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**Title**

Statins have beneficial effects on platelet free radical activity and intracellular distribution of GTPases in hyperlipidaemia.

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**Short title:** Statins and platelet free radical activity.

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**Aims:** In addition to lowering cholesterol, statins may alter endothelial release of the vasodilator nitric oxide and harmful superoxide free radical. Statins also reduce cholesterol intermediates including isoprenoids. These are important for post-translational modification of substances including the GTPases Rho and Rac. By altering membrane association of these molecules, statins affect intra-cellular positioning and hence activity of a multitude of substances. These include endothelial nitric oxide synthase which produces nitric oxide (inhibited by Rho) and the nicotinamide dinucleotide (phosphate) oxidase which produces superoxide (dependent on Rac). Statins may improve endothelial function by enhancing production of nitric oxide while decreasing superoxide production.

**Methods:** 40 hypercholesterolaemic patients were randomised to treatment with atorvastatin or placebo. 20 normolipidaemic patients were also studied. Platelet nitrite, nitric oxide and superoxide were examined as was cellular distribution of the GTPases Rho and Rac at baseline and after eight weeks of treatment.

**Results:** Following atorvastatin therapy platelet nitric oxide was increased (3.2 pmol/10<sup>8</sup>platelets) and superoxide output attenuated (-3.4pmol/min/10<sup>8</sup> platelets) when compared with placebo. The detection of both Rho and Rac was significantly reduced in the membranes of platelets implying reduced activity.

**Conclusion:** This study shows altered nitric oxide/superoxide production following statin therapy. A potential mechanism for this is the change in distribution of intracellular GTPases which was felt to be secondary to decreases in isoprenoid intermediates, suggesting that activity of the former had been affected by atorvastatin.

## Introduction

Hydroxy methylglutaryl coenzyme A (HMG CoA) reductase inhibitors (statins) reduce cardiovascular mortality and morbidity [1, 2]. The chief mechanism by which they produce benefit is by lowering low density lipoprotein cholesterol (LDL-C), however analysis of several randomised trials [1, 2] and a wealth of human and animal cellular data [3, 4] suggest that statins have additional 'pleiotropic' activity.

One of the earliest indicators of vascular disease is the development of endothelial dysfunction (ED) that acts as the substrate for the accelerated development of atherosclerosis. A principle factor in the development of ED is a reduction in the bioactivity of nitric oxide (NO). This may be because of decreased production or enhanced degradation by reactive oxygen species (ROS) most importantly the superoxide anion ( $O_2^-$ ) [5,6]. Statins not only lower LDL-C [7] but improve endothelial function, and this may contribute to the improved clinical outcomes associated with administration of these drugs [8].

Statins decrease intracellular levels of all the intermediates of the intrinsic lipid production pathway, including cholesterol [9]. Several of these intermediates, particularly the isoprenoids farnesyl pyrophosphate and geranylgeranyl pyrophosphate (FPP and GGPP), are thought to have significant biological activity [10]. Their actions have been well established, particularly with respect to intracellular trafficking and signalling [11]. The molecules have been shown to adhere to cellular proteins at a post-translational stage and facilitate the interaction with intracellular organelles or the cell membrane resulting in alteration of activity.

The GTPases Rac and Rho are of particular importance in relation to endothelial function as they can influence production of NO and  $O_2^-$ . NO production by endothelial nitric oxide synthase (eNOS) has been shown to be reduced by Rho in several models [12] whilst the primary source of  $O_2^-$ , namely nicotinamide dinucleotide (phosphate)(NAD(P)H) oxidases depends on Rac for conformational change and subsequent activation [13, 14].

The aims of this study were to examine the release of NO, nitrite and  $O_2^-$  from platelets (used as a compartmentalised tissue model for endothelial cells (EC)) in patients with hypercholesterolaemia and control subjects, and determine the effects of atorvastatin administration. This model permitted the study of the relative concentration of Rho and Rac GTPases in the membrane (where activity is enhanced) and cytosol (where activity is decreased) of platelet samples at baseline and after treatment.

## Methods

### Patient recruitment & trial design

40 patients with hyperlipidaemia and 20 normolipidaemic age and sex-matched controls were recruited. Hyperlipidaemic patients were aged between 30 and 70 years and had a total cholesterol level of at least 6.5 mmol/l. Individuals were excluded if they had any history of cardiovascular disease (CVD), hypertension (or blood pressure  $> 140/80$  mmHg), diabetes mellitus (or fasting plasma glucose  $\geq 7.0$  mmol/L) or if they were taking anti-platelet, anti-hypertensive or anti-anginal medication. Patients with marked hypertriglyceridaemia ( $> 2.5$  mmol/L) were also excluded as were those treated with a statin in the 6 weeks prior to the study. 98% of patients were statin naïve at the time of enrolment. All patients received written information and signed consent forms. The study was approved by the Queen University Belfast Research Ethics committee and the investigation conformed with the principles outlined in the Declaration of Helsinki.

Blood sampling and ultrasound investigations were performed in the morning, in a temperature-controlled laboratory. Subjects fasted for at least 12 hours and refrained from caffeine, alcohol, cigarettes and over-the-counter drug preparations for 24 hours prior to study. Blood was drawn with minimal agitation and was separated into whole blood (lipid, glucose) and platelet samples (NO,  $O_2^-$  and GTPases).

Patients were randomised in a double-blind manner to receive atorvastatin initially at 10mg daily increased to 20mg after 4 weeks, or placebo for an eight week period (20 patients per group). Studies were repeated after 8 weeks of treatment. Control subjects (cholesterol  $< 5.2$  mmol/L) underwent a baseline study only.

### Measurement of biochemical parameters

Total cholesterol, triglycerides and HDL-C were measured using the Roche/Hitachi 911 analyser (Roche Diagnostics GmbH, Mannheim, Germany). Total cholesterol was quantified enzymatically using the Trinder reaction. LDL-C concentration was calculated using the Friedwald formula.

### Platelet preparation

Platelets were prepared for analysis as previously described [15]. Collection tubes were centrifuged at 600 rpm for 17 minutes. The platelet rich plasma fraction was removed and centrifuged at 1000 rpm for 10 minutes. It was passed through a 17 x 50 mm Sepharose-2B column equilibrated with albumin-free Tyrodes/ HEPES buffered saline to create gel filtered platelet fractions. Platelet counts were calculated in an automated counter. The fresh platelet fractions were used immediately for NO and  $O_2^-$  detection.

### NO analysis

Release of NO from biological systems is most sensitively examined using an electrochemical microprobe as first described by Shibuki [16] and later adapted for platelet NO measurement [17]. NO diffuses through a gas-permeable membrane on the probe and is oxidised by the metallic electrode resulting in an electrical current.

### Measurement of platelet NO release

The release of NO was measured using an NO meter and ami NO-700 NO-selective micro-electrode (Innovative Instruments Inc., Florida, USA). The inter-day variation in our laboratory was less than 7%. Methods utilised in our laboratory have been fully described previously [15].

In brief, to each platelet sample was added 20  $\mu\text{L}$  each of 120  $\mu\text{M}$  calcium and magnesium. The first sample of platelets was not activated. The second "native" sample was tested for absolute NO determination following activation. 50  $\mu\text{L}$  of 1 mM of the eNOS inhibitor  $\text{N}^G$ - monomethyl-L-Arginine (L-NAME) was added to the third sample and 20  $\mu\text{L}$  of 100uM/ml superoxide dismutase (SOD) was added to the fourth tube. An electrical current was then displayed digitally on the attached PC and allowed to stabilise. Platelet activation and stimulated NO release was produced by the addition of 24  $\mu\text{L}$  of 200  $\mu\text{M}$  ADP and 36  $\mu\text{L}$  of fibrinogen (10  $\mu\text{g}/\mu\text{l}$  strength), and change in current measured for a 15 minute period. Calculation of platelet NO release was quantified by measuring the height of the NO peak post-platelet stimulation in pico-amps (pA) in comparison with the calibration standard for that day and expressed as the NO release corrected for platelet content in picomoles per  $10^8$  platelets. 250  $\mu\text{L}$  aliquots of test samples were mixed with 250  $\mu\text{L}$  of 6M Tris buffer and frozen for nitrite analysis.

### Measurement of platelet nitrite

Due to the short half life of NO and relative difficulty of analysis, platelet nitrite was also quantified to ensure reliability of results. Nitrite is an indirect measure of NO release and is a more stable oxidized form. Total platelet nitrite was quantified in samples frozen after NO analysis. Frozen samples were thawed and nitrite was reduced to NO using 1500 $\mu\text{L}$  of sulphuric acid and potassium iodide solution (0.5% w/v). A cadmium reducing wire was placed in the platelet samples and mixed for five minutes equilibrated at 37°C. Nitrite was then detected as for real time NO release.

### $\text{O}_2^-$ analysis

Platelet release of  $\text{O}_2^-$  was measured using lucigenin-enhanced chemiluminescence [18]. Earlier criticism for this method centred on the discovery that lucigenin itself can produce  $\text{O}_2^-$ , whilst later studies in our own and other laboratories have confirmed that the lower doses of lucigenin (i.e. 20  $\mu\text{M}$ ) employed are not associated with redox cycling [19].

### Measurement of platelet $O_2^{\cdot-}$ release

Samples of gel-filtered platelets were diluted and 10  $\mu$ L of 1800  $\mu$ M lucigenin was added, yielding a solution concentration of 20  $\mu$ M. The sample was given time to dark adapt and placed in a Sirius Luminometer (Berthold Detection Systems, Pforzheim, Germany). Inter-day variation in the assay in our laboratory is 8.4%.

The platelet sample was activated using 100  $\mu$ L of 10  $\mu$ M phorbol-12-myristic-13-acetate (PMA). Basal release of superoxide, determined as chemiluminescence units per minute, was recorded for up to 20 minutes to achieve steady-state. This method was used to determine  $O_2^{\cdot-}$  release from platelet samples containing diluent and lucigenin (Native sample), 5  $\mu$ L of 500  $\mu$ M L-NAME (L-NAME sample) and from a sample containing 10  $\mu$ L of 100  $\mu$ M DPI and 1 mM quinacrine (DPI/Quinacrine sample) [14, 15]. The plateau height of the DPI/Quinacrine curve was subtracted from the native and the L-NAME curves. Areas under the curve were calculated and compared with the daily calibration curves adjusted for the known efficiency of the reaction which is 30%. These values were then expressed as  $O_2^{\cdot-}$  in pmoles/min/ $10^8$  platelets.

### Determination of cytosolic and membrane associated Rac and Rho in platelet samples

Platelet samples were treated fresh to obtain separate membrane and cytosolic fractions. Methods used were as employed previously in our laboratory and in accordance with those described in the literature [20, 21]. The platelet pellet was isolated by centrifugation and the purity of each collection confirmed by flow cytometry. The platelet pellet was washed, sonicated and centrifuged. The supernatant was transferred to each of two cryovials and stored at  $-80^\circ\text{C}$ . The pellet was washed and buffer added. The sample was incubated on ice for 15 minutes and centrifuged. 55  $\mu$ L of supernatant was transferred to each of 2 cryovials and stored at  $-80^\circ\text{C}$ .

### Western blotting and measurement of Rac and Rho distribution

A standard amount (10  $\mu$ g) of each fraction (cytosol or membrane) was diluted with 2  $\mu$ L of 1:1 mercaptoethanol:loading buffer and, in addition, a protein molecular weight marker was loaded onto a 12% polyacrylamide gel. The proteins were separated by molecular weight using sodium dodecyl sulphate polyacrylamide gel electrophoresis. Following protein separation, the proteins within the gel were transferred onto a PVDF membrane (Immobilon P, Millipore, USA).

After washing and incubation with blocking buffer, the samples were incubated with primary antibody - anti RhoA and anti Rac1 (Santa Cruz Biotechnology, USA). The membrane was incubated for 1 hour at room temperature with a 1:10000 dilution of horseradish peroxidase (HRP) labelled secondary antibody (anti-rabbit HRP, Biorad Laboratories, Hercules, CA.). The labelled protein was detected using enhanced chemiluminescence (ECL Plus, Amersham Biosciences) and exposed to X-ray film (Fotochemische, Werke, Germany) in a dark room. Densitometry was used to quantify each band (Analytical Image Systems, Imaging Research Inc.) [20, 21].

### Statistical analysis

Analysis of parameters was performed using SPSS version 12.0.1 for Windows (SPSS Inc, IL, USA). Results were expressed as mean  $\pm$  95% confidence limits throughout unless otherwise stated. Normally distributed variables were compared using Student's t-tests. Variables not normally distributed were analysed by nonparametric means.

The distribution of both GTPases was studied in the cell membrane and cytosol for patients with hyperlipidaemia both before and after atorvastatin and placebo. Due to the skewed distribution, median figures  $\pm$  1<sup>st</sup> and 3<sup>rd</sup> interquartile ranges are used in preference to mean  $\pm$  95%CI. Correlations between change in serum cholesterol and NO and O<sub>2</sub><sup>-</sup> were made using Spearman's Rank correlation test.

## **Results**

### Patient Baseline characteristics

Patient characteristics are summarised in table 1. The patients were well matched for age and gender. There were no significant differences in weight, Body Mass Index (BMI) or baseline serum glucose between groups. The patient groups were also well matched for blood pressure. There were more smokers in the atorvastatin group compared to placebo. There was a highly significant statistical difference between the hypercholesterolaemic patient groups and control subjects in relation to baseline total cholesterol, LDL-cholesterol and cholesterol/HDL ratio. No significant difference in HDL-C was detected between the three groups. There was also a significant difference between TG levels in the active and placebo groups compared with controls (table 2).

### Changes in lipid parameters

Changes in lipid profile over the study period are shown in table 2. Treatment with atorvastatin resulted in a significant reduction in total cholesterol, LDL-C, cholesterol/HDL ratio and TG. No difference in HDL-C was observed. In the placebo group, there was no significant change in any of the lipid parameters over the study period.

### Platelet Nitric Oxide release

Changes in platelet nitric oxide release are shown in table 2. Treatment with atorvastatin resulted in a significant increase in NO release of 3.2pmol /10<sup>8</sup> platelets (95%CI 0.55, 7.83)(p=0.05) (see figure 1a). There was no significant change in NO release in the placebo group. L-NAME inhibited the production of NO in all samples with no statistically significant differences in any group. SOD was associated with an increase in NO detection. No statistically significant correlation was found between changes in platelet NO and alterations in total cholesterol at trial end.

### Platelet nitrite content

Treatment with atorvastatin resulted in a significant increase in platelet nitrite of 26.4 pmoles/10<sup>8</sup> platelets (95%CI 9.6-43.2)  $p=0.002$ , whereas treatment with placebo resulted in a non-significant decrease of 6.4 pmoles/10<sup>8</sup> platelets (95%CI -17.01 – 4.19) confirming the changes observed in NO.

### Platelet O<sub>2</sub><sup>-</sup> release

Changes in O<sub>2</sub><sup>-</sup> release are shown in table 2. Baseline O<sub>2</sub><sup>-</sup> levels were significantly lower in the control group compared to patient groups ( $p=0.04$ ). Treatment with atorvastatin resulted in a significant reduction in platelet O<sub>2</sub><sup>-</sup> release of 3.4 pmol/min/10<sup>8</sup> platelets (95%CI 1.4, 4.9)( $p=0.001$ )(figure 1b). No significant change was seen with placebo. There was no correlation between change in platelet O<sub>2</sub><sup>-</sup> and change in total cholesterol.

### Rho GTPase

There was no statistically significant difference in the quantification of Rho in either the membrane or cytosol at baseline. Treatment with atorvastatin resulted in a statistically significant reduction in quantification of Rho in the platelet cell membranes [1380 densitometry units (DU) (IQ range 1090, 1825) at trial start; 641 DU (IQ range 106, 800) at trial end ( $p<0.001$ )]. There was an increase in Rho in the platelet cell membrane in the placebo group [1365 DU (IQ range 1107, 1911) at trial start; 1740 DU (IQ range 1464, 1923) at trial end ( $p=0.02$ )]. Treatment with atorvastatin also resulted in a statistically significant increase in the amount of Rho found in the cell cytosol [1105 DU (IQ range 710, 1273) at trial start; 1445 DU (IQ range 1062, 2003) at trial end ( $p=0.04$ )] The amount of Rho in the cell cytosol was not affected by treatment with placebo [1222 DU (IQ range 891, 1868) at trial start; 1129 DU (IQ range 946, 1418) at trial end] (figure 3)

### Rac GTPase (figure 2b)

No significant difference was found at baseline between groups when comparing the relative distribution of Rac GTPase in the platelet membranes and cytosol. Treatment with atorvastatin resulted in a statistically significant reduction in cell membrane Rac [2592 DU (IQ range 1757, 3700) at trial start; 1941 DU (IQ range 76, 2796) at trial end] ( $p=0.017$ ) compared with placebo [2775 DU (IQ range 2094, 3507) at trial start; 2740 DU (IQ range 2490, 3689) at trial end], where no significant alteration in membrane Rac was found. Cytosolic Rac GTPase showed a trend toward increase with atorvastatin [1568 DU (IQ range 1288, 1880) at trial start; 1888 DU (IQ range 1387, 2372) at trial end] but this did not reach significance. Platelet samples from the placebo group showed no change in cytosolic Rac [1793 DU (IQ range 1584, 2833) at trial start; 1675 DU (IQ range 1363, 2136) at trial end] (figure 4).

### **Discussion**

This study is, to our knowledge, the first human study demonstrating clear changes in NO and  $O_2^-$  release in conjunction with altered Rho and Rac localisation in samples from patients following treatment with atorvastatin at a commonly prescribed dose.

ED is now considered a very early stage in the development of CVD and attenuated NO bioavailability has been shown to occur with a number of CVD risk factors including hyperlipidaemia [22]. Other investigators have previously shown that platelets contain the same pathways for NO production that are found in EC [23, 24]. Platelet-derived NO is also thought to be diminished in the presence of certain CVD risk factors [25] and the actions of platelet NO have been identified as complementary to EC NO [26]. Statins have been associated with enhanced NO bioactivity both in EC [27] and in platelets [28].

Patients with hyperlipidaemia demonstrated a marked attenuation of NO release that was significantly improved following eight weeks treatment with atorvastatin, and this was accompanied by a significant increase in nitrite, a stable NO metabolite. No change in NO release was associated with placebo suggesting that the alteration in detectable NO was related to statin administration. No relationship was demonstrated between the magnitude of TC/LDL reduction and enhanced NO release.

A significant change was identified in membrane association of the eNOS inhibitory factor Rho GTPase with statin therapy. This implies that atorvastatin also altered the environment within the platelets to influence Rho membrane attachment and free radical production. Previous studies have shown that membrane association of Rho relate to isoprenoid levels, in particular GGPP [12]. Atorvastatin may not only reduce intracellular cholesterol but also intermediates such as GGPP that alter proteins which depend on isoprenoid molecules for their trafficking and ultimate activity.

A marked increase in the platelet  $O_2^-$  was found in patients with hyperlipidaemia that was suppressed after treatment with atorvastatin. This may be secondary to decreased activation of the NAD(P)H oxidase as previously demonstrated by our group [14, 15] and others [29]. Although previous studies have shown that lipid lowering alone can produce this improvement in ROS levels [29] we found no significant relationship between lipid alteration and reduced levels of oxidative stress in our study.

The detection of Rac GTPase in the membranes of platelet samples was markedly reduced following statin treatment. Rac is an integral part of the NAD(P)H Oxidase system being crucial to the conformational changes that are necessary following activation and hence  $O_2^-$  release [13, 14]. Therefore the decreased Rac detected in the cell membranes may have lead to a reduction in the ability of NAD(P)H Oxidase to form  $O_2^-$  with statin therapy.

In these and previous studies our group have employed platelets as an easily accessible ex-vivo compartmentalised model for endothelial cells. Platelets represent an

excellent source for this purpose, given the fact that platelets and EC share identical eNOS [23, 24] and NAD(P)H Oxidase subtypes, in contrast to alternative models such as neutrophils which differ significantly particularly with respect to NAD(P)H Oxidase [14, 15].

The results suggest that mechanisms in addition to atorvastatin's lipid-altering properties may be important in humans. There are a number of limitations within this study worthy of mention. Whilst the implication is that Rho and Rac alterations are secondary to changes in isoprenylation this was not specifically tested in this trial. Furthermore, there was no alternative lipid lowering arm to further test the hypothesis that the described actions occur secondary to intrinsic statin properties. However, other groups have described alteration in various factors independently of lipid lowering with statins not found with other hypolipidaemic medications [30]. Despite these limitations this work suggests that statin therapy can alter not only effectors of endothelial function but intracellular trafficking of vitally important cellular proteins. Further, these changes were found in human subjects receiving an orally administered and commonly prescribed preparation in doses that are highly relevant to everyday clinical practice.

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**Disclosures**

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**Conflict of interest**

None declared.

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**Figure Legends***Figure 1*

Platelet nitric oxide (a) and superoxide (b) in controls and patients pre and post atorvastatin and placebo. (\*  $p=0.05$ , † $p=0.001$ )

*Figure 2*

Relative change in membrane and cytosolic Rho GTPase (a) and Rac GTPase (b) after atorvastatin and placebo.

*Figure 3*

Representative Western blot demonstrating changes in Rho GTPase pre- and post-statin.

*Figure 4*

Representative Western Blot demonstrating changes in Rac GTPase pre- and post-statin.

Table 1: Patient Baseline Characteristics

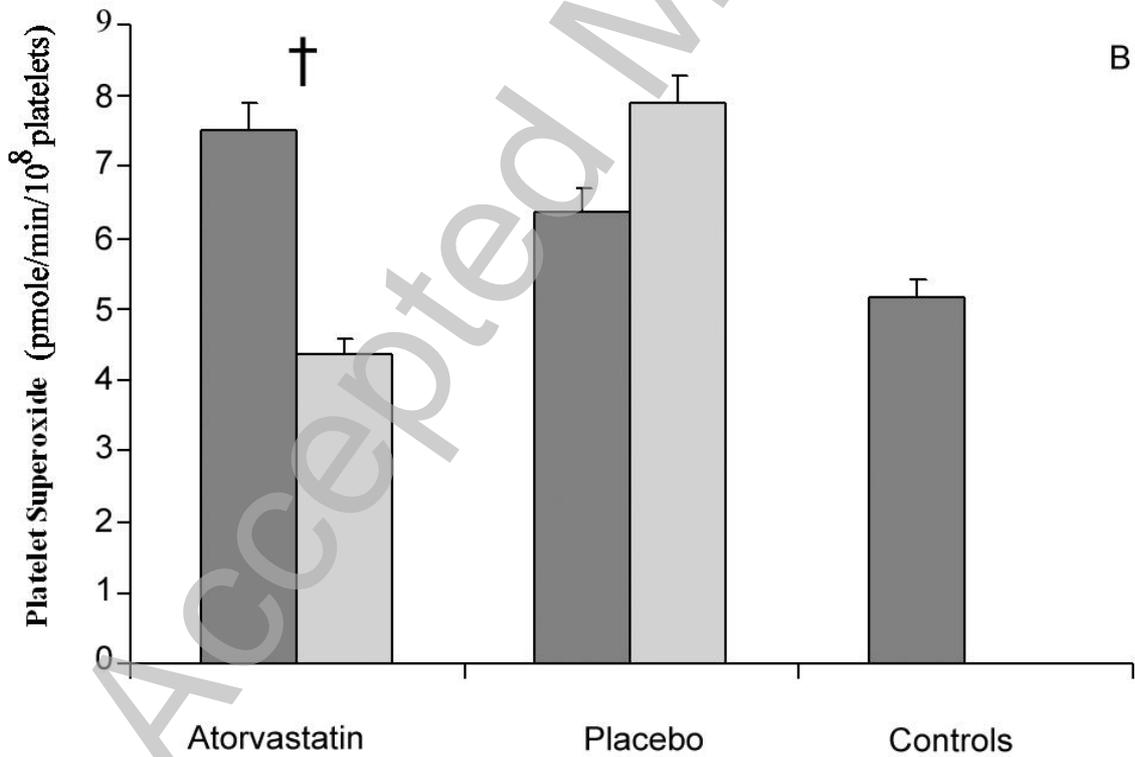
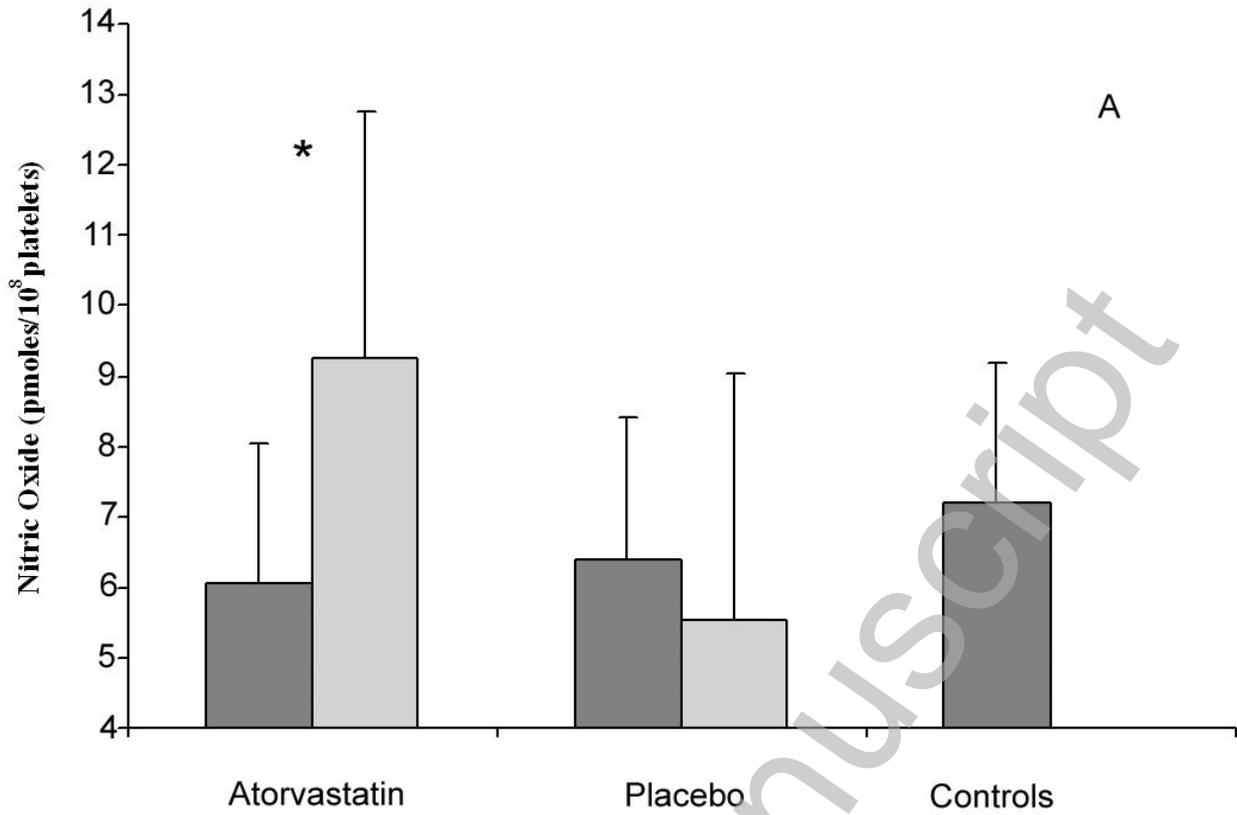
	Atorvastatin	Placebo	Control
<b>Age (years)</b>	49.7 (34-64)	50.7 (37-69)	49.7 (34-63)
<b>Gender</b>	Male 10 Female 10	Male 11 Female 9	Male 10 Female 10
<b>Body Mass Index (kg/m<sup>2</sup>)</b>	26.2 (24.9-27.6)	25.4 (24.6-26.3)	25.5 (23.9-27.2)
<b>Cigarette smokers</b>	6 /20*	3 /20*	4 /20
<b>Systolic BP (mmHg)</b>	123 (118-129)	124 (119-130)	122 (116-128)
<b>Diastolic BP (mmHg)</b>	72 (69-76)	74 (70-77)	71 (67-74)
<b>Glucose (mmol/L)</b>	4.2 (4-4.4)	4.2 (4-4.4)	4.3 (4.1-4.5)

\*p<0.05 for a difference in smoking status between the active treatment and placebo groups.

Table 2: Alterations in biochemical parameters in patients and controls

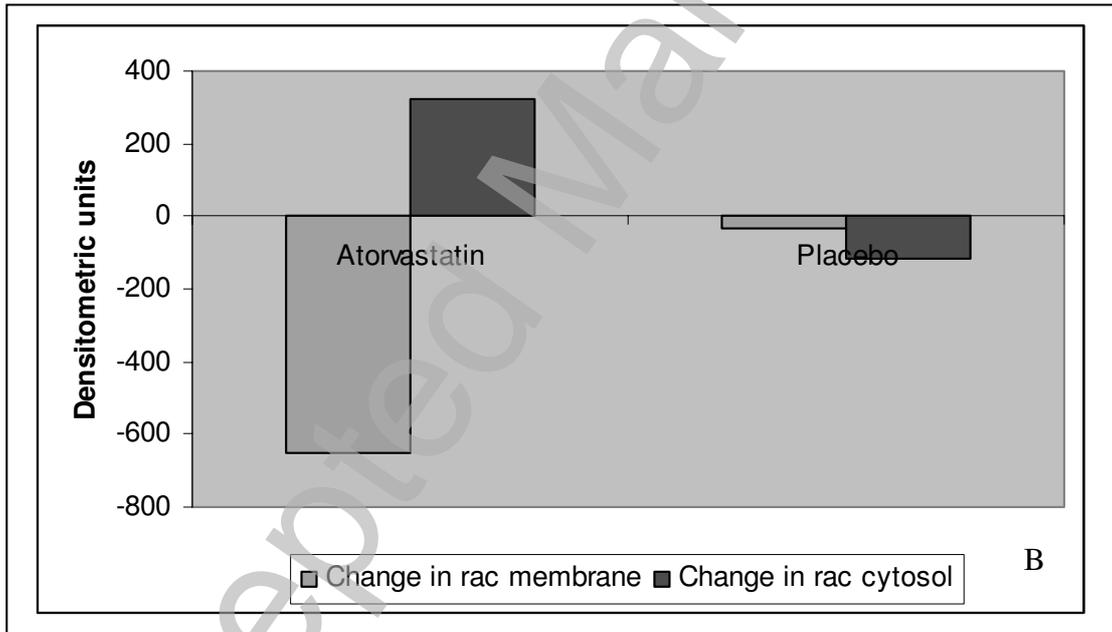
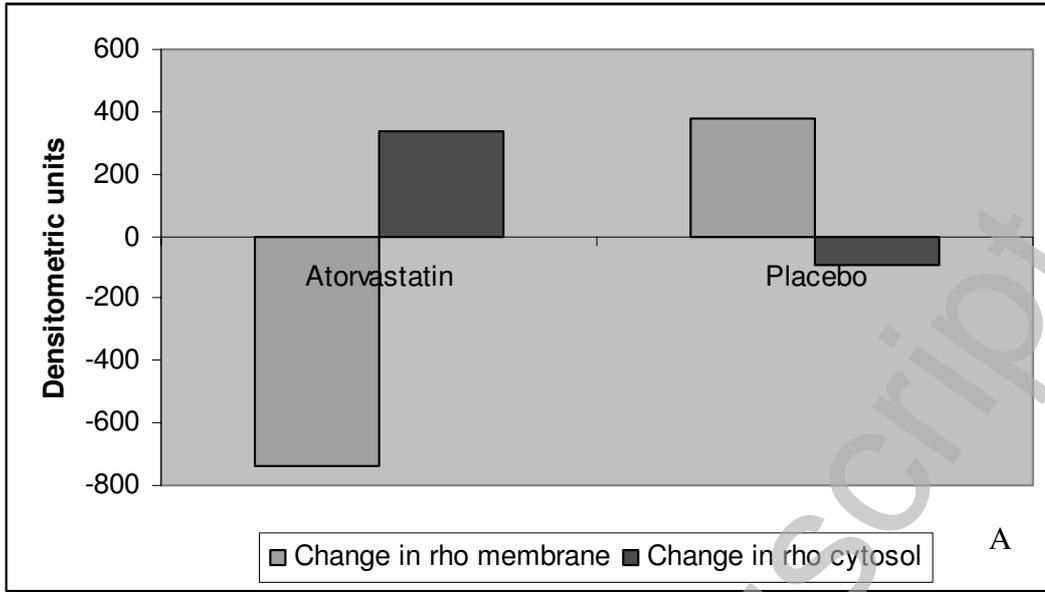
	Atorvastatin		Placebo		Controls
	Trial Start	Trial End	Trial Start	Trial End	
<b>Blood lipid parameters</b>					
<b>Cholesterol (mmol/L)</b>	7.4 (6.9-7.8)	5.0 <sup>†</sup> (4.8-5.3)	7.9 (7.4- 8.5)	7.3 (6.7-7.9)	5.2* (4.8-5.5)
<b>LDL-Cholesterol (mmol/L)</b>	5.0 (4.6-5.5)	2.8 <sup>†</sup> (2.5-3.1)	5.7 (5.1-6.4)	5.3 (4.6-6.1)	2.7* (2.4-3.2)
<b>HDL-Cholesterol (mmol/L)</b>	1.4 (1.2-1.6)	1.4 (1.3-1.6)	1.4 (1.3-1.6)	1.4 (1.3-1.6)	1.5 (1.3-1.6)
<b>Triglyceride (mmol/L)</b>	2.2 (1.9-2.6)	1.8 <sup>‡</sup> (1.5-2.2)	1.8 (1.5-2.3)	1.8 (1.5-2.2)	1.4 <sup>§</sup> (1.1-1.6)
<b>Total cholesterol/ HDL-Cholesterol ratio</b>	5.6 (4.9-6.3)	3.8 <sup>†</sup> (3.3-4.3)	5.8 (5.0-6.5)	5.8 (4.9-6.7)	3.5* (3.1-3.9)
<b>Platelet NO release (pmoles/10<sup>8</sup> platelets)</b>					
<b>Native</b>	6.05 (3.8-8.35)	9.25 <sup>  </sup> (5.8-12.7)	6.4 (3.95-8.8)	5.55(2.2-8.85)	7.2 (5.0-9.4)
<b>L-NAME</b>	1.5 (1.05-1.95)	1.25 (0.7-1.85)	1.3 (0.95-1.6)	1.2 (0.85-1.5)	0.7 (0.45-0.9)
<b>SOD</b>	7.0 (6.2-7.8)	8.0 (6.1-9.95)	6.6 (5.15-8.05)	8.9 (7.1-10.65)	6.35 (4.05-8.65)
<b>Platelet nitrite content (pmoles/10<sup>8</sup> platelets)</b>					
<b>Native</b>	37.39 (27.03-47.75)	63.79 ±(43.76-83.82)	34.08 (23.19-44.95)	27.66 (22.13-33.19)	43.05 (34.54-51.58)
<b>Platelet superoxide anion release (pmoles/min/10<sup>8</sup> platelets)</b>					
<b>Native</b>	9.3 (6.7-11.9)	5.9 <sup>  </sup> (4.0-7.7)	7.8 (5.7-10.0)	9.6 <sup>§</sup> (5.9-13.3)	6.6 <sup>£</sup> (4.9-8.3)
<b>L-NAME</b>	8.0 (5.6-10.5)	5.5 (3.8-7.2)	7.3 (5.3-9.3)	8.3 (5.2-11.4)	6.5 (4.7-8.2)
<b>DPI/Quinacrine</b>	1.8 (1.4-2.2)	1.5 (1.3-1.8)	1.5 (1.2-1.8)	1.7 (1.4-2.1)	1.5 (1.2-1.7)
<b>Native – DPI</b>	7.5 (5.1-9.9)	4.4 <sup>  </sup> (3.7-6.1)	6.3 (4.3-8.4)	7.9 (4.5-11.3)	5.2 <sup>£</sup> (3.7-6.6)

\* $p < 0.01$  versus patient groups, <sup>†</sup> $p < 0.001$  versus trial start, <sup>‡</sup> $p = 0.002$  versus trial start. <sup>§</sup> $p = 0.01$  versus patient groups. <sup>||</sup> $p = 0.05$  versus trial start. <sup>¶</sup> $p = 0.001$  versus trial start. <sup>§</sup> $p = 0.01$  versus trial start. <sup>£</sup> $p = 0.04$  versus patient groups. DPI: diphenyliodonium. HDL: high-density lipoprotein. LDL: low-density lipoprotein. L-NAME: N<sup>G</sup>-monomethyl-L-Arginine. SOD: Superoxide dismutase.



■ Day 1 □ Day 2

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/CS20090388



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**Untreated**

**Medicated**

Cytosol Membrane

Cytosol Membrane

