** Percentage decrease in the LDL oxidation rate in the propagation phase in the presence of HDL3c and HDL3b subfractions, and total HDLs from arterial and venous plasmas (n = 16). **B.** Percentage increase in the duration of the propagation phase during LDL oxidation in the presence of HDL3b, HDL3c and total HDL subfractions from arterial and venous plasmas. **C.** Percentage decrease in the maximal concentration of conjugated dienes during LDL oxidation in the presence of HDL3c and HDL3b subfractions, and total HDLs from arterial and venous plasmas.

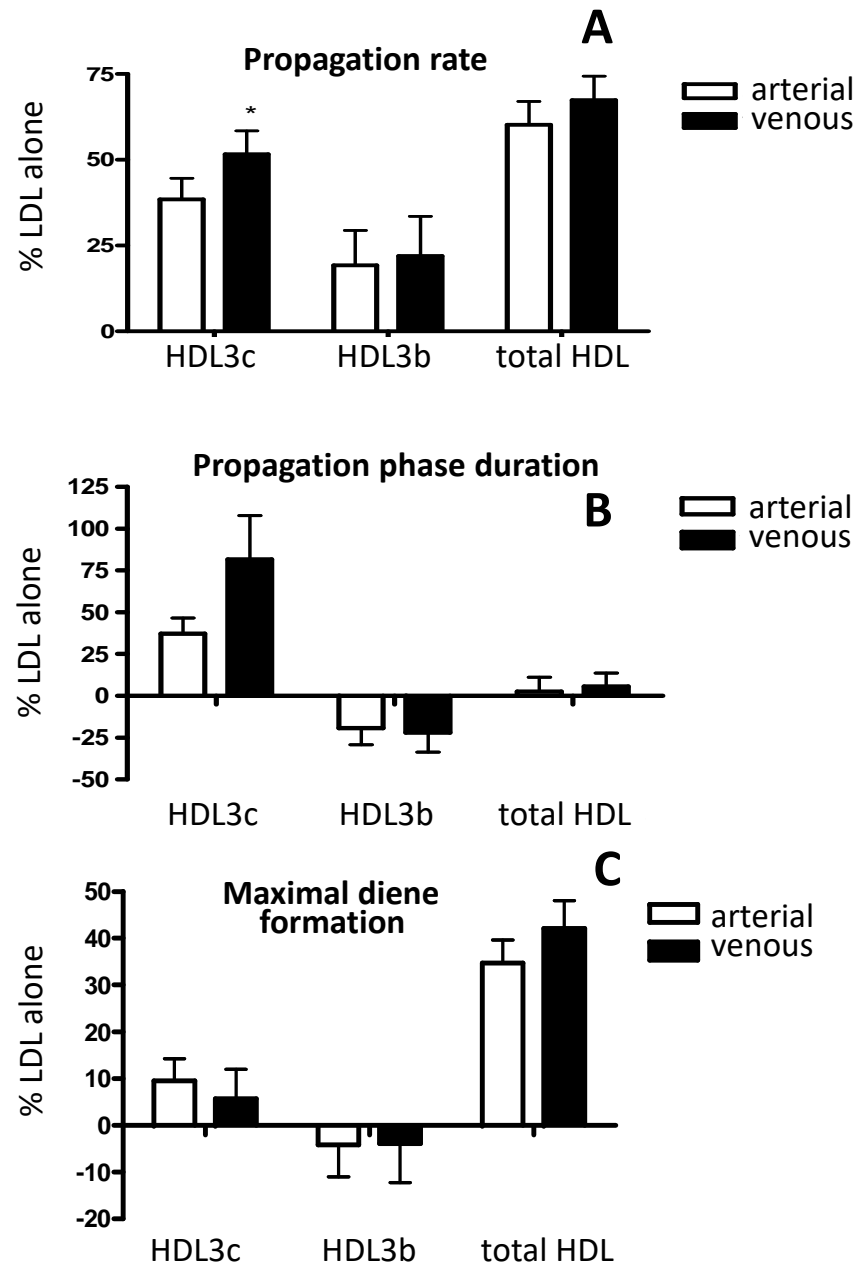
\* p < 0.05 vs. arterial blood plasma

Figure 2. Antioxidative activity of HDL3c subfractions during *in vitro* LDL oxidation in patients treated with statins (n= 10) vs. untreated patients (n = 6). **A.** Percentage of the decrease of LDL oxidation rate in the propagation phase in the presence of HDL3c subfractions from arterial and venous blood plasmas. **B.** Duration of the propagation phase during the oxidation of LDL in the presence of HDL3c subfractions from arterial and venous blood plasmas.

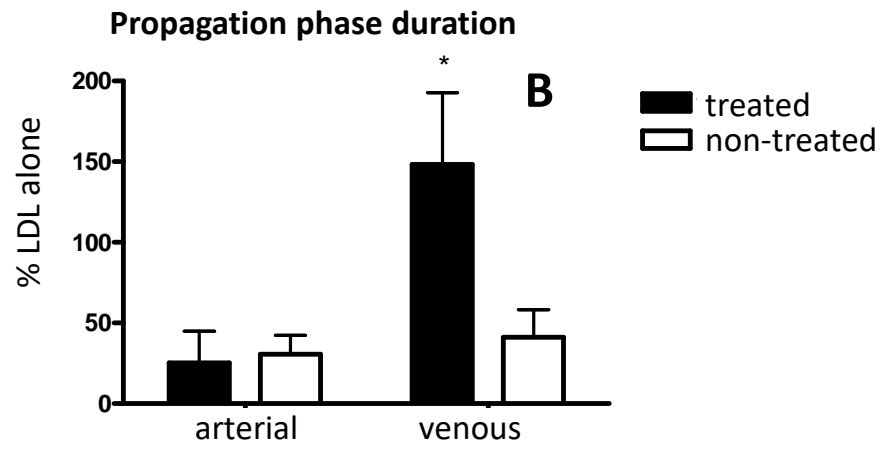
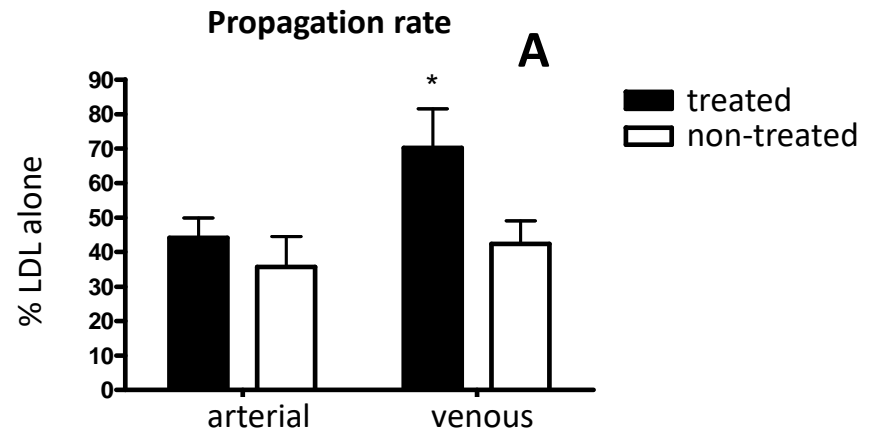
\* p < 0.05 vs. HDL3 subfractions from venous plasma in untreated patients.

Figure 3. Arterial and venous serum lipid profiles (**A:** HDL-cholesterol, **B:** LDL-cholesterol; **C:** triglycerides; **D:** Lp-PLA2) of patients treated (n=10) and untreated (n=6) by statins.

\*\* p < 0.01 vs. arterial blood plasma



**Figure 1**



**Figure 2**

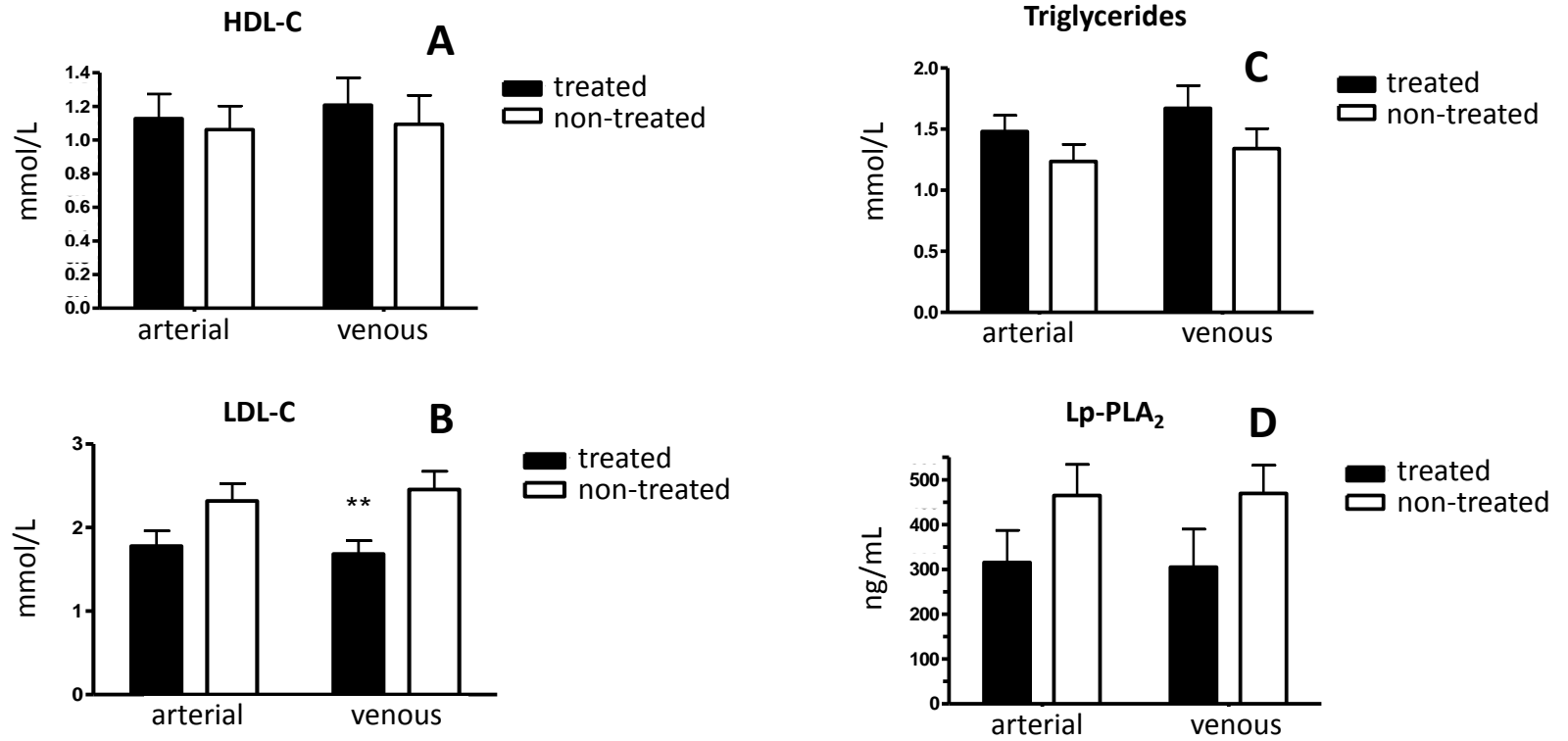


Figure 3