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# Ezetimibe's effect on platelet aggregation and LDL tendency to peroxidation in hypercholesterolaemia as monotherapy or in addition to simvastatin

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## WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Statins demonstrate a pleiotropic effect which contributes beyond the hypocholesterolaemic effect to prevent atherosclerosis.

## WHAT THIS STUDY ADDS

- Ezetimibe has an antioxidative effect when given as monotherapy or as an add-on to the statin, simvastatin.

## AIMS

To investigate the effect of lowering low-density lipoprotein-cholesterol (LDL-C) on platelet aggregation and LDL tendency to peroxidation by ezetimibe alone or with simvastatin in hypercholesterolaemia.

## METHODS

Sixteen patients with LDL-C >3.4 mmol $l^{-1}$ received ezetimibe for 3 months (Part I). Twenty-two patients on fixed simvastatin dose with LDL-C >2.6 mmol $l^{-1}$ were enrolled (Part II). Part II patients continued simvastatin treatment 20 mg $day^{-1}$ for 6 weeks, then received 20 mg $day^{-1}$ simvastatin combined with ezetimibe 10 mg $day^{-1}$ for another 6 weeks. The tendency of LDL to peroxidation measured by lag time in minutes required for initiation of LDL oxidation and by LDL oxidation at maximal point (plateau) was measured before and after ezetimibe treatment.

## RESULTS

Part I: Ezetimibe 10 mg daily for 3 months decreased plasma LDL-C level 16% ($P = 0.002$), prolonged lag time to LDL oxidation from 144 ± 18 min to 195 ± 16 min ($P < 0.001$), decreasing maximal aggregation from 83 ± 15% to 60 ± 36% ($P = 0.04$). Part II: Serum level LDL-C decreased 23% ($P = 0.02$) and lag time in minutes to LDL oxidation was prolonged from 55.9 ± 16.5 to 82.7 ± 11.6 ($P < 0.0001$) using combined simvastatin–ezetimibe therapy. There were no differences in platelet aggregation.

## CONCLUSIONS

Ezetimibe was associated with decreased platelet aggregation and LDL tendency to peroxidation. Treatment with ezetimibe in addition to simvastatin has an additive antioxidative effect on LDL.

## Introduction

Hypercholesterolaemia is a major risk factor for atherosclerosis. In hypercholesterolaemic patients, reduced low-density lipoprotein (LDL)-receptor activity in the liver contributes to increased plasma low-density lipoprotein-cholesterol (LDL-C) concentrations. Plasma LDL in these patients is more 'aged' and thus more susceptible to oxidative modifications than is LDL derived from healthy individuals [1, 2].

Activation of platelets occurs in hypercholesterolaemic patients. Enhanced platelet responsiveness has been noted when exposed to aggregatory agonists *ex vivo* [3, 4]. Reducing plasma cholesterol level by lovastatin decreases the platelet cholesterol/phospholipid molar ratio, normalizing both decreased platelet fluidity and increased platelet aggregation [5]. Oxidatively modified LDL triggers platelet activation and enhances platelet aggregation and secretion via specific binding sites for the lipoprotein on the platelet surface. These sites differ from the classical apolipoprotein B/E receptors on fibroblasts [6].

Complex interactions among plasma lipoproteins, platelets and arterial wall monocyte-derived macrophages are of major importance in atherogenesis. Platelets are responsible for the maintenance of haemostasis. Their activation state can be measured by determination of platelet adhesion, degranulation and aggregation. The observation of aggregated platelets and cholesterol-rich lipoproteins in the atherosclerotic plaque suggests that these lipoproteins and activated platelets participate in the pathogenesis of atherosclerosis. Atherogenic plasma lipoproteins, LDL and very-low-density lipoprotein (VLDL) have been shown to enhance platelet activity, whereas increased plasma high-density lipoprotein (HDL) shows the opposite effect. Increased concentrations of plasma LDL-C or VLDL-C and decreased plasma HDL-C concentrations could account for enhanced platelet activity observed in dyslipidaemic patients.

Ezetimibe is the first cholesterol absorption inhibitor that potently inhibits the absorption of biliary and dietary cholesterol from the small intestine [7]. The consequences of cholesterol absorption inhibition include decreased cholesterol delivery to the liver, reduced hepatocyte cholesterol stores, increased LDL production [8], but still increased LDL clearance from the serum [9] and, subsequently, decreased serum LDL-C levels. Clinical trials in humans have demonstrated that ezetimibe lowers LDL-C and triglycerides and raises HDL-C [10]. Ezetimibe plus simvastatin (pooled doses: 10–80 mg day$^{-1}$) provided an incremental 13.8% LDL-C reduction, 2.4% HDL-C increase and 7.5% triglyceride reduction compared with pooled simvastatin alone. Co-administration of ezetimibe and simvastatin provided LDL-C reductions of 44–57%, triglyceride reductions of 20–28% and HDL-C increases of 8–11%, depending on the simvastatin dose. Ezetimibe 10 mg plus simvastatin 10 mg and simvastatin 80 mg alone each provided a 44% LDL-C reduction [11].

Several studies have shown reduced LDL oxidizability after statin therapy. Simvastatin and pravastatin decreased the maximal rate of diene production and total diene production, but not the lag time required for the initiation of $CuSO_4$-induced LDL oxidation [12]. In another study, lag time was prolonged during fluvastatin treatment [13]. Statins can reduce the production of reactive oxygen species and increase the resistance of LDL to oxidation. Fluvastatin has been shown to possess better antiperoxyl radical properties and simvastatin better antihydroxyl radical properties *in vitro* [14]. Moreover, fluvastatin has radical scavenging activity and is effective against oxidative modification of LDL *ex vivo* [13].

Several hypolipidaemic drugs, including statins as well as plasma pheresis, have been shown to reduce platelet tendency to aggregation [15–17]. Platelet deactivation by atorvastatin is related to CD36 and lectin-like oxidized LDL receptor-1 expression reduction before significant LDL level changes [18]. Simvastatin treatment (20–40 mg day$^{-1}$) for 8 months normalized the enhanced platelet aggregation in hypercholesterolaemic patients [19].

Ezetimibe as a monotherapy has demonstrated no effect on arterial stiffness or high-sensitivity C-reactive protein (hsCRP), whereas the administration of simvastatin at 40 mg day$^{-1}$ improved arterial stiffness and CRP. Increasing the dose of simvastatin or administering ezetimibe in combination with simvastatin had no beneficial effects on arterial stiffness [20].

The aim of the current study was to investigate the effect of LDL lowering by ezetimibe alone or in addition to simvastatin on platelet aggregation and on LDL tendency to peroxidation in hypercholesterolaemic patients (*ex vivo* and *in vitro* studies).

## Patients and methods

### *Ezetimibe monotherapy (Part I)*

Sixteen hypercholesterolaemic patients with LDL-C >3.4 and triglycerides <3.4 mmol l$^{-1}$ without a history of cardiocerebrovascular disease were enrolled. Five healthy volunteers were enrolled as the control group.

*Criteria for inclusion*

**1** Hypercholesterolaemic patients without hypolipidaemic therapy and on 3 months' American Heart Association (AHA) Step 1 diet.
**2** Age ≥18 years.
**3** No cardiocerebrovascular disease.
**4** Creatine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ glutamyl transferase (GGT) <1.5× upper limit of normal at baseline.

*Criteria for exclusion*

1 Women receiving cyclical hormones.
2 Treatment with psyllium, other fibre-based laxatives, phytosterol margarines or other over-the-counter drugs that affect serum lipids, unless treated with a stable regimen for >6 weeks and the patient agrees to continue this regimen for the duration of the trial.
3 Oral corticosteroids unless used as replacement therapy for pituitary/adrenal disease and a stable regimen for ≥6 weeks.
4 Lipid-lowering agents, including fish oils or cholestyramine taken within 6 weeks.
5 Active coronary heart disease: unstable angina, acute myocardial infarction, coronary artery bypass graft (CABG) or percutaneous transluminal coronary angioplasty (PTCA) within the last 3 months.
6 Pregnant women and women with childbearing potential unless on safe contraception.
7 Psychiatric disease with defects in judgement.
8 Severe renal or hepatic disease.
9 Uncontrolled hypo- or hyperthyroidism.
10 Contraindication for ezetimibe treatment.

The patients were treated with ezetimibe 10 mg day$^{-1}$ for 3 months. Blood tests for lipids, liver (ALT, AST, GGT, alkaline phosphatase and bilirubin) and renal function tests (urea and creatinine), complete blood count, general urine test, LDL tendency to peroxidation, platelet aggregation and electrocardiogram were done at baseline and after 3 months for the patients and controls.

## *Ezetimibe in addition to simvastatin (Part II study)*

Twenty-two hypercholesterolaemic patients on a fixed dose of simvastatin (20 mg day$^{-1}$) with LDL-C >3.4 or >2.6 mmol l$^{-1}$ and triglycerides <3.4 mmol l$^{-1}$ without active coronary heart disease (CHD) were enrolled. Eight healthy volunteers were enrolled as controls.

*Criteria for inclusion* Hypercholesterolaemic patients on stable simvastatin dose (20 mg day$^{-1}$) for = 1 month.

1 Age ≥18 years on stable AHA Step 1 diet.
2 Patients without CHD or with one risk factor; LDL >3.4 mmol l$^{-1}$ and for patients with stable CHD or CHD risk equivalent (clinical manifestation of noncoronary forms of atherosclerotic disease) or with two risk factors [cigarette smoking, hypertension (blood pressure ≥140/90 mmHg or on antihypertensive medication), HDL-C <1 mmol l$^{-1}$, family history of premature CHD; LDL-C >2.6 mmol l$^{-1}$].
3 CK, ALT and AST <1.5× upper limit of normal at baseline.

*Criteria for exclusion*

1 Lipid-lowering agents other than simvastatin including fish oils and cholestyramine taken within 6 weeks.
2 Active CHD: unstable angina, acute myocardial infarction, CABG or PTCA within the last 3 months.
3 Contraindication for ezetimibe or simvastatin treatment.
4 Other exclusion criteria as in Part I study.

Patients continued treatment with simvastatin for 6 weeks, followed by the same dose of simvastatin combined with ezetimibe 10 mg day$^{-1}$ for an additional 6 weeks. The same tests were done as in the Part I study at 6 weeks on simvastatin therapy and after 6 weeks on combined therapy: simvastatin 20 mg day$^{-1}$ and ezetimibe 10 mg day$^{-1}$ (week 12).

## *LDL tendency to peroxidation*

Blood samples collected into sodium ethylene diaminetetraacetic acid ($Na_2EDTA$) (1 mM) from subjects and controls at baseline and after ezetimibe therapy were centrifuged at 1500 *g* for 10 min at room temperature. LDL was separated from the plasma by discontinuous gradient ultracentrifugation [21] at 4°C and was dialysed against saline $Na_2EDTA$ (1 mM). LDL protein concentration was determined by the method of Lowry [22].

LDL oxidation studies were performed each time on fresh LDL samples. Before oxidation, LDL was dialysed against saline 1.006 with $Na_2EDTA$ 1 mM, pH 7.4 and was then dialysed against phosphate-buffered saline (PBS) at 4°C to remove $Na_2EDTA$. It was then diluted with PBS to 0.2 mg of protein per ml. The lipoprotein was incubated in the presence of 5 μM $CuSO_4$ at 37°C. Measuring conjugated dienes formation at 234 nm continuously monitored the kinetics of LDL oxidation [23]. For each LDL sample, we received a curve consisting of three consecutive phases: lag phase (lag time in minutes required for the initiation of $CuSO_4$-induced LDL oxidation), propagation phase and a final plateau phase. LDL oxidation at the maximal point (plateau) was analysed by the thiobarbituric acid reactive substances assay, which measures malondialdehyde (MDA) equivalents [24].

## *Platelet separation*

For platelet studies, venous blood (30 ml) was collected through siliconized syringes into sodium citrate, 3.8% at a ratio of 9 : 1 (v:v) for platelet-rich plasma (PRP) preparation or into acid citrate dextrose solution (1.4% citric acid, 2.5% sodium citrate and 2% dextrose) at a ratio of 9 : 1 (v:v) for washed platelet (WP) preparation. PRP was prepared by low-speed centrifugation (100 *g* for 10 min) at 25°C, and the remaining sample was recentrifuged at 1000 *g* for 10 min to obtain platelet-poor plasma (PPP). Platelets in PRP were counted and diluted with PPP to achieve a uniform concentration of $3 \times 10^8$ platelets ml$^{-1}$. WP was prepared by centrifugation at 240 *g* for 20 min. The platelet

pellet was washed twice in 5 mmol HEPES buffer, pH 7.4 (140 mmol NaCl, 2 mmol KCL, 1 mmol $MgCl_2$, 5 mmol HEPES, 12 mmol $NaHCO_3$ and 5.5 mmol of glucose). For the preparation of WP suspension, 15 µl of acetic acid (1 mol $l^{-1}$) was added to 1 ml of platelet suspension throughout the WP preparation in order to ensure the acidic conditions required for platelet resuspension. This procedure reduced the medium pH to 6.5 without influencing the aggregation response of the WP.

### *Platelet aggregation*

Collagen (Nycomed, Konstanz, Germany), adenosine diphosphate (ADP) and epinephrine (at concentrations of 1 and 4 µg $ml^{-1}$) were used as the aggregating agents. The results at 4 µg $ml^{-1}$ were introduced in this study because they were more prominent. Platelet aggregation was performed at 37°C in an aggregometer (PACKS-4 Platelet Aggregation Chromogenic Kinetic System-4; Helena Laboratories, Beaumont, TX, USA) using HEPES as a reference system. Results were expressed as the extent of maximal aggregation (% of maximal amplitude).

### *Cholesterol content in platelet membranes*

Platelets were washed three times with HEPES buffer and then sonicated twice for 20 s at 80 W. Platelet lipids were extracted with hexane:isopropanolol (3 : 2, v : v). The cholesterol content was measured in the dried hexane phase by the method of Chiamori *et al.* [25]. Platelet protein was determined using the method of Lowry [22].

For *in vitro* studies, platelets were incubated *in vitro* with increasing concentrations of ezetimibe for 1 h and aggregation was then measured after adding collagen or epinephrine.

In the second *in vitro* experiment, native LDL was incubated with increasing concentrations of ezetimibe, and then $CuSO_4$ was added to measure LDL tendency to oxidation by measuring lag time to LDL oxidation and MDA level in the plateau phase.

Ezetimibe concentrations were 10, 50, 100, 200, 500 ng $ml^{-1}$ as total ezetimibe $C_{max}$ is 72 ng $ml^{-1}$ (ezetimibe glucoronide $C_{max}$ is 68.9 ng $ml^{-1}$ and ezetimibe $C_{max}$ is 5.23 ng $ml^{-1}$) [26].

*In vitro* studies were done by triplicates.

### *Statistical analysis*

Normal distributions were described using the mean and standard deviation (SD) and other distributions using medians and interquartile ranges (IQR). Student's *t*-test was used for comparing the two means. Serum levels of triglycerides are presented as mean and IQR and analysed after log transformation. Pearson's correlation was done and considered significant at the 0.05 level (two-tailed). Multivariate analysis was carried out using SPSS for windows, version 14.0 (SPSS Inc., Chicago, IL, USA).

The protocol was reviewed and approved by the Ziv Medical Centre Institutional Review Board, and all patients provided written informed consent.

## Results

### In vitro *studies*

Upon *in vitro* incubation of LDL with increasing concentrations of ezetimibe (0, 10, 100 and 500 ng $ml^{-1}$), no significant effect on the levels of MDA in the plateau phase of LDL oxidation could be observed ($r = -0.24$, $P$ = NS). Similarly, there was no significant effect in this *in vitro* system on the lag time required for LDL oxidation ($r = -0.5$, $P$ = NS).

There was no significant effect of ezetimibe on the platelet aggregation *in vitro* system of platelets as measured by the slope of the aggregation curve (cm $min^{-1}$) or by the extent of maximal aggregation (% of maximal amplitude) when induced with collagen ($r = 0.08$, $P$ = NS and $r = 0.02$, $P$ = NS, respectively).

### *Part I study*

Demographic and clinical characteristics of the patients are presented in Table 1.

Treatment with ezetimibe daily for 3 months decreased plasma total cholesterol by 12% ($P = 0.0008$) and this was related to a decreased plasma LDL-C level of 16% ($P = 0.002$). HDL-C and triglyceride plasma levels were not affected. After log transformation, the serum triglyceride

**Table 1**

Demographic and clinical characteristics of patients and control in Part I study: Monotherapy with ezetimibe

| Parameter | Ezetimibe ($n = 16$) | Control ($n = 5$) |
|---|---|---|
| Mean age (years) | 52.1 ± 4.9 | 54.2 ± 5.3 |
| Gender: male/female | 12/4 | 3/2 |
| Mean systolic blood pressure (mmHg) | 120 ± 8 | 119 ± 8 |
| Mean diastolic blood pressure (mmHg) | 82 ± 5 | 80 ± 4 |
| Mean body mass index (kg $m^{-2}$) | 25.7 ± 2 | 26.7 ± 0.9 |
| Coronary heart disease | 0 | 0 |
| Cerebrovascular disease | 0 | 0 |
| Diabetes mellitus | 0 | 0 |
| Hypertension | 11 | 0 |
| Smoker | 2 | 0 |
| Aspirin use | 9 | 0 |
| Angiotensin converting enzyme inhibitor | 5 | 0 |
| Calcium channel blockers | 2 | 0 |
| Angiotensin receptor blocker | 1 | 0 |
| Hormone replacement therapy | 0 | 0 |
| Diuretics | 3 | 0 |
| β-Blockers | 1 | 0 |

## Table 2

Results of control group and hypercholesterolaemic patients at baseline and 3 months after ezetimibe monotherapy

| Parameter | Baseline | Ezetimibe | *P* ezetimibe *vs.* baseline | Control | *P* control *vs.* baseline |
|---|---|---|---|---|---|
| Total cholesterol (mmol $l^{-1}$) | 7.4 ± 1.3 | 6.6 ± 1.1 | 0.0009 | 4.6 ± 0.4 | <0.0001 |
| LDL-C (mmol $l^{-1}$) | 5.2 ± 1.4 | 4.4 ± 1.2 | 0.002 | 2.7 ± 0.6 | <0.0001 |
| HDL-C (mmol $l^{-1}$) | 1.3 ± 0.3 | 1.2 ± 0.3 | NS | 1.0 ± 0.1 | 0.01 |
| Triglycerides (mmol $l^{-1}$) | 2.3 ± 1.2 | 2.0 ± 1.2 | NS | 1.9 ± 0.3 | NS |
| Lag time to LDL oxidation (min) | 144 ± 18 | 195 ± 16 | <0.001 | 179 ± 23 | 0.035 |
| % of platelet aggregation (PRP)-collagen | 83 ± 15 | 60 ± 36 | 0.04 | 50 ± 8 | <0.0001 |
| % of platelet aggregation (PRP)-ADP | 79 ± 13 | 63 ± 24 | 0.004 | 46 ± 9 | 0.0001 |
| % of platelet aggregation (PRP)-epinephrine | 56 ± 31 | 66 ± 24 | NS | 47 ± 9 | NS |
| % of platelet aggregation (WP)-collagen | 83 ± 9 | 60 ± 25 | 0.04 | 46 ± 5 | 0.009 |
| Platelet cholesterol content (μmol $mg^{-1}$ protein) | 2.5 ± 0.2 | 1.9 ± 0.2 | 0.00005 | 1.4 ± 0.1 | 0.00001 |

LDL-C, Low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein cholesterol; PRP, platelet-rich plasma; ADP, adenosine diphosphate; WP, washed platelets.

level was 0.30 ± 0.24 (IQR = 0.44) and 0.22 ± 0.25 (IQR = 0.26) ($P = 0.4$) before and after therapy, respectively. The lag time required for LDL oxidation was prolonged by 35% ($P < 0.001$) (Table 2). Linear regression between plasma LDL-C reduction and lag time prolongation revealed a correlation factor of $r = 0.34$ ($P = 0.205$). In parallel, the extent of maximal platelet aggregation with collagen was decreased by 28% ($P = 0.04$) (Table 2). Linear regression between plasma LDL-C reduction and between platelet cholesterol content reduction resulted in $r = 0.64$ ($P = 0.045$). Linear regression between decrease in platelet cholesterol content and extent of maximal platelet aggregation with collagen resulted in $r = 0.41$ ($P =$ NS). $r$ was −0.1 ($P =$ NS) when the linear regression was between the decrease in plasma LDL-C and the extent of maximal platelet aggregation with collagen.

### *Part II study*

Demographic and clinical characteristics of the patients are presented in Table 3. Liver function tests, serum creatinine and CK levels were normal during simvastatin and simvastatin–ezetimibe therapy.

### *Effect on serum levels of lipids*

Serum levels of total cholesterol and LDL-C were reduced significantly by the addition of ezetimibe 10 mg $day^{-1}$, in addition to simvastatin therapy, by 15% ($P = 0.03$) and 23% ($P = 0.02$), respectively. HDL-C and triglyceride plasma levels were not changed by the addition of ezetimibe. After log transformation, the serum triglyceride level was 0.28 ± 0.23 (IQR = 0.36) and 0.28 ± 0.19 (IQR = 0.28) ($P =$ NS) before and after therapy, respectively.

### *Effect on LDL tendency to peroxidation*

Lag times in minutes required for the initiation of LDL oxidation in the presence of $CuSO_4$ was prolonged by 48% by adding ezetimibe to simvastatin therapy ($P < 0.0001$). MDA equivalents content in LDL measured at the maximal point (plateau) was reduced by 34% upon adding ezetimibe to simvastatin therapy ($P = 0.049$) (Table 4). There were no significant correlations between differences in serum LDL levels, LDL MDA contents and lag times required for the initiation of LDL oxidation.

## Table 3

Demographic and clinical characteristics of patients and control group in Part II study: Treatment with ezetimibe in addition to simvastatin therapy

| Parameter | Simvastatin ($n = 22$) | Control ($n = 8$) |
|---|---|---|
| Mean age (years) | 59 ± 10 | 58 ± 8 |
| Gender: male/female | 15/7 | 7/1 |
| Mean systolic blood pressure (mmHg) | 132 ± 12 | 118 ± 9 |
| Mean diastolic blood pressure (mmHg) | 82 ± 6 | 78 ± 6 |
| Mean body mass index (kg $m^{-2}$) | 28 ± 19 | 27 ± 1.5 |
| Coronary heart disease | 7 | 0 |
| Cerebrovascular disease | 3 | 0 |
| Diabetes mellitus | 2 | 0 |
| Hypertension | 15 | 0 |
| Smoker | 5 | 0 |
| Aspirin use | 14 | 0 |
| Angiotensin converting enzyme inhibitor | 11 | 0 |
| Calcium channel blockers | 7 | 0 |
| Angiotensin receptor blockers | 0 | 0 |
| Hormone replacement therapy | 0 | 0 |
| Diuretics | 3 | 0 |
| β-Blockers | 7 | 0 |

### *Effect on platelet aggregation*

After combining ezetimibe therapy with statin, there were no differences in platelet aggregation measured by lag time to initiation of aggregation, extent of maximal aggregation (% of maximal amplitude) or the slope of the aggregation curve (cm $min^{-1}$) after activation of platelets by

## Table 4

Results of control group and hypercholesterolaemic patients on 6 weeks of simvastatin therapy and 6 weeks of ezetimibe in addition to simvastatin therapy

| Parameter | Simvastatin period | Simvastatin–ezetimibe period | Control |
|---|---|---|---|
| **Total cholesterol (mmol $l^{-1}$)** | 5.6 ± 1.6 | 4.8 ± 1.2† | 5.4 ± 1.1 |
| **LDL-C (mmol $l^{-1}$)** | 3.4 ± 1.4 | 2.6 ± 1.0† | 3.4 ± 1.1 |
| **HDL-C (mg $dl^{-1}$)** | 1.2 ± 0.3 | 1.2 ± 0.3 | 1.4 ± 0.2 |
| **Triglycerides (mg $dl^{-1}$)** | 2.2 ± 1.0 | 2.0 ± 0.8** | 1.2 ± 0.5* |
| **Lag time to LDL oxidation (min)** | 55.9 ± 16.5 | 82.7 ± 11.6† | 80 ± 11* |
| **MDA content in LDL (nmol MDA $mg^{-1}$ LDL protein)** | 11.3 ± 6.7 | 7.5 ± 5.6† | 7.2 ± 2.6* |
| **Platelet cholesterol content (μmol $mg^{-1}$ protein)** | 1.9 ± 0.1* | 2.0 ± 0.1** | 1.8 ± 0.1 |

†$P < 0.05$ simvastatin and ezetimibe *vs.* simvastatin; *$P < 0.05$ simvastatin *vs.* control; **$P < 0.005$ simvastatin and ezetimibe *vs.* control. LDL-C, Low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein cholesterol; MDA, malondialdehyde.

collagen, ADP or epinephrine. Platelet cholesterol content did not change between patients on simvastatin or on simvastatin combined with ezetimibe.

On multivariate regression, there were no significant correlations between either MDA equivalents contents in LDL or lag times and between serum levels of total cholesterol, triglycerides, LDL and HDL.

## Discussion

In the present study, ezetimibe decreased platelet aggregation, but not when added to simvastatin therapy. Ezetimibe as monotherapy or in addition to simvastatin therapy decreased LDL tendency to peroxidation measured by lag time in minutes required for the initiation of LDL oxidation in the presence of $CuSO_4$ and MDA equivalents content in LDL measured at the maximal point.

LDL tendency to peroxidation and increased platelet aggregability have emerged as two of the major processes in enhancing atherosclerosis [4]. In hypercholesterolaemic patients, reduced LDL-receptor activity in the liver contributes to increased plasma LDL concentration. Plasma LDL in these patients is more 'aged' and thus more susceptible to oxidative modifications than is LDL derived from healthy individuals [1, 2].

The major effect of ezetimibe therapy is the prevention of absorption of cholesterol from the small intestine, thus reducing the half-life ($t_{1/2}$) of LDL in the plasma as a result of increased uptake of the lipoprotein, mainly by liver cells. This results in the enrichment of the LDL particles with a 'younger' LDL population (as the 'aged' LDL is rapidly taken up by the liver), which is less prone to oxidative stress than the 'old' LDL population. The LDL-associated antioxidants are therefore less consumed by the oxidative stress in the 'younger' *vs.* the 'old' LDL particles, leaving lipoproteins which are enriched with antioxidants [2]. This may explain the reduced tendency of LDL to peroxidation during ezetimibe treatment as monotherapy. Linear regression between serum LDL-C level reduction and lag time prolongation showed a weak nonsignificant positive correlation ($r = 0.34$). In a recent study, ezetimibe inhibited the progression of carotid artery and aortic atherosclerosis lesion cross-sectional area under Western, low-fat and cholesterol-free dietary conditions in Apo $E^{-/-}$ mice [27]. This effect may be due to the hypocholesterolaemic and indirect antioxidative mechanisms.

In an assessment of 432 patients in two phase II studies monotherapy, ezetimibe 10 mg administration to patients with primary hypercholesterolaemia significantly increased HDL-C by 3.5% ($P < 0.05$) within 2 weeks of treatment compared with placebo [10]. HDL-associated paraoxonase can protect LDL from oxidation [28], thus contributing to LDL resistance to oxidation observed under ezetimibe treatment. In the present study, the serum HDL level was not affected by ezetimibe treatment. Ezetimibe reduced significantly the concentrations of all LDL particles, but this was more pronounced in the small, dense LDL subfractions [29]. Small, dense LDL is more prone to oxidation than large LDL [30]. The reduction in small, dense LDL by ezetimibe can reduce the tendency of LDL to oxidation. The negative results of *in vitro* studies preclude the possibility of a direct effect of ezetimibe to reduce LDL tendency to peroxidation.

Several studies have shown that statins have a protective antioxidative effect on LDL [12–14]. It has been demonstrated that simvastatin does not increase the lag time of the copper-induced oxidation of LDL *in vitro*, whereas it reduces significantly the rate of oxidation [31]. Moreover, simvastatin can significantly reduce circulating ox-LDL levels in subjects undergoing both primary and secondary prevention of CHD [32]. Simvastatin treatment in hyperlipidaemic patients decreased aldehyde production derived from lipoprotein oxidation [33]. In a recent study, atorvastatin and simvastatin therapy did not decrease the concentration of circulating oxidized LDL particles, apart from an initial transient reduction at 12 weeks [34]. The diversity of results in different studies may be due to different methods of measurements. In this study, patients on simvastatin therapy still had a greater tendency of LDL to peroxidation than normal volunteers. Ezetimibe co-administered with simvastatin resulted in significant

incremental decreases in hsCRP, possibly consistent with an additional anti-inflammatory effect compared with simvastatin therapy [35]. In the present study, addition of ezetimibe to simvastatin further decreased LDL tendency to peroxidation. It appears that the mechanism results from LDL lowering, without direct effect as was demonstrated by negative results in *in vitro* studies. The reduction in hsCRP via the combination of ezetimibe and simvastatin [35] may be also due to reduced oxidative stress, and not a direct effect on the cascade of inflammation.

In a recent study, 4 weeks of simvastatin treatment improved endothelial function independently of LDL-C lowering, at least in part by reducing oxidant stress. On the other hand, ezetimibe treatment for 4 weeks reduced LDL by 15.4%, but did not affect endothelial dysfunction [36]. In hypercholesterolaemic patients, acute LDL-C reduction by >60% with apheresis [37], or by dietary and cholestyramine therapy resulting in >30% LDL-C reduction [38], has been shown to improve endothelium-dependent vasodilation, suggesting that an increased cholesterol level contributes to endothelial dysfunction. The current study has shown that ezetimibe on the top of simvastatin does protect LDL from peroxidation. The main difference in the present study is that LDL lowering by ezetimibe on top of simvastatin was 23%, compared with 15.4% in the study by Landmesser *et al.* [36]. A meta-analysis including 5039 patients has shown that ezetimibe on top of statin reduced LDL further by 23.6% [39], as in the present study.

Increased hypersensitivity and hyperaggregability of platelets from hypercholesterolaemic patients detected *in vivo* and *in vitro* suggest that high levels of LDL may alter platelet function [40]. LDL sensitizes platelets *in vitro* to a variety of stimulating agents such as ADP, collagen and thrombin [41].

LDL and HDL bind to a single class of lipoprotein-binding proteins in the platelet membrane. The lipoprotein binding sites have shown higher affinity for HDL than LDL, and the lipoproteins interfered with binding of each other in a noncompetitive manner [42]. LDL increases the exposure of fibrinogen binding sites on platelets. Platelets from familial hypercholesterolaemia patients bind more fibrinogen than platelets from normolipidaemic patients [43].

In the present study, a decreased plasma LDL concentration under ezetimibe therapy was associated with decreased cholesterol content in the platelets, which may increase the fluidity of these membranes. In contrast, decreased fluidity correlates with increased aggregation of platelets [5]. Moreover, decreased LDL tendency to peroxidation due to ezetimibe treatment may reduce platelet aggregation as thoroughly oxidized LDL activated platelets and accelerated adhesion, as reported in one [44] but not in a second study [45].

Mildly oxidized or minimally modified LDL enhanced platelet aggregability and release reaction to a greater extent than heavily oxidized LDL [45, 46]. The extent of LDL oxidizability in the present study was not addressed. The correlation between plasma LDL-C reduction and the reduction of platelet cholesterol content was prominent ($r = 0.64$, $P = 0.045$), supporting the lowering of serum LDL-C and platelet cholesterol content by ezetimibe as the mechanism of the antiplatelet effect. Addition of ezetimibe to simvastatin showed no additive effect on platelet aggregation activated by ADP, collagen or epinephrine.

The neutral effect of ezetimibe on platelet aggregation in patients treated by simvastatin may be due to the failure of ezetimibe to decrease further the platelet cholesterol content. Another possibility is that the result with ezetimibe alone was due to the chance, broad scattering of results. To sum up, treatment with ezetimibe in addition to simvastatin has an additive antioxidative effect on LDL.

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