The Atherogenic Lipoprotein Subfraction

Studies in Patients with Familial Hypercholesterolaemia

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Declaration

This is to certify that this thesis is my own work and has not been presented for any degree to another University. I am the senior author in the majority of the publications included in this thesis and have performed the bulk of the clinical studies, laboratory investigations, statistical analysis of data and drafting of the manuscripts myself.

This thesis is dedicated to my wife, Anne, and Philippa and Nicholas, our two children.

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Abstract

Familial hypercholesterolaemia (FH) is an inherited disorder caused by mutations in the low-density lipoprotein (LDL) receptor which lead to diminished clearance of cholesterol from the circulation and, consequently, to markedly elevated LDL-cholesterol (LDL-C) levels. The resultant hypercholesterolaemia predisposes these patients to severe premature atherosclerosis, particularly coronary artery disease (CAD). The concentration of LDL-C and lifetime vascular exposure to raised plasma LDL-C concentrations are major determinants of atherosclerosis, but there remains a considerable variability in the extent of atherosclerosis present and in the expression of clinical disease in these patients. *Qualitative* differences in LDL such as LDL particle size and susceptibility to lipid oxidation may play a role, as may other biochemical risk factors.

The purpose of this thesis was to determine whether such qualitative differences in LDL are important determinants of the extent and severity of atherosclerosis and to determine whether it is mainly the reduction in LDL-C that is of benefit, or whether antioxidant therapy would also be effective in preventing progression of atherosclerosis in FH subjects.

FH patients were found to have large, buoyant LDL particles, which are less susceptible to lipid oxidation than smaller, denser particles. In the absence of other causes of insulin resistance, patients with FH have normal fasting insulin and triglyceride levels, normal postprandial lipaemia, and do not have microalbuminuria. They, therefore, usually show no features of the metabolic syndrome despite severe, accelerated atherosclerosis.

Similarly, the role of lipid oxidation in the pathogenesis of atherosclerosis in FH remains uncertain. LDL isolated from FH patients is more resistant to oxidation, and antioxidant therapy appears to be of little or no benefit in preventing progression of atherosclerosis in these hypercholesterolaemic subjects. Particularly in severely hypercholesterolaemic subjects, more conclusive proof of a protective effect of antioxidants from large prospective studies presently in progress is needed before antioxidant therapy can be advocated for the treatment and prevention of atherosclerosis.

In subjects with FH, *quantitative* rather than qualitative differences in LDL are associated with accelerated atherosclerosis. Therapy in FH should therefore be aimed primarily at reducing LDL-C levels.

List of Papers

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List of abbreviations

CAD – coronary artery disease

FH – familial hypercholesterolaemia

HDL – high-density lipoprotein

HDL-C – high-density lipoprotein cholesterol

LDL – low-density lipoprotein

LDL-C – low-density lipoprotein cholesterol

Introduction

Familial hypercholesterolaemia (FH) is an inherited disorder caused by mutations in the low-density lipoprotein (LDL) receptor which lead to diminished clearance of cholesterol from the circulation and, consequently, to markedly elevated LDL-cholesterol (LDL-C) levels.[1] The resultant hypercholesterolaemia predisposes these patients to severe premature atherosclerosis, particularly coronary artery disease (CAD). FH is particularly common in the Afrikaner, Jewish and Indian populations of South Africa with an estimated prevalence of heterozygous FH of greater than 1%.[2] Individuals with heterozygous FH usually present with CAD in the third to fifth decade whereas those with homozygous FH often die from accelerated atherosclerosis before the age of 30 years.[1] The concentration of LDL-C and lifetime vascular exposure to raised plasma LDL-C concentrations are major determinants of atherosclerosis, but there remains a considerable variability in the extent of atherosclerosis present and in the expression of clinical disease in these patients.[3] Susceptibility to CAD is partly related to particular types of LDL-receptor gene mutations, receptor negative mutations being associated with more severe expression of CAD compared to binding-defective alleles.[4,5] In addition to genetic variability at the LDL-receptor locus, the expression of CAD can be influenced by numerous established concomitant risk modulators such as hypertension, diabetes mellitus and cigarette smoking which significantly increase the risk for developing CAD, particularly in males.[6,7] Biochemical risk factors that have been associated with both the onset and progression of CAD include elevated levels of lipoprotein(a) [8], fibrinogen [9], insulin [10] and homocysteine [11].

Qualitative differences in LDL such as LDL particle size and susceptibility to lipid oxidation may also play a role. LDL consists of a heterogeneous population of particles that differ in their size, density and metabolic properties.[12] Two major LDL subclass patterns can be identified on gradient gel electrophoresis or ultracentrifugation, the small dense subclass pattern (pattern B) being associated with an increased risk of myocardial infarction.[13] Small dense LDL particles are considered more atherogenic as they are transported more rapidly into the subendothelial space, bind more avidly to proteoglycans and are more susceptible to oxidation.[14] A predominance of small dense LDL is linked to the metabolic syndrome or atherogenic lipoprotein phenotype - a pattern of dyslipidaemia characterised by hypertriglyceridaemia, low HDL-C levels, enhanced post prandial lipaemia and insulin resistance.[15]

The purpose of this thesis was to determine whether such qualitative differences in LDL are important determinants of the extent and severity of atherosclerosis and to determine whether it is mainly the reduction in LDL-C that is of benefit, or whether antioxidant therapy would also be effective in preventing progression of atherosclerosis in FH subjects.

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Paper 1

The susceptibility of LDL to oxidation can be studied in vitro by incubating LDL with endothelial cells or with redox-active ions. Measurement of the susceptibility of LDL to oxidation by copper ions as initially described by Esterbauer [1] has been widely used as a measure of its atherogenicity.

This paper describes a method that utilises a rapid preparation of LDL that is quick, simple and compares favourably with Esterbauer's method. This method was used for assessing the susceptibility of LDL to oxidation in several of the studies presented in this thesis.

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33rd FSASP Congress

Rapid method for measuring copper induced LDL oxidation

A.J. Areias[a], J. Richardson[a], F.J. Raal[b]

Abstract *Background.* Recent evidence has shown that oxidatively modified LDL (Ox-LDL) is pro-atherogenic. Increased susceptibility of LDL to oxidation may therefore be an important determinant of risk for atherosclerosis. Present methods used for the measurement of Ox-LDL are impractical as routine assays.

Method. We describe a modified method that utilizes single step vertical ultracentrifugation and continuous monitoring of conjugated diene production after copper induced LDL oxidation.

Results. In a group of healthy subjects (n=15) we observed lag times of 64.5 (±5.9) min and the time to peak conjugated diene formation was 140.5 (±6.9) min (values are mean ± standard error of the mean).

Conclusions. The proposed method is quick (±6 hours), simple and compares favourably with previously published data. *(Eur J Lab Med 1995;1:87-89).*

Introduction

Increased total plasma cholesterol concentration, especially the low density lipoprotein (LDL) cholesterol has been shown to be an important cause of coronary artery disease[1]. Recent evidence has shown that oxidatively modified LDL (OxLDL), which is a substrate for the scavenger receptor on macrophages may be important in the formation of the fatty streak, and is pro-atherogenic[2]. Increased susceptibility of LDL to oxidation has been shown to promote premature coronary atherosclerosis in individuals with triglyceride enriched LDL[3] and may therefore be an important determinant of risk for atherosclerosis. Present methods used for the measurement of oxidized lipoproteins and lipids are either non-specific or time consuming (i.e. cannot be completed within the same day) and are unsuitable as routine tests (reviewed by Chait[4]). We describe a method that utilizes a rapid preparation of LDL and quantitates the susceptibility of LDL to oxidation with results obtainable in 5-6 hours. Plasma LDL is separated by single step vertical ultracentrifugation (modification of Chung *et al.*'s method[5]) and OxLDL is measured by continuous monitoring of conjugated diene production after copper induced LDL oxidation (modification of Esterbauer's method[6]). Results are expressed as "lag time", which can be defined as the time required to overcome the inherent ability of LDL to resist oxidation.

Materials and Methods

Heparinized blood was collected after a 14 hour fast and immediately centrifuged at 1000 g for 10 min. The plasma density was adjusted to 1.21 g/mL by adding 0.37 g desiccated NaBr to 1.3 mL plasma in a Quick-Seal ultracentrifuge tube (Beckman Instruments) and this was carefully overlaid with 3.5 mL of 0.15 M NaCl pH 7.4. Vertical ultracentrifugation was carried out at 65000 rpm in a Beckman L8-70 centrifuge for 45 minutes at 10 °C using a VTi 65 rotor. The ultracentrifuge was set to slow acceleration and no brake[5]. The LDL fraction, visible as a distinct band was collected by piercing the side of the tube with a needle and gently aspirating the band (±0.5 mL). This was desalted on a Sephadex G25M (Pharmacia) column (bed volume 9 mL) with 0.01 M PBS pH 7.4 as the eluent.

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The cholesterol content was assayed with the CHOD-PAP method (Boehringer Mannheim) and the concentration adjusted to 80 μg/mL with PBS (final volume 2.5 mL). Oxidation was induced by the addition of Cu^{2+} (sulphate) in 0.15 M NaCl pH 7.4 at a final concentration of 10 μM. Conjugated diene production was measured by monitoring the change in absorbance at 234 nm[7] from zero to three hours at 15 min intervals on a Shimadzu UV 120-02 with a LKB 2209 multitemp circulator set to 25 °C. Protein was assayed by the Lowry method as modified by Hartree[8]. The purity of the LDL fraction was assessed by lipoprotein and protein gel electrophoresis using the Paragon system (Beckman). Prior to the latter the LDL fraction was concentrated (5-10x) using Centriflo cones (Amicon CF-25).

Lipid peroxides were measured by the ferrous oxidation-xylenol (FOX) assay[9] at various times during the induced oxidation.

Results

LDL was the only band visible on lipoprotein electrophoresis. On protein electrophoresis two bands corresponding to albumin and apolipoprotein B were visible. Gel densitometry showed that the albumin fraction represented less than 15% of the total protein present. This corresponded to <15 μg/mL of albumin in our fraction (total protein was 72.2 ± 6.2 μg/mL (mean±SD)). To verify that albumin at this concentration did not interfere with our assay we added albumin (BSA. Sigma) at different concentrations (15, 37.5 and 75 μg/mL). There was no effect on either the lag time or the peak conjugated diene production (Figure 1). Due to the contamination of the LDL fraction by albumin we opted to standardize our assay according to cholesterol concentration. Varying the concentration of cholesterol (60-100 μg/mL) did not appreciably alter the lag time (Figure 2). and we standardized to 80 μg/mL because this gives a maximal absorbance of approximately 1.

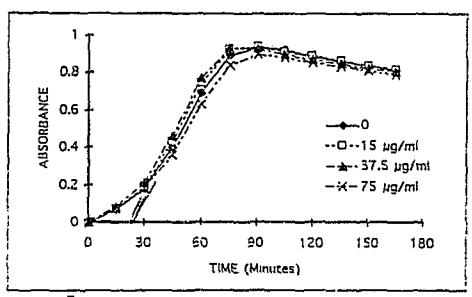

Figure 1. Adding albumin at different concentrations (to the same specimen) up to 5 times the concentration present in the LDL fraction does not have an effect on the lag time or on the conjugated diene production.

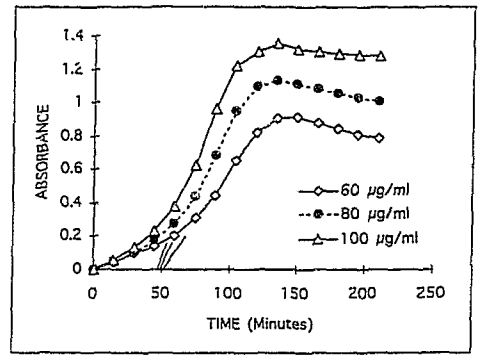

Figure 2. Varying the cholesterol concentration (i.e. LDL concentration) did not affect the lag time but there was an increase in the peak conjugated diene production.

Higher incubation temperatures reduced assay time (quicker lag times) but decreased the intraindividual reproducibility. When performed at 25 °C we obtained an intraindividual CV of 5.5%.

Comparisons with: a) Esterbauer's original method[6] showed a good correlation (r=0.96; n=6) and, b) the FOX method which measures lipid hydroperoxides showed that the peak conjugated diene production and lipid peroxide formation were at similar times (Figure 3).

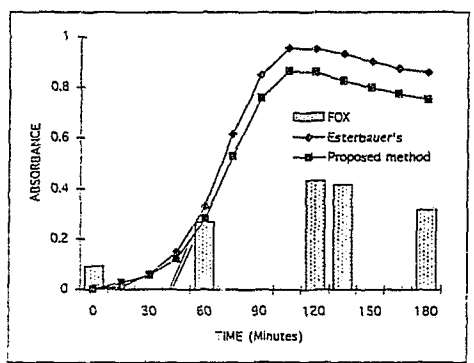

Figure 3. Comparison of a specimen analyzed by the proposed rapid method, the FOX method and the original Esterbauer method. A good correlation was observed (r=0.96; n=6) in the lag times of Esterbauer's and the proposed method. The FOX assay does not allow for continuous monitoring. The assay was performed by sampling a specimen in which copper mediated oxidation was induced at 5 timed intervals. The peak lipid peroxide production and peak conjugated diene production were similar.

EDTA is normally used as the anticoagulant and antioxidant. The drawback is that it binds copper and can therefore inhibit oxidation[10] and must be completely removed. We compared heparin and EDTA specimens. the LDL fraction obtained from EDTA plasma was dialyzed in 100 volumes of 0.01 M PBS pH 7.4. Good correlation was obtained for lag times (r=0.98: n=4).

Using our method. the lag time in a group of healthy subjects (n=15) was 64.5 (±5.9) min and the time to peak conjugated diene formation was 140.5 (±6.9) min (values are mean ± standard error of the mean).

Discussion

The rapid processing time of our method (i.e. ±2 hours prior to inducing oxidation) decreases the risk of sample auto-oxidation and allows us to use heparin instead of EDTA as the anticoagulant. The sample obtained from single spin vertical ultracentrifugation is pure except for contamination by albumin. which has been shown to act as an antioxidant[11]. We have shown that adding albumin (75 µg/mL) at 5x the levels present in our sample does not interfere with our assay. Cholesterol concentration was used as a measure of LDL because of this contamination. When different concentrations of cholesterol were used we noticed that although the lag time was unchanged the peak conjugated diene showed a proportional increase. This may be explained by the hypothesis of Esterbauer and Jurgens[12] that copper binds to discrete sites on apo B and these forms the centre for repeated radical production. Therefore the lag time will be dependent on the susceptibility of each LDL molecule to oxidation and the peak production will depend on the total number of molecules. A final copper concentration of 10 µM was chosen because we did not want copper availability to be a limiting factor. It has also been shown by Kleinveld *et al.*[13] that at low copper concentration there is an inverse relationship between maximal rate of diene production and lag time with the curves plateauing at 10 µmol/L Using the LKB Multitemp circulator we are able to maintain a constant 25 °C irrespective of the room temperature and equipment heat.

In conclusion the proposed method using vertical ultracentrifugation is quick (±6 hours). simple and lag time results compare favorably with previously published data (reviewed in 12).

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Paper 2

The aim of this study was to determine whether the susceptibility of LDL to *in vitro* oxidation is increased in FH subjects. Unexpectedly, LDL isolated from both homozygous and heterozygous FH patients was found to be less susceptible to oxidation compared to LDL isolated from normocholesterolaemic control subjects. Serum and LDL vitamin E levels were also found to be higher in the FH subjects.

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ATHEROSCLEROSIS

Susceptibility of low density lipoprotein to oxidation in familial hypercholesterolaemia

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Abstract

There is increasing evidence that oxidative modification of low density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis. Subjects with familial hypercholesterolaemia (FH) have elevated concentrations of LDL and develop premature atherosclerosis. The aim of the study was to determine whether the susceptibility of LDL to in vitro oxidation is increased in FH subjects. LDL was isolated from 15 FH homozygotes (mean age ± SD, 19 ± 10 years; mean LDL-cholesterol 16.86 ± 3.55 mmol/l), 15 FH heterozygotes (38 ± 13 years; LDL-cholesterol 5.53 ± 1.78 mmol/l) and 15 normocholesterolaemic subjects (31 ± 8 years; LDL-cholesterol 3.07 ± 0.77 mmol/l). Susceptibility of LDL to in vitro copper-mediated oxidation was assessed by measuring conjugated diene production at 234 nm, the lag phase being a measure of the resistance of LDL to oxidation. Unexpectedly, the mean duration of the lag phase was 2.2 fold longer in the FH homozygotes (123.8 ± 45.0 min) and 1.75-fold longer in the FH heterozygotes (99.9 ± 40.6 min) than in the controls (57.1 ± 27.9 min; $P < 0.0001$). Serum and LDL vitamin E levels were higher in the FH patients, but not when expressed relative to LDL-cholesterol concentration. There was also no correlation between LDL vitamin E concentration and duration of the lag phase. LDL bulk rather than the susceptibility of LDL to oxidation is probably the more important factor for the initiation and progression of atherosclerosis in FH patients.

Keywords: Familial hypercholesterolaemia; Low density lipoprotein; Lipid oxidation: Vitamin E

1. Introduction

There is increasing evidence that oxidative modification of low density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis [1,2]. Oxidized LDL is taken up more rapidly by scavenger receptors and, unlike native LDL, can lead to macrophage cholesterol accumulation and foam cell formation. Patients with familial hypercholesterolaemia (FH) develop premature and often severe atherosclerosis, and in these patients the atherosclerotic lesions characteristically contain many cholesterol-laden foam

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cells [3]. The aim of the present study was to determine whether LDL isolated from these patients is more susceptible to in vitro oxidation than normal LDL.

2. Patients and methods

Patients with homozygous and heterozygous FH attending the lipid clinic at the Johannesburg Hospital were asked to participate in the study. The diagnosis of homozygous FH was based on (i) serum total cholesterol levels consistently greater than 15 mmol/l; (ii) the appearance of xanthomas in the first decade of life; (iii) documentation in both parents of hypercholesterolaemia or clinical signs indicative of the heterozygous state; and (iv) confirmation by DNA analysis of FH LDL receptor mutations common in South Africa [4]. Diagnosis of heterozygous FH was based on the the presence of a family history of hypercholesterolaemia, clinical signs of FH, together with an elevated serum total cholesterol level and confirmed by DNA analysis. Healthy normolipidaemic subjects with no history of hypercholesterolaemia or coronary artery disease were used as controls. Any subject taking vitamin supplements was excluded from the study. To avoid the possible effects of diet on the susceptibility of LDL to oxidation, all subjects were advised on a standard low cholesterol (< 200 mg/day), low fat ($< 30\%$ of total calories) diet which they were asked to adhere to for at least 6 weeks prior to sample collection. All recruited subjects gave their consent to the study which was approved by the University of the Witwatersrand's committee for research on human subjects.

2.1. Preparation and oxidation of low density lipoprotein

Our procedure for isolation of LDL was adapted from the method of Chung et al. with some modifications [5]. Heparinized blood (10 ml) was centrifuged immediately after collection at 1000 × *g* for 10 min. Plasma (1.3 ml) was added to 0.37 g of sodium bromide in a Quickseal ultracentrifuge tube (Beckman Instruments). The salt was dissolved by gentle mixing, and the solution was then overlaid with 3.5 ml of 0.15 mol/l NaCl, pH 7.4. The balanced and sealed tubes were then vertically ultracentrifuged at 65 000 rev./min (370 000 × *g*) in a VTi 65 rotor using a Beckman L8-70 ultracentrifuge for 45 min at 10°C. The LDL fraction (± 0.5 ml) was aspirated by piercing the side of the tube with a needle. This was then desalted by gel filtration on a Sephadex G25M column (Pharmacia) using 0.01 mol/l phosphate buffered saline (PBS), pH 7.4, as eluent. The isolated LDL fraction was free of detectable amounts of vitamin C as assessed by high performance liquid chromatography (HPLC), as well as other lipoprotein classes as assessed by agarose gel electrophoresis. LDL cholesterol was adjusted to a concentration of 80 μg/ml and then 10 μmol/l copper sulphate was added as the pro-oxidant. The susceptibility of LDL to copper-mediated oxidation was assessed by measuring conjugated diene formation at 25°C, monitored spectrophotometrically at 234 nm according to the method of Esterbauer et al. [6]. Using this method, LDL oxidation can be divided into 3 phases: a lag phase, a propogation phase and a decomposition phase [6]. During the lag phase, antioxidants are consumed, but no significant oxidation of fatty acids occurs. The propogation phase occurs once LDL is depleted of its antioxidants and fatty acids are rapidly oxidized to form conjugated dienes. Levels of conjugated dienes then slowly fall during the decomposition phase. The lag phase was defined as the intercept of a tangent drawn to the steepest segment of the propogation phase to the horizontal axis, and is a measure of the resistance of LDL to oxidation. All samples were run in duplicate.

2.2. Vitamin E content

Serum and LDL vitamin E was measured by HPLC according to a modification of the method described by Lehmann et al. [7]. Briefly, vitamin E was extracted from 0.2 ml plasma or LDL with 0.8 ml of acetone and 1 ml of petroleum ether (40–60°C). After removing the organic phase, the extraction was repeated twice. The organic phases were then pooled and evaporated to dryness under nitrogen. The residue was then dissolved in 0.1 ml ethanol, and 20 μl was injected onto a Ultremex 5 μm particle C-18 column (150 × 4.6

mm). Detection was performed using a 464 electrochemical detector (Waters). We used tocopherol acetate as the external standard.

2.3. Other measurements

Total cholesterol (total-C) and triglyceride (TG) were quantified by enzymatic methods using automated techniques [8]. High density lipoprotein cholesterol levels (HDL-C) were determined by the phospho-tungstate-magnesium precipitation procedure. LDL cholesterol (LDL-C) levels were calculated using the Friedewald formula [9]. Protein content of LDL was measured by the method of Lowry et al. [10].

2.4. Statistics

Comparisons were performed using a one-way analysis of variance to test for a significant difference between the three groups. Pairwise comparisons were then used to determine which groups were significantly different from each other. Correlation coefficients were determined according to Spearmann's rank correlation method. In all comparisons, $P < 0.05$ was considered significant. Except where otherwise stated, results are expressed as mean $\pm$ SD.

3. Results

Fifteen patients with homozygous and heterozygous FH and 15 normolipaemic controls participated in the study (Table 1). None of the homozygous FH patients were receiving lipid-lowering therapy at the time of the study. Eight of the 15 heterozygous FH patients were on statin therapy (simvastatin 20–40 mg/day). As expected, the mean serum LDL-C levels were much higher in the homozygous and heterozygous FH patients than in the controls (16.86 $\pm$ 3.55 vs. 5.58 $\pm$ 1.78 vs. 3.07 $\pm$ 0.77 mmol/l, respectively; $P <$ 0.0001). Before being subjected to in vitro oxidation, the isolated LDL was standardised according to cholesterol concentration to allow for these differences. LDL was standardised according to lipid rather than protein, as the first step in lipid oxidation after consumption of antioxidants present in LDL is peroxidation of polyunsaturated fatty acids present in the LDL lipids, rather than protein [5]. However, standardising according to protein content rather than lipid content did not alter the findings (data not shown).

Unexpectedly, the resistance of LDL to oxidation, as measured by the duration of the lag phase during copper-mediated oxidation, was greater in the patients with homozygous and heterozygous FH than in the controls (Fig. 1). The mean duration of the lag phase was 2.2-fold longer in the FH homozygotes (123.8 $\pm$ 45.0 min) and 1.75-fold longer in the FH heterozygotes (99.9 $\pm$ 40.6 min) than in the controls (57.1 $\pm$ 27.9 min; $P <$ 0.0001). In the heterozygous FH patients there was no difference in lag phase between those receiving statin therapy and those on no therapy (103 $\pm$ 45 vs. 96 $\pm$ 38 min; $P = 0.82$), despite

Table 1
Characteristics of the patients with familial hypercholesterolaemia (FH) and the normolipidaemic controls

	Controls $n = 15$	FH heterozgotes $n = 15$	FH homozygotes $n = 15$
Sex (M/F)	11/4	4/11	8/7
Age (years)	31 (25–52)	38 (13–53)	19 (4–36)
T-chol (mmol/l)	4.70 (0.94)	7.39 (2.26)	17.79 (3.59)*
TG (mmol/l)	1.02 (0.52)	1.00 (0.74)	0.62 (0.38)
HDL-C (mmol/l)	1.17 (0.34)	1.36 (0.56)	0.65 (0.22)**
LDL-C (mmol/l)	3.07 (0.77)	5.58 (1.78)	16.86 (3.55)*

Data are expressed as mean (range) or mean (S.D.).
T-chol, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.
* Significant difference between all three groups: $P < 0.0001$.
** HDL-C of FH homozygotes significantly less than other two groups: $P < 0.001$.

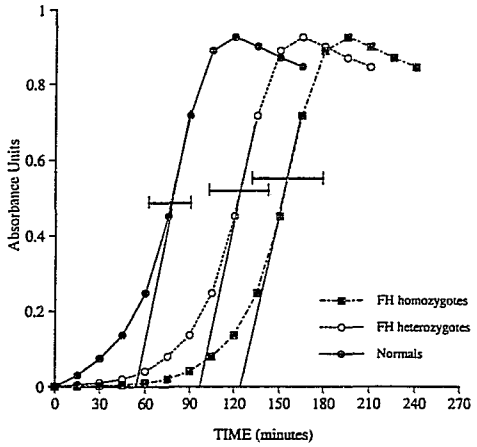

Fig. 1. Mean conjugated diene formation measured as the change in absorbance at 234 nm following copper-mediated oxidation of LDL in the patients with familial hypercholesterolaemia (FH) and in the normolipidaemic controls. LDL (80 μg cholesterol/ml) was incubated with 10 μM copper ions at 25°C. Differences in the time course of conjugated diene formation as measured by the duration of the lag phase (■—■ = mean ± S.D.) were significant: $P < 0.0001$.

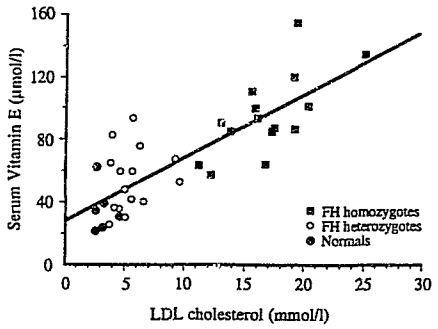

Fig. 2. Relationship between the serum vitamin E and LDL-cholesterol concentration in the patients with familial hypercholesterolaemia (FH) and in the normolipaemic controls. Vitamin E correlated with LDL cholesterol concentration, $r = 81$; $P < 0.0001$.

the difference in LDL-C levels (4.47 ± 0.69 vs. 6.85 ± 1.83 mmol/l, $P = 0.008$). The three groups differed in their age and sex distribution (Table 1). However, there was no correlation between lag phase and sex or age in any of the groups or in the three groups combined.

Serum and LDL vitamin E levels were much higher in the homozygous and heterozygous FH patients, and there was a good correlation between vitamin E and serum LDL-cholesterol levels (Fig. 2). However, these differences were not significant when expressed relative to cholesterol concentration (Table 2). In addition, there was no correlation between LDL vitamin E content and duration of the lag phase in any of the groups.

4. Discussion

There is increasing evidence that oxidative modification of LDL occurs in vivo and increases LDL atherogenicity. Enhanced susceptibility of LDL to oxidation may therefore predispose to atherosclerosis. In fact, some studies have shown that the susceptibility of LDL to oxidation is correlated with the severity of atherosclerosis [11,12].

A number of factors can influence susceptibility of LDL to oxidation. An early step in LDL oxidation is peroxidation of polyunsaturated fatty acids. Dietary intake may alter LDL fatty acid composition and influence the susceptibility of LDL to oxidation. Diets enriched in linoleate increase the susceptibility of LDL to oxidation, whereas diets enriched in oleate and depleted of linoleate reduce susceptibility [13]. All subjects were prescribed a standard diet low in saturated fat and cholesterol for at least 6 weeks prior to sample collection, and a major difference in fatty acid intake between the groups is therefore unlikely. A second important determinant of LDL susceptibility to oxidation is the content of endogenous antioxidants. Under pro-oxidant conditions, the antioxidants contained in LDL are first destroyed, and only then do the polyunsaturated fatty acids in LDL begin to undergo lipid peroxidation. Vitamin E is the major lipid-soluble antioxidant present within LDL and accounts for approximately 80% of the antioxidant capacity of LDL [14,15]. Several studies have shown that enrichment with vitamin E, both in vitro and in vivo, significantly reduces LDL susceptibility to oxidation [16–18]. Vitamin E is transported mainly in LDL cholesterol [19]. The finding of

Table 2
Vitamin E status of the patients with familial hypercholesterolaemia (FH) and the normolipidaemic controls

	Controls	FH heterozygotes	FH homozygotes
Serum vitamin E (μmol/l)	35.5 (14.8)	54.5 (20.2)	95.7 (26.4)*
LDL vitamin E (μmol/l)	14.1 (5.5)	30.2 (8.8)	85.4 (23.4)*
% Vitamin E bound to LDL	45.6 (12.6)	61.4 (11.1)	87.5 (14.2)*
LDL vitamin E per LDL-C (μmol/mmol)	4.94 (1.6)	4.84 (1.5)	5.03 (1.5)

LDL, low density lipoprotein; LDL-C, low density lipoprotein-cholesterol.
* Significant difference between all three groups, $P < 0.0001$.

elevated vitamin E levels in our FH patients is therefore not unexpected as they have markedly elevated LDL levels. There was, however, no difference in the amount of vitamin E relative to LDL cholesterol between the groups. In addition, there was no correlation in any of the groups between vitamin E levels and susceptibility of LDL to oxidation. Therefore, differences in vitamin E content cannot explain the reduced susceptibility of LDL to oxidation in FH in our study.

Other lipid-soluble antioxidants present in LDL such as ubiquinol-10, lycopene and β-carotene were not measured in our study. These antioxidants are present in amounts of only 1/20th to 1/300th of that of vitamin E, and thus contribute little to the lipoprotein's total antioxidant capacity [14,20]. In addition, supplementation with β-carotene has failed to increase the resistance of LDL to oxidation [18,21]. Vitamin C appears to act indirectly to protect LDL against oxidation by preventing the loss of vitamin E and β-carotene [22]. Vitamin C is water soluble and is extracted during preparation of the LDL and therefore cannot explain the differences in oxidative resistance.

In addition to compositional features, particle size and density have been shown to influence susceptibility of LDL to oxidation. LDL is known to include multiple distinct subclasses that differ in size, density and chemical composition [23]. Recent reports have shown that smaller, more dense LDL display enhanced susceptibility to copper-induced oxidation when compared to larger, more buoyant lipoprotein particles [24,25]. In addition, a predominance of small, dense LDL particles is associated with an increased risk of coronary heart disease [26]. Greater uptake by macrophages of dense LDL as a result of enhanced susceptibility to oxidation may explain this finding [24]. Subjects with FH have larger, more buoyant LDL particles, which are relatively cholesterol enriched and protein depleted [27,28]. Our findings of a decreased susceptibility of LDL to oxidation in FH are in keeping with these observations, and contradict previous reports which have demonstrated increased susceptibility of LDL to oxidation in hypercholesterolaemic subjects [29,30].

The results of this study challenge, but do not disprove, the oxidative hypothesis of atherosclerosis. Currently, it is thought that LDL particles do not undergo any significant degree of oxidation within the circulation because of the presence of abundant antioxidant defences. However, LDL particles are continuously entering and leaving the arterial wall; some of these particles become entrapped in the interstitial matrix, and because of prolonged resident time, may be subjected to sustained oxidative stress. Thus, although initially more resistant, once lipid peroxidation has been initiated, a larger, more cholesterol-enriched LDL, such as that typically found in patients with familial hypercholesterolaemia, may generate more total oxidized lipid within the arterial wall with pathologic consequences [31]. In fact, oxidized LDL has been demonstrated in xanthomata from patients with FH [32].

It is now well established that elevated plasma concentrations of LDL are associated with accelerated atherogenesis. In subjects with FH, quantitative rather than qualitative differences in LDL are therefore probably more important in causing accelerated atherosclerosis, LDL bulk rather than the susceptibility of LDL to oxidation being the

more important factor for the initiation and progression of atherosclerosis in these subjects.

Acknowledgements

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Paper 3

In this study the effect of long-term high dose vitamin E supplementation on xanthoma regression in subjects with homozygous FH was evaluated. Although vitamin E supplementation increased serum vitamin E levels and reduced the susceptibility of LDL to oxidation, vitamin E had no demonstrable effect on xanthoma regression in any of the subjects studied.

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Lack of effect of high dose vitamin E on xanthoma regression in homozygous familial hypercholesterolaemia

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Abstract

There is increasing evidence that oxidative modification of low-density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis. Homozygous familial hypercholesterolaemia (HFH) is characterized by premature, severe atherosclerosis. Drugs available at present are ineffective in lowering the markedly elevated LDL levels in this condition; antioxidant therapy to protect the LDL against oxidation may be of benefit. Probucol, the only drug shown to induce xanthoma regression in HFH, is a potent antioxidant, but it also lowers high-density lipoprotein cholesterol (HDL-C) levels, causing some concern. Vitamin E is a naturally occurring antioxidant that does not affect HDL-C levels. We have therefore evaluated the effect of long-term high dose vitamin E on xanthoma regression in HFH. Ten subjects with HFH, mean age 17 years (range 4–34), received vitamin E (400–1000 mg/dl alpha-tocopherol acetate/day) for a period of 23 months (range 12–27). There was a 4.2-fold increase in the mean serum vitamin E level (mean (S.D.) 49.7 (19.9) to 177.9 (45.6) μmol/l; $P < 0.005$), but no change in serum lipid or lipoprotein concentrations. Although there was an increase in the in vitro resistance of LDL to oxidation as determined by the duration of the lag phase during copper-mediated oxidation (116 (8.34) vs. 141.5 (9.23) min; $P < 0.005$) there was no xanthoma regression; in fact they progressed in 4 subjects. Unlike probucol, high dose long-term vitamin E has no demonstrable effect on xanthoma regression in HFH.

Key words: Vitamin E; Antioxidant; Lipid peroxidation; Xanthoma; Homozygous familial hypercholesterolaemia

1. Introduction

It is well established that elevated concentrations of low-density lipoprotein (LDL) are associated with the development of atherosclerosis and that the cellular uptake of LDL from the plasma is mediated mainly by the LDL receptor [1]. How-

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ever, subjects with homozygous familial hypercholesterolaemia (HFH) develop premature and severe atherosclerosis despite a lack of LDL receptor activity [2]. This suggests that arterial uptake of LDL giving rise to foam cells is mediated by a pathway independent of the LDL receptor. There is now increasing evidence that scavenger receptor uptake of oxidatively modified LDL plays an important role in the pathogenesis of atherosclerosis [3]. Oxidized LDL is taken up by macrophages three to ten times more rapidly than is native LDL and, unlike native LDL, can lead to foam cell formation. In addition, oxidized LDL is chemotactic for monocytes, induces endothelial cell damage, and stimulates cytokine and growth factor release from cells present in the arterial wall [4]. Oxidized LDL has been identified in atherosclerotic lesions, and circulating autoantibodies to epitopes of oxidized LDL are found in patients with atherosclerosis [5].

The use of antioxidants to inhibit the oxidation of LDL may therefore retard or prevent the atherogenic process. The only drug shown to induce xanthoma regression in HFH is probucol [6,7]. Probucol is a potent antioxidant but, because it lowers high-density lipoprotein cholesterol (HDL-C) levels concomitantly, it has been regarded with caution by many physicians [8]. Vitamin E is a potent natural antioxidant that does not effect HDL-C levels [9]. Therefore, if probucol causes xanthoma regression because of its antioxidant effect, vitamin E may also be beneficial.

In this study we evaluated the effect of long-term high dose vitamin E administration on xanthoma regression in HFH. In addition we assessed whether vitamin E supplementation could decrease the susceptibility of LDL to in vitro oxidation in this disease.

2. Patients and methods

Patients with HFH attending the Lipid Clinic at the Johannesburg Hospital were asked to participate in the study. The diagnosis of HFH was based on the presence of (i) serum total cholesterol levels consistently greater than 15 mmol/l; (ii) the appearance of xanthomas in the first decade of life; (iii) documentation in both parents of hypercholesterolaemia or clinical signs indicative of the heterozygous state; and (iv) confirmation by DNA analysis of familial hypercholesterolaemia LDL receptor mutations common in South Africa. Only patients who had not received probucol, or in whom probucol therapy had been discontinued for at least 6 months, were eligible. No participants were taking vitamin supplements when recruited, and all were instructed to avoid supplemental vitamins.

After detailed explanation, all recruited patients gave their written informed consent to the study, which was approved by the University of the Witwatersrand's committee for research on human subjects.

Ten patients, 4 males and 6 females, agreed to participate in the study (Table 1). Seven patients were homozygotes or compound heterozygotes for FH Afrikaner-1, -2 or -3. These three founder-type mutations together account for more than 80% of FH in Afrikaners [10]. Two subjects fulfilled the clinical criteria for HFH but were heterozygous for the common FH Afrikaner mutations. One patient was a homozygote for the FH664 mutation. The mean age of the patients was 17 years (range 4–34); their body mass index 20.8 kg/m^2 (range 15.5–24.8). Three patients had previously undergone coronary artery bypass surgery and were taking aspirin. One of them was also receiving atenolol and diltiazem. None of the patients were on lipid-lowering medication. Only one of the patients smoked cigarettes.

All patients were kept on a standard low-cholesterol, low-saturated-fat diet. Their diets were not altered during the study, to avoid the effect of diet on susceptibility of LDL to lipid oxidation. Vitamin E was administered orally as dl-alpha-tocopherol acetate provided by Hoffman-La Roche. Eight of the patients received 1000 mg/day and two, aged 4 and 7 years, received 400 and 600 mg/day, respectively. Patients were seen 3-monthly. Fasting lipograms, after a 10 h overnight fast, as well as serum vitamin E levels were measured at each visit. Compliance with vitamin E therapy was assessed by tablet counts and by measurement of serum vitamin E levels, the vitamin E

Table 1
Patient characteristics

Patient no.	Age (years)	Sex	BMI (kg/m^2)	FH genotype	Fasting lipogram (onset, mmol/l)				Other
					Total-C	TG	LDL-C	HDL-C	
1	14	F	19.4	FH664	15.12	1.15	13.8	0.81	
2	32	F	22.7	Afrik 2/2	17.65	0.75	16.0	0.94	PCS age 16; CABG age 26
3	14	M	23.5	Afrik 1/2	19.34	0.92	18.3	0.57	CABG age 9
4	14	F	18.8	Afrik 1/1	17.82	1.02	16.5	0.83	
5	14	F	23.5	Afrik 1/1	13.57	1.66	12.6	0.24	
6	17	M	22.9	Afrik 1/3	15.69	0.87	14.9	0.44	
7	34	M	21.6	Afrik 1/1	19.32	1.73	18.0	0.62	CABG age 30
8	18	F	24.8	Afrik 2/2	22.60	0.51	21.5	0.91	PCS age 5
9	7	F	15.6	Afrik 1/?	16.12	1.30	15.0	0.50	
10	4	M	15.5	Afrik 1/?	15.68	1.54	14.5	0.43	

Mean (S.D.) 17 (9.1) — 20.8 (3.2) — 17.3 (2.5) 1.15 (0.4) 16.1 (2.5) 0.63 (0.2)
BMI, body mass index; CABG, coronary artery bypass; FH genotype, genotype of identified familial hypercholoesterolaemia mutations; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; PCS, portacaval shunt; TG, triglycerides; total-C, total cholesterol.

level of each patient during supplementation being determined as the mean of all vitamin E levels measured after the start of vitamin E therapy.

The susceptibility of LDL to oxidative modification in vitro was assessed at the onset of the study and after at least 3 months of vitamin E supplementation.

2.1. Preparation and oxidation of LDL

The procedure for preparation and oxidation of LDL was adapted from the method of Esterbauer et al. with some modifications [11]. Heparinized blood (10 ml) was centrifuged immediately after collection at 1000 × *g* for 10 min. Plasma (1.3 ml) was added to 0.37 g of sodium bromide in a Quickseal ultracentrifuge tube (Beckman Instruments). The salt was dissolved by gentle mixing, and the solution was overlayered with 3.5 ml of 0.15 mol/l NaCl, pH 7.4. The balanced and sealed tubes were then vertically ultracentifuged at 65 000 rev./min (370 000 × *g*) in a VTi 65 rotor using a Beckman L8-70 ultracentrifuge for 45 min at 10°C. The LDL fraction (± 0.5 ml) was aspirated by piercing the side of the tube with a needle. This was then desalted by gel filtration on a Sephadex G25 M column (Pharmacia) using 0.01 mol/l phosphate buffered saline, pH 7.4, as eluent. LDL cholesterol was adjusted to a concentration of 80 μg/ml and then 10 μmol/l copper sulphate was added as the pro-oxidant. The susceptibility of LDL to copper sulphate-mediated oxidation was assessed by measuring conjugated diene formation at 25°C, monitored spectrophotometrically at 234 nm. Each sample was run in duplicate. Using this method, LDL oxidation can be divided into three phases: a lag phase, a propagation phase and a decomposition phase [11]. During the lag phase antioxidants are consumed, but no significant oxidation of fatty acids occurs. The propagation phase occurs once LDL is depleted of its antioxidants and fatty acids are rapidly oxidized to form conjugated dienes. Levels of conjugated dienes then slowly fall during the decomposition phase. The lag phase was defined as the intercept of a tangent drawn to the steepest segment of the propagation phase to the horizontal axis, and is a measure of the resistance of LDL to oxidation.

Lipid peroxides generated during LDL oxidation with 10 μmol/l copper sulphate in phosphate buffered saline for 0–4 and 24 h were also measured, by means of the FOX assay. Unlike the TBARS assay, this method, in which ferrous ions are oxidized in the presence of xylenol orange, is specific for lipid peroxides [12]. Briefly, 0.1 ml ali-

qouts of LDL were mixed with 0.9 ml FOX reagent, incubated for 30 min at room temperature, and then measured spectrophotometrically at 560 nm. An extinction coefficient of 4.52×10^4/M per cm was used to calculate concentrations of lipid peroxides from the absorbance [12].

2.2. Other measurements

Total cholesterol (total-C) and triglyceride (TG) were quantified by enzymatic methods using automated techniques [13]. HDL-C was determined by the phospho-tungstate-magnesium precipitation procedure. LDL cholesterol (LDL-C) levels were calculated using the Friedewald formula [14].

Serum vitamin E was measured by high-pressure liquid chromatography [15].

2.3. Assessment of xanthomas

Xanthomas were assessed 6-monthly by xeroradiography of the Achilles tendons at the level of the tibiocalcaneal joint, and by standardized photography of all visible cutaneous xanthomas. Coronary angiography was not performed on all patients for ethical reasons. Two of the patients had, however, undergone coronary angiography within 6 months prior to the onset of the study, and they underwent repeat angiography at the end of the study.

2.4. Statistics

Comparisons were performed using one-way analysis of variance for multiple samples or Student's *t*-test for paired data. The signed rank test was used for non-parametric data where appropriate. In all comparisons, $P < 0.05$ was considered significant. Except where otherwise stated, results are expressed as mear (S.D.) in the text and mean (S.E.) in the figures.

3. Results

Patients were followed for a mean of 23 months (range 12–27). No adverse clinical or biochemical effects of vitamin E therapy occurred during this period, confirming the safety of oral vitamin E even at high doses [16].

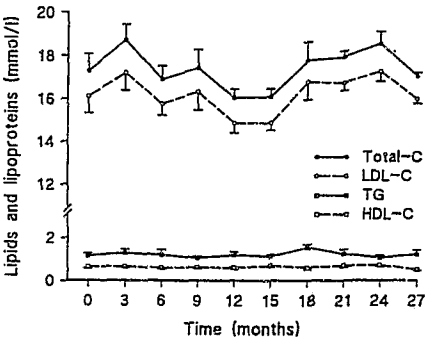

Fig. 1. Mean (S.E.) serum lipid and lipoprotein concentrations in HFH patients during the study period. No significant changes occurred in any variable.

Compliance with therapy, as assessed by tablet counts and serum vitamin E levels, was well maintained throughout the study. The mean serum vitamin E level prior to vitamin E supplementation was 49.7 (19.9) μmol/l. This is two to three times as high as the mean level found in normocholesterolaemic controls in our laboratory (unpublished data). On average, there was a 4.2-fold increase in the mean serum vitamin E level to

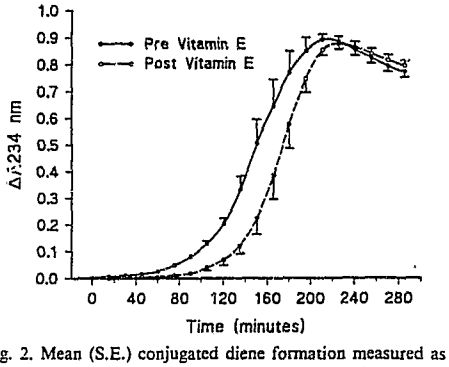

Fig. 2. Mean (S.E.) conjugated diene formation measured as change in absorbance at 234 nm (ΔA 234 nm) following copper-mediated oxidation of LDL in HFH patients before and after vitamin E supplementation. Prolongation of the time course of conjugated diene formation with vitamin E supplementation was significant; $P < 0.005$.

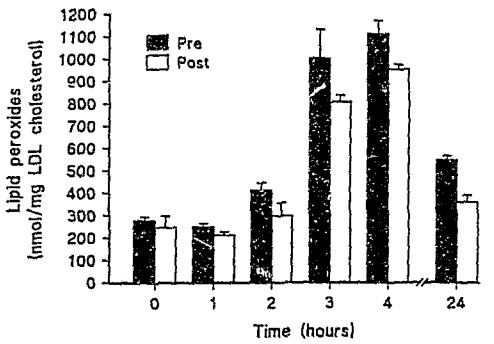

Fig. 3. Mean (S.E.) lipid peroxide formation in HFH patients following copper-mediated oxidation of LDL before and after vitamin E supplementation. Differences were not significant.

177.9 (45.6) μmol/l with supplementation; $P < 0.005$. There was no change in mean serum total-C, TG, HDL-C or LDL-C concentrations during the study (Fig. 1).

The resistance of LDL to oxidation, as measured by the duration of the lag phase during copper sulphate-mediated oxidation, increased from 116 (8.34) to 141.5 (9.23) min, $P < 0.005$ (Fig. 2). Similarly, there was a decrease in the amount of lipid peroxides generated during copper-mediated oxidation but this was not significant (Fig. 3).

There was no change in the thickness of Achilles tendons as assessed by xeroradiography during the study. There was also no evidence of regression of cutaneous xanthomata in any of the patients. In fact, cutaneous xanthomas progressed in four of the ten patients. Repeat coronary angiography in the two subjects who had undergone coronary angiography prior to the onset of the study showed progression of coronary atherosclerosis in both.

4. Discussion

HFH is a rare but serious disorder, most patients dying from coronary artery disease before the age of 30 years [2]. It remains a very difficult condition to treat. The present treatment of choice, namely LDL-apheresis or liver transplantation, is unfortunately not readily available at our academic complex. As currently available drugs are also ineffective in lowering the markedly elevated LDL, we lack effective measures for reducing the markedly elevated LDL levels in these patients. Antioxidant therapy to protect the LDL against lipid oxidation may, however, be of some benefit.

Vitamin E, the major peroxyl-radicle trapping antioxidant present in human blood, is carried mainly in LDL. It increases the resistance of LDL to oxidation, the LDL being oxidized only once all vitamin E is consumed [17]. LDL isolated from subjects given vitamin E supplementation shows increased resistance to oxidation [18,19]. Epidemiological studies have shown an inverse correlation between vitamin E consumption and risk for coronary artery disease [20,21]. Animal studies have also suggested that vitamin E supplementation not only can delay progression, but may even cause regression of atherosclerosis [22].

In our study, administration of high dose vitamin E over a 2 year period had no demonstrable effect on xanthoma regression in HFH. In fact, progression of xanthomas occurred in several of the patients. Cutaneous and tendon xanthomas in familial hypercholesterolaemia contain foam cells indistinguishable from those found in atherosclerotic lesions in the arterial wall. It is therefore likely that vitamin E also failed to cause regression of arterial atherosclerosis. This is supported by the demonstration of progression of coronary atherosclerosis in the two patients who underwent repeat coronary angiography.

LDL resistance to oxidation in vitro increased with vitamin E supplementation, but this increase was only modest. Vitamin E carried in LDL is delivered to cells mainly via the LDL receptor [23]. In HFH subjects, who lack LDL receptors, the LDL may therefore already be 'saturated' with vitamin E. This may explain why the plasma levels of vitamin E prior to vitamin E supplementation were 2–3-fold higher than those found in normal subjects.

The results of this study do not disprove the oxidative hypothesis of atherosclerosis. Currently, it is thought that because of the presence of abun-

dant antioxidant defences, LDL particles do not undergo any significant degree of oxidation within the circulation. However, LDL particles are continuously entering and leaving the arterial wall; some of these particles become entrapped in the interstitial matrix, and because of prolonged resident time may be subjected to sustained oxidative stress. Thus, although initially more resistant, once lipid peroxidation has been initiated a larger, more cholesterol-enriched LDL, such as that typically found in patients with HFH, may generate more total oxidized lipid within the arterial wall with pathologic consequences [24]. Our findings do, however, indicate the need for further trials before antioxidant therapy can be routinely advocated for the therapy of atherosclerosis in general.

We, and others, have shown marked regression of cutaneous and tendon xanthomas in subjects with HFH treated with probucol [6,7]. This regression is out of proportion to the degree of lowering of LDL and is evident within 6–12 months of therapy. This would imply that probucol is either a more potent antioxidant than vitamin E or that it causes xanthoma regression by some other mechanism. On a molar basis, probucol, which is transported within LDL, has an antioxidant potential three to seven times that of vitamin E [8]. Unlike vitamin E, probucol markedly decreases HDL-C and increases the activity of cholesteryl ester transfer protein, effects consistent with an increase in reverse cholesterol transport [25]. In fact, in subjects with HFH, reduction in the size of xanthomas correlates best with the reduction in plasma HDL-C levels [6]. Probucol also affects the macrophage directly and can reduce cell-mediated oxidation of LDL [26,27]. Xanthoma regression by probucol may well occur by these mechanisms rather than by antioxidation.

In summary, our study has shown that a 2 year trial of high dose vitamin E therapy in 10 patients with HFH produced no effect on xanthoma regression, a modest increase in the resistance of LDL to oxidation, and no significant decrease in the amount of lipid peroxides generated in vitro. Whether administration of mega-doses of the vitamin, or combination with other naturally occuring antioxidants (such as β-carotene and vitamin C), would be of greater benefit to such patients remains to be established.

5. Acknowledgements

This work was supported by the South African Medical Research Council. We would like to thank Hoffman-La Roche, South Africa, for supplying the vitamin E for the study. Professor D.R. van der Westhuyzen and Dr. G.A. Coetzee, University of Capetown, are acknowledged for performing the DNA analysis and Dr. E. Ebstein, University of the Witwatersrand, for assessing the xeroradiograms. Dr. S. Wolff, University College, London, kindly demonstrated the FOX assay.

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Paper 4

In this paper the role of lipid oxidation and antioxidant therapy in FH subjects is reviewed. The hypothesis is put forward that *qualitative* differences in LDL, such as LDL susceptibility to oxidation, are less important than the amount or *quantity* of LDL in determining the extent and progression of atherosclerosis in these subjects.

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redox report

Review article

Low density lipoproteins and atherosclerosis – quantity or quality?

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SUMMARY. **Oxidative modification of low density lipoprotein (LDL) appears to be important in the pathogenesis of atherosclerosis. Inhibiting the oxidation of LDL may retard or prevent the atherogenic process. However, susceptibility of LDL to oxidation in vitro and its atherogenicity in vivo may not always correlate. Subjects with familial hypercholesterolaemia (FH) develop severe, premature atherosclerosis despite having large, bouyant LDL particles which are less susceptible to oxidation. High dose, long-term vitamin E increases the resistance of LDL to oxidation but, unlike probucol, has no effect on xanthoma regression in homozygous FH. In FH, the quantity of LDL takes priority and the main aim of therapy is reduction of LDL bulk. Individuals with small, dense LDL particles are at increased risk for atherosclerosis despite desirable plasma LDL cholesterol levels. Small, dense LDL particles are more susceptible to oxidation and in these subjects antioxidant therapy may be of greater benefit. In subjects with atherosclerosis, current management should be aimed primarily at reducing the LDL cholesterol level. In the future antioxidant therapy may complement our management of hypercholesterolaemia.**

Low density lipoproteins (LDL) are the major cholesterol-carrying lipoproteins in plasma. It is now well established that elevated concentrations of LDL are associated with the development of atherosclerosis and that the cellular uptake of LDL from the plasma is mediated mainly by the LDL receptor.[1] However, incubation of macrophages in the presence of high concentrations of native LDL fails to cause accumulation of cholesterol ester, and does not result in foam cell formation.[2] In addition, subjects with homozygous familial hypercholesterolaemia (HFH) develop premature and severe atherosclerosis despite a lack of LDL receptor activity.[3] This suggests that arterial uptake of LDL giving rise to foam cells, the hallmark of the atherosclerotic lesion, is mediated by a pathway independent of the LDL receptor. There is now increasing evidence that modification of LDL, mainly by peroxidation within the intima, allows for uptake of LDL by scavenger receptors and plays an important role in the pathogenesis of atherosclerosis.[4] All four major cell types within atherosclerotic lesions (endothelial cells, smooth-muscle cells, macrophages and lymphocytes) can oxidise LDL but the macrophage seems to be the most active. Oxidized LDL is taken up by macrophages 3–10 times more rapidly than is native LDL and, unlike native LDL, can lead to foam cell formation. Once formed, oxidized LDL has many other potential atherogenic effects. Oxidized LDL increases the adherence and migration of monocytes; induces endothelial cell damage; and stimulates cytokine and growth factor release from cells present in the arterial wall.[5] There is also a growing body of evidence to show that oxidative modification of LDL does indeed occur in vivo. Oxidized LDL has been identified in atherosclerotic lesions, and circulating autoantibodies to epitopes of oxidized LDL are found in patients with atherosclerosis.[6] In addition lipid peroxide concentrations have been found to be higher in subjects with atherosclerosis.[7]

If oxidative modification of LDL is important for the development of atherosclerosis, then antioxidant therapy may retard or prevent the atherogenic process. In experimental studies involving hypercholesterolaemic rabbits and primates, dietary supplementation with natural or synthetic lipophilic antioxidants can lead to profound

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reductions in the extent of atherosclerosis, independent of any effect on plasma lipid levels.[8,9] However, the potential benefits of antioxidant therapy in humans remain uncertain. Several studies have shown that oral supplementation with vitamin E or probucol increases the resistance of LDL to oxidation.[10,11] Epidemiological data suggest an inverse correlation between plasma levels of vitamin E or B-carotene and the incidence of coronary heart disease (CAD).[12,13] Two large prospective studies have recently shown an association between a lower risk of CAD and high intake of vitamin E in both men and women.[14,15] However, other studies have found no association, or even an increase in the rate of death from CAD in the supplemented group.[16,17] Definitive proof that oxidized LDL is causally related to atherogenesis and that antioxidant therapy is of benefit is therefore still awaited and several studies are in progress to confirm or refute the oxidative hypothesis of atherosclerosis.

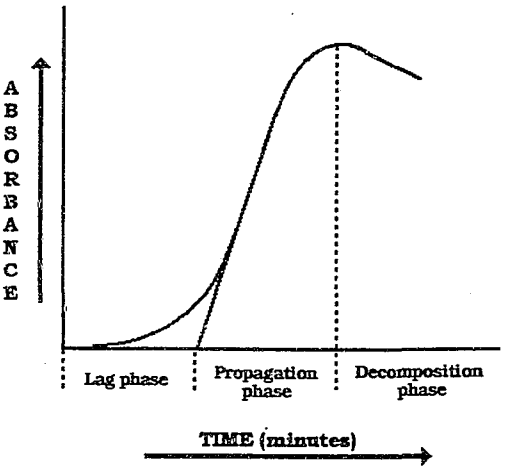

Fig. 1—Conjugated diene formation measured spectrophotometrically as the change in absorbance at 234 nm following copper-mediated oxidation of LDL in vitro. The lag phase is a measure of the resistance of LDL to oxidation.

SUSCEPTIBILITY TO OXIDATION

The susceptibility of LDL to lipid peroxidation can be studied in vitro by incubating LDL with endothelial cell or with redox-active ions. Measurement of susceptibility of LDL to oxidation by copper ions has been widely used as a measure of its atherogenicity.[18] During peroxidation double bonds in polyunsaturated fatty acids rearrange to form conjugated double bonds; these can be detected spectrophotometrically by an increase in absorbance at 234 nm, thus providing an effective method of continuously monitoring oxidation of LDL in vitro. Using this method, LDL oxidation can be divided into 3 phases: a lag phase, a propagation phase and a decomposition phase. During the lag phase antioxidants are consumed, but no significant oxidation of fatty acids occurs. The propagation phase occurs once LDL is depleted of its antioxidants and fatty acids are rapidly oxidized to form conjugated dienes. Levels of conjugated dienes then slowly fall during the decomposition phase. The lag phase can be defined as the intercept of a tangent drawn to the steepest segment of the propagation phase to the horizontal axis, and is a measure of the resistance of LDL to oxidation (Fig. 1). Some studies have shown that the susceptibility of LDL to oxidation is correlated with the severity of atherosclerosis.[19,20] However, susceptibility of LDL to oxidation and its in vivo atherogenicity may not always correlate and extrapolations from results obtained from in vitro oxidation alone are likely to be erroneous.

LDL PEROXIDATION IN FAMILIAL HYPERCHOLESTEROLAEMIA

Familial hypercholesterolaemia (FH) is an autosomal codominant disorder caused by mutations that interfere with the function of the LDL receptor, leading to diminished removal of cholesterol from the circulation and consequently to greatly elevated LDL levels. Patients with FH develop premature and often severe atherosclerosis, and in these patients the atherosclerotic lesions characteristically contain many cholesterol-laden foam cells.[3] If lipid oxidation is important in the pathogenesis of atherosclerosis one might expect patients with FH to have LDL that is more susceptible to peroxidation.

However, we have recently demonstrated that the susceptibility of LDL to in vitro oxidation is decreased in both homozygous and heterozygous FH subjects.[21] LDL was isolated from 15 FH homozygotes (mean [SD], age 19 [10] years; LDL-cholesterol 16.86 [3.55] mmol/l); 15 FH heterozygotes (age 38 [13] years; LDL-cholesterol 5.58 [1.78] mmol/l) and 15 normocholesterolaemic subjects (age 31 [8] years; LDL-cholesterol 3.07 [0.77] mmol/l). The mean duration of the lag phase following copper-mediated oxidation of LDL in vitro was 2.2-fold longer in the FH homozygotes (123.8 [45.0] min) and 1.75-fold longer in the FH heterozygotes (99.9 [40.6] min) than in the controls (57.1 [27.9] min; $P < 0.0001$). Subjects with FH have large buoyant LDL

particles, which are relatively cholesterol enriched.[22,23] Several recent reports have shown that smaller, more dense LDL display enhanced susceptibility to copper-induced oxidation when compared to larger, more buoyant lipoprotein particles.[24,25] Our findings of a decreased susceptibility of LDL to oxidation in FH are in keeping with these observations, and contradict previous reports which have demonstrated increased susceptibility of LDL to oxidation in FH subjects.[26,27]

We have also evaluated the effect of long-term high dose vitamin E on xanthoma regression in subjects with homozygous familial hypercholesterolaemia (HFH).[28] Vitamin E was used in the study as it is the major peroxyl-radical trapping antioxidant present in human blood and has been shown to increase resistance of LDL to oxidation.[29] Ten subjects received vitamin E (400–1000 mg dl-alpha-tocopherol acetate/day) for a mean period of 23 months (range 12–27). There was a 4.2-fold increase in the mean serum vitamin E level (49.7 [19.9] to 177.9 [45.6] μmol/l; $P<0.005$), but no change in serum lipid or lipoprotein concentrations. Although there was an increase in the in vitro resistance of LDL to oxidation as determined by the duration of the lag phase during copper mediated oxidation, this increase was only modest (116 [8.34] vs 141.5 [9.23] min; $P<0.005$). There was no xanthoma regression; in fact xanthoma progressed in 4 subjects. High dose long-term vitamin E therefore had no demonstrable effect on xanthoma regression in HFH. Possibly the degree of protection afforded by vitamin E was insufficient, and a more potent antioxidant is required to protect LDL in this condition.

The only drug shown to induce xanthoma regression in HFH is probucol. We, and others, have shown marked regression of cutaneous and tendon xanthomas in subjects with HFH treated with this drug.[30,31] This regression is out of proportion to the degree of lowering of LDL and is evident within 6–12 months of therapy. This would imply that probucol is either a more potent antioxidant than vitamin E, or that it causes xanthoma regression by some other mechanism. On a molar basis, probucol, which is transported within LDL, has an antioxidant potential 3–7 times that of vitamin E.[11] We have recently evaluated the effect of vitamin E compared to probucol on in vitro LDL oxidation in HFH. LDL was isolated from 8 HFH patients pre- and post mega-dose vitamin E supplementation (6000 mg α-tochoperol acetate/day for 6 weeks) and then after probucol (1000 mg/day for 6 weeks). Vitamin E caused only a modest increase in the resistance of LDL to oxidation, whereas probucol was much more effective (unpublished data; Fig. 2). However, unlike vitamin E, probucol markedly decreases HDL-cholesterol (HDL-C) and

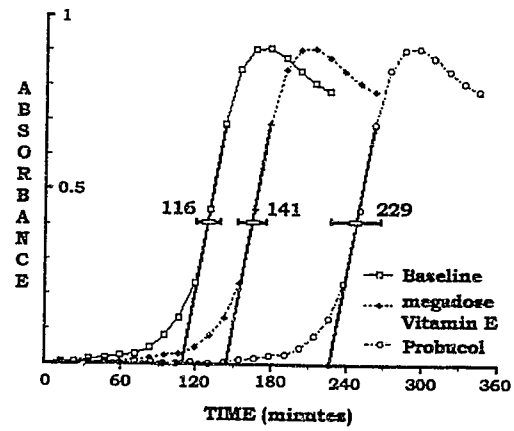

Fig. 2—Mean conjugated diene formation measured spectrophotometrically as the change in absorbance at 234 nm following copper-mediated oxidation of LDL isolated from 8 homozygous familial hypercholesterolaemia patients at baseline, and following megadose vitamin E (6000 mg/day for 6 weeks) and probucol (1000 mg/day for 6 weeks) therapy. Differences in the time course of conjugated diene formation, as measured by the duration of the lagphase, were significant; $P<0.001$. ⊢⊣=mean±SD.

increases the activity of cholesteryl ester transfer protein, effects consistent with an increase in reverse cholesterol transport.[32] In fact, in subjects with HFH, reduction in the size of xanthomas correlates best with the reduction in plasma HDL-C levels.[30] Probucol also affects the macrophage directly and can reduce cell-mediated oxidation of LDL.[33] Xanthoma regression by probucol may well occur by these mechanisms rather than by antioxidation and this requires further study.

The results of these studies in patients with FH challenge but do not disprove the oxidative hypothesis of atherosclerosis. Although LDL isolated from FH subjects is less susceptible to oxidation in vitro, it is not necessarily less atherogenic in vivo. Currently, it is thought that because of the presence of abundant anti-oxidant defences, LDL particles do not undergo any significant degree of oxidation within the circulation.[34] However, LDL particles are continuously entering and leaving the arterial wall; some of these particles become entrapped in the interstitial matrix, and because of prolonged resident time, may be subjected to sustained oxidative stress.[35] Thus, although initially more resistant, once lipid peroxidation has been initiated, a larger, more cholesterol-enriched LDL, such as that typically found in patients with HFH, may generate more total oxidized lipid within the arterial wall with pathogenic consequences (Fig. 3). In addition LDL size may be important in modifying its metabolism by changing its

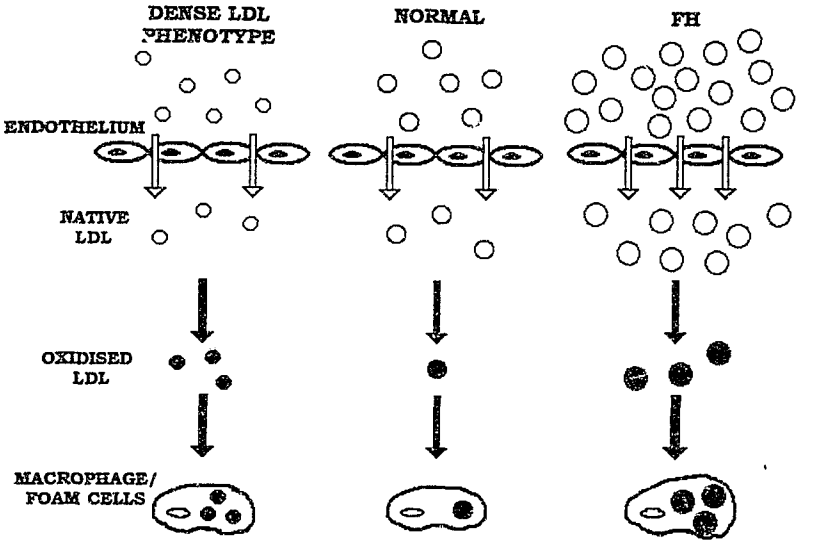

Fig. 3—Lipid oxidation within the arterial wall. Small, dense LDL is more susceptible to oxidation rendering it more atherogenic despite limited arterial influx. Familial hypercholesterolaemic (FH) subjects have larger, more bouyant LDL which is more resistant to oxidatative modification. However, because of greater LDL bulk, the total amount of oxidised LDL generated is greater resulting in foam cell formation.

affinity for the LDL receptor and directing it towards scavenger pathways in the arterial wall. Nigon et al have described that lighter (d=1.024–1.029 g/ml) and denser (d=1.035–1.043 g/ml) fractions of LDL have lower receptor binding affinities than LDL of intermediate density.[36] Decreased binding of large LDL has also been attributed to steric hindrance produced by the crowding of LDL particles on receptor lattices.[37]

LIPID PEROXIDATION IN SUBJECTS WITH ATHEROSCLEROSIS AND DESIRABLE CHOLESTEROL LEVELS

It is well known that only a minority of patients with coronary artery disease (CAD) have LDL concentrations above the 95th percentile, and the frequency distribution of LDL-cholesterol in patients with CAD is not greatly different from that of the general population.[36] Approximately 50% of primary myocardial infarctions occur in subjects with SC<6.5 mmol/L, and 20% afflict those with desirable levels (<5.2 mmol/L).[38]

Quantitative differences in LDL may well play a more important role in this group of patients. Until recently LDL have been considered a single entity. It is now known that LDL exists in multiple discrete forms that can be separated on the basis of particle size and buoyant density.[39] In most individuals LDL can be categorized as either predominantly large and buoyant (pattern A) or small and dense (pattern B). Pattern B, present in approximately 30% of the population, is associated with a more atherogenic type of lipid profile, with increased levels of triglyceride and apoprotein B and lower levels of HDL and apoprotein A1 despite normal or only moderately elevated LDL levels.[40] The dense LDL phenotype is also associated with the insulin resistance syndrome - a cluster of metabolic disorders including glucose intolerance, non-insulin dependent diabetes mellitus, hypertension, and abdominal obesity – which is associated with a high prevelance of coronary artery disease.[41] Interestingly, in the Kaiser Permanente Women Twins Study there was a graded association between the number of manifestations of the insulin resistance syndrome and the prevalence of LDL pattern B.[42]

There is now substantial evidence to show that individuals with small, dense LDL particles are at increased risk for coronary artery disease despite having desirable plasma LDL levels.[43,44] These subjects have a threefold increased risk of myocardial infarction independent of age, sex or relative weight. In addition, the LDL of young male subjects undergoing angiography for coronary

artery disease and that of post-myocardial infarction patients is low in molecular weight and displaced toward the denser part of the LDL density range.[45,46]

The association between dense LDL and atherosclerosis may be due to the greater susceptibility of dense LDL to modifying processes that render it more atherogenic. A progressive increase in susceptibility to peroxidation has been demonstrated with decreasing LDL diameter.[24,25,47] In addition, small dense LDL particles demonstrate reduced affinity for the LDL receptor and bind more avidly to proteoglycans and glycosaminoglycans which may result in their being trapped on extracellular matrix components in the artery wall where they may be vulnerable to oxidative modification.[36,48]

LDL QUANTITY OR QUALITY: A HYPOTHESIS

These studies suggest that in subjects with established atherosclerosis, the more desirable the LDL cholesterol level, the more likely one is to find LDL that is more susceptible to lipid peroxidation. In subjects with markedly elevated LDL levels, such as in FH, susceptibility of LDL to oxidation is less important and LDL bulk takes priority. In these subjects the main aim of therapy remains reduction of LDL quantity.

In subjects with desirable cholesterol levels, qualitative rather than quantitative differences in LDL become increasingly more important. These subjects are more likely to have small dense LDL which is more susceptible to oxidation, and in these subjects antioxidant therapy may well be of greater benefit. However, the role of other biochemical atherogenic factors, such as hyperlipoprotein (a), homocysteinuria and hyperfibrinogenaemia, must also be considered.

In subjects with atherosclerosis, current management should still be aimed primarily at reducing the LDL cholesterol level. As our understanding increases we may be able to add selective antioxidant therapy that will complement the management of hypercholesterolaemia.

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Jenny Pieters is thanked for typing the manuscript. The author's original work (FJR) which has been described in this review forms part of a PHD thesis which is to be submitted to the University of the Witwatersrand. Financial support was provided by the South African Medical Research Council.

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Paper 5

This study shows that, in the absence of other causes of insulin resistance, FH subjects have normal fasting glucose and insulin levels and that they are not insulin resistant. Insulin resistance therefore appears to play little role in the pathogenesis of accelerated atherosclerosis in FH subjects.

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Atherosclerosis seems not to be associated with hyperinsulinaemia in patients with familial hypercholesterolaemia

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Abstract. Raal FJ, Panz VR, Pilcher GJ, Joffe BI. (Carbohydrate and Lipid Metabolism Research Group, Johannesburg, South Africa). Atherosclerosis seems not to be associated with hyperinsulinaemia in patients with familial hypercholesterolaemia. *J Intern Med* 1999; 246: 75–80.

Objective. To study the relationship between hyperinsulinaemia, insulin resistance, leptin and atherosclerosis in subjects with familial hypercholesterolaemia (FH).

Design. Case-control cross-sectional study.

Setting. Lipid clinic, Johannesburg Hospital, South Africa.

Subjects and methods. Fasting serum lipid, glucose, insulin and leptin levels were measured in 24 homozygous FH subjects: 20 FH heterozygotes without coronary artery disease (CAD); 22 heterozygotes with documented CAD; and 20 healthy normocholesterolaemic subjects. Insulin resistance was calculated using the homeostasis model assessment (HOMA) formula.

Results. Mean glucose and insulin levels were similar in all 4 groups. There was no significant difference in calculated insulin resistance between any of the groups. There was also no relationship between the degree of insulin resistance and total or LDL-cholesterol levels. Using Spearman's correlation coefficient (R_s) calculated insulin resistance correlated with triglyceride ($R_s = 0.27$; $P < 0.05$) and inversely with HDL-cholesterol ($R_s = -0.26$; $P < 0.05$). Fasting insulin concentrations and calculated insulin resistance were similar in FH subjects with overt CAD compared to those without. Leptin levels were higher in the FH subjects with CAD. However, these subjects were older and had a larger body mass index (BMI), and when adjusted for age and BMI, only BMI correlated with leptin levels (multiple $r = 0.65$; $P < 0.001$).

Conclusions. In the absence of other causes of insulin resistance, FH subjects have normal fasting insulin levels and, in general, they are not insulin resistant. Insulin resistance appears to play little role in the pathogenesis of accelerated atherosclerosis in FH.

Keywords: atherosclerosis, familial hypercholesterolaemia, insulin resistance, insulin, leptin.

Introduction

It has been suggested that hyperinsulinaemia and insulin resistance may play a pathophysiological role in the atherosclerotic process [1, 2]. Epidemiological prospective studies have shown a positive association between insulin concentrations and the prevalence of coronary artery disease (CAD) [3–5]. Patients with angiographically documented CAD have also been shown to be insulin resistant and have fasting hyperinsulinaemia [6–8]. However, insulin resistance and hyperinsulinaemia often coexist with a cluster of metabolic factors including hypertension, dyslipidaemia, central obesity and impaired fibrinolysis, which are also associated with increased risk of atherosclerosis [9]. It remains uncertain whether it is the insulin resistance *per se* or whether it is its coexistence with these other metabolic abnormalities that promotes atherosclerosis.

Leptin, the product of the *ob* gene, is highly correlated with body fat mass and has been shown

to play a significant role in the regulation of body weight [10]. Several investigators have demonstrated a positive correlation between insulin and leptin levels even after correction for body fat mass [11, 12], suggesting that insulin may play a role in the regulation of leptin production. Insulin has also been shown to upregulate leptin mRNA expression and chronic hyperinsulinaemia increases leptin production in both animals and man [13, 14]. Elevated leptin levels would therefore be expected if atherosclerosis is a chronic hyperinsulinaemic state.

Familial hypercholesterolaemia (FH) is an inherited disorder caused by mutations in the low-density lipoprotein (LDL)-receptor which leads to diminished removal of cholesterol from the circulation and, consequently, to markedly elevated LDL-cholesterol levels [15]. The resultant hypercholesterolaemia predisposes these patients to severe premature atherosclerosis, particularly CAD. Individuals with heterozygous FH usually present with CAD in the third to fifth decade whereas those with homozygous FH often die from accelerated atherosclerosis before the age of 30 years [15]. If atherosclerosis is an insulin-resistant state, patients with FH should be markedly insulin resistant, have hyperinsulinaemia and have elevated leptin levels independent of body mass.

In this study we evaluated fasting glucose, insulin, and leptin concentrations, as well as insulin resistance in patients with homozygous and heterozygous FH with and without overt CAD, and compared them to a group of healthy normocholesterolaemic subjects with no evidence of cardiovascular disease.

Subjects and methods

Subjects

Patients with FH were recruited from the lipid clinic at the Johannesburg Hospital. They comprised 24 homozygous FH patients (12 M, 12 F, mean age ± SD = 24 ± 9 years; mean body mass index [BMI] ± SD = 23.3 ± 5.0 kg m^{-2}) of whom 8 had CAD, 20 heterozygous patients without documented CAD (9 M, 11 F; mean age 39 ± 11 years; mean BMI 25.6 ± 3.8 kg m^{-2}), and 22 heterozygotes with CAD (13 M, 9 F; mean age 55 ± 12 years; mean BMI 26.1 ± 4.5 kg m^{-2}). In addition, 20 healthy subjects (10 M, 10 F; mean age 28 ± 5 years; mean BMI 22.3 ± 2.3 kg m^{-2}) were randomly selected from the staff of the University of the Witwatersrand.

The diagnosis of homozygous FH was based on (i) serum total cholesterol levels consistently > 15 mmol L^{-1}; (ii) the appearance of xanthomas in the first decade of life; (iii) documentation in both parents of hypercholesterolaemia or clinical signs indicative of the heterozygous state; and (iv) confirmation by DNA analysis of FH LDL-receptor mutations common in South Africa [16]. Diagnosis of heterozygous FH was based on the presence of a family history of hypercholesterolaemia, clinical signs of FH, together with an elevated serum total cholesterol level, and confirmation by DNA analysis. CAD was considered to be present if the subject had suffered a definite myocardial infarction, had undergone coronary artery bypass surgery or coronary angioplasty or had angiographic evidence of coronary atherosclerosis. All FH patients had been advised to adhere to a standard low-cholesterol, low-saturated-fat diet. The homozygous FH patients and heterozygous FH patients without overt CAD were not on any lipid-lowering medication at the time of blood sampling. None of the patients were receiving LDL-apheresis. The FH heterozygous patients with CAD were all being treated with HMG-CoA reductase inhibitors (statins) as it was considered unethical to stop lipid-lowering medication in this group. None of the patients had a history of hypertension or diabetes mellitus and none were receiving medication such as beta-blockers or thiazide diuretics, which are known to aggravate insulin resistance. Twenty healthy normocholesterolaemic subjects with no history of hypercholesterolaemia or CAD were used as controls. These subjects were age-, sex- and weight-matched to the FH homozygotes, the group that was expected to have the most severe atherosclerosis. All subjects gave informed consent to participate in the study which was approved by the committee for research on human subjects of the University of the Witwatersrand.

Methods

Venous blood samples were taken after an overnight fast of at least 10 h. The samples were centrifuged and the separated serum aliquots were stored at −70°C until analysed. Radioimmunoassay kits were

used to measure leptin (Linco Research, St. Charles, USA) and insulin (Pharmacia AB, Uppsala, Sweden). The minimal detectable dose of insulin was 3.0 mU L^{-1}. Glucose was measured by the glucose oxidase method and enzymatic, colourimetric methods were used to measure total cholesterol, high-density lipoprotein (HDL)-cholesterol and triglycerides, employing a Hitachi autoanalyser and reagents supplied by Boehringer Mannheim, Mannheim, Germany. Intra-assay variation for each of these assays was < 5%. LDL-cholesterol values were calculated according to the formula of Friedewald *et al.* [17]. Although the method of choice to estimate insulin resistance is the hyperinsulinaemic euglycaemic clamp [18], the homeostasis model assessment (HOMA) has been strongly correlated with independent measures of insulin resistance using this method [19]. Therefore, the HOMA model was used to calculate insulin resistance in the patients in this study from their fasting glucose and insulin concentrations. In nondiabetic subjects, insulin levels generally reflect the degree of insulin resistance [20].

Statistical analysis

Comparisons were made by one-way analysis of variance followed by Student's *t*-test with the Bonferroni correction. The Kruskal–Wallis one-way analysis of variance and the Wilcoxon's rank sum test were used for nonparametric data where appropriate. Spearman's correlation coefficient (R_s) was computed between selected variables using the GraphPad PrismTM program (GraphPad Software Inc., San Diego, USA). A value of $P < 0.05$ was considered significant. Results are expressed as mean ± SD.

Results

Mean age and weight were similar in the FH homozygotes and healthy subjects. The FH heterozygotes with and without CAD were older and had a larger BMI. Fasting serum biochemical concentrations of the 4 groups are shown in Table 1. As expected, mean total cholesterol and LDL-cholesterol concentrations were significantly higher in the FH homozygotes than in the heterozygotes both with and without CAD, which were in turn higher than the healthy subjects ($P < 0.001$). The lower total and LDL-cholesterol concentrations found in the heterozygous FH patients with CAD compared with those without CAD were probably a result of the lipid-lowering therapy in this group. HDL-cholesterol value was significantly lower in the FH homozygotes ($P < 0.01$). Triglyceride levels were similar in all 4 groups.

Interestingly, there was no significant difference in fasting insulin concentrations or calculated insulin resistance between any of the groups. There was also no relationship between the degree of insulin resistance and total or LDL-cholesterol levels. Calculated insulin resistance did, however, correlate with fasting triglyceride ($R_s = 0.27$; $P < 0.05$) and

Table 1 Fasting serum biochemical concentrations in familial hypercholesterolaemic patients with and without coronary artery disease, and healthy subjects

	Healthy subjects (n = 20)	FH homozygotes (n = 24; CAD = 8)	FH heterozygotes without CAD (n = 20)	FH heterozygotes with CAD (n = 22)
Total cholesterol (mmol L^{-1})	4.7 ± 0.7	16.0 ± 3.0**	9.9 ± 1.3**	7.0 ± 1.6**
HDL-cholesterol (mmol L^{-1})	1.4 ± 0.4	0.7 ± 0.3*	1.2 ± 0.3	1.1 ± 0.3
LDL-cholesterol (mmol L^{-1})	2.7 ± 0.7	14.5 ± 2.9**	8.0 ± 1.3**	5.0 ± 1.5**
Triglyceride (mmol L^{-1})	1.2 ± 0.6	1.5 ± 0.8	1.6 ± 0.8	2.0 ± 1.0
Glucose (mmol L^{-1})	4.6 ± 0.7	4.7 ± 0.6	4.8 ± 0.4	5.1 ± 0.6
Insulin (mU L^{-1})	10.5 ± 3.5	11.8 ± 3.9	10.4 ± 4.3	12.0 ± 5.6
Leptin (mg L^{-1})	7.7 ± 6.4	11.8 ± 14.6	13.3 ± 11.4	15.9 ± 14.9
Insulin resistance (calculated)	2.1 ± 0.8	2.5 ± 1.0	2.3 ± 1.1	2.7 ± 1.4

Data are Mean ± SD; FH, Familial Hypercholesterolaemia; CAD, Coronary Artery Disease.
*$P < 0.01$; **$P < 0.001$ compared to healthy subjects

inversely with HDL-cholesterol ($R_s = -0.26$: $P < 0.05$). There was also no difference in fasting insulin concentrations or insulin resistance in those subjects with overt CAD compared to those without.

Based on gender, mean leptin values were within the reference range determined in our laboratory ($M = 3.1 \pm 1.9$; $F = 10.6 \pm 3.8$ $\mu g\ L^{-1}$) for the healthy subjects. FH homozygotes and FH heterozygotes without CAD. Leptin levels for the FH heterozygous with CAD were higher ($M = 9.1 \pm 5.4$; $F = 25.9 \pm 18.9$ $\mu g\ L^{-1}$) but these subjects were older and had a larger BMI. When simple correlations were performed, leptin correlated significantly with BMI ($r = 0.59$: $P < 0.01$), and calculated insulin resistance ($r = 0.36$: $P < 0.01$) (Fig. 1), as well as with age ($r = 0.26$: $P < 0.02$), but not with total cholesterol or LDL-cholesterol ($r = 0.06$: $P = 0.61$). However, using multiple stepwise regression analysis to adjust for insulin resistance and age, their significance fell away and only BMI remained significantly correlated with leptin (multiple $r = 0.65$: $P < 0.001$).

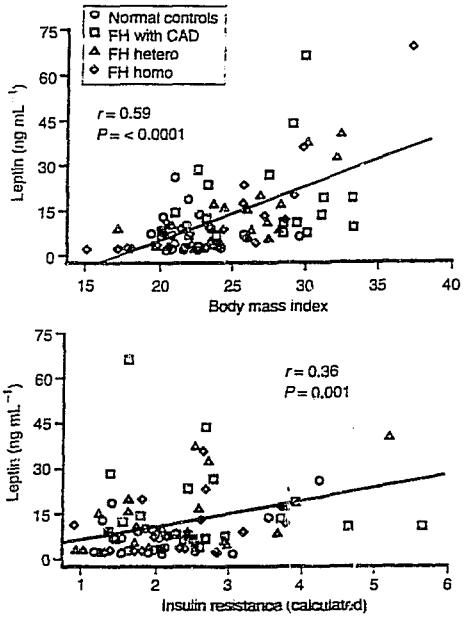

Fig. 1 Scatter plots showing the correlations between leptin, body mass index and insulin resistance.

Discussion

There has been much controversy about whether hyperinsulinaemia and insulin resistance are independent risk factors for atherosclerosis. Earlier prospective studies identified hyperinsulinaemia as a risk factor for CAD [3–5]. More recently, Després *et al.* [21], in a large prospective study, reported an association between insulin and CAD independent of other known cardiovascular risk factors. Fasting hyperinsulinaemia and insulin resistance also have a positive correlation with atherosclerosis as assessed by carotid ultrasonography [22, 23] or coronary angiography [6–8]. Not all studies have confirmed this association, however, and a recent meta-analysis did not support the notion that hyperinsulinaemia is a major risk factor for atherosclerosis [24].

Insulin resistance and hyperinsulinaemia often coexist with a cluster of metabolic factors referred to as the metabolic syndrome or 'syndrome X' [1, 25]. Numerous studies have now confirmed the increased risk for atherosclerosis in patients with this syndrome [26]. However, although subjects with insulin resistance and the metabolic syndrome may be predisposed to atherosclerosis, the converse is not necessarily true.

Previous studies in subjects with isolated hypercholesterolaemia have been contradictory. A study by Paolisso *et al.* [27] showed patients with hypercholesterolaemia to be insulin resistant. Other studies in such patients have demonstrated normal resistance to insulin as measured by the hyperinsulinaemic euglycaemic clamp technique [28–30]. However, these studies did not include subjects with homozygous FH who are known to suffer from the most severe, accelerated atherosclerosis [15]. We therefore studied subjects with both homozygous and heterozygous FH, both with and without CAD, who had isolated severe hypercholesterolaemia. Our study demonstrates that these subjects have normal fasting insulin levels and that they are not insulin resistant as assessed by the HOMA model, supporting the notion that isolated hypercholesterolaemia is not an insulin-resistant state [31]. It should be noted, however, that the HOMA model is limited by the precision of the insulin assay, making it difficult to produce precise estimates for individual subjects taken from a single fasting sample. Therefore, HOMA estimates should

be considered as a relative rather than an absolute value for insulin resistance.

There was also no relationship between insulin resistance and total or LDL-cholesterol levels as shown by others [32]. This suggests that in subjects with the metabolic syndrome, hyperinsulinaemia and insulin resistance are more of a marker for a cluster of metabolic abnormalities including hypertension, hyper-triglyceridaemia, low HDL-cholesterol levels, enhanced postprandial lipaemia, a preponderance of small, dense LDL particles, as well as impaired fibrinolysis associated with increased risk for atherosclerosis and CAD, rather than being a marker of atherosclerosis *per se* [33]. FH subjects, on the other hand, have normal or only mildly elevated triglyceride levels, normal postprandial lipaemia [34] and large, rather than small, LDL particles [35], and thus have few, if any, features of the metabolic syndrome. This is also supported by the findings in a previous study in which we found no association between microalbuminuria, another proposed marker of the metabolic syndrome which is also considered to be a predictor of cardiovascular disease, and atherosclerosis in subjects with homozygous FH [36]. Our study also shows that leptin levels are not influenced by the degree of hyperlipidaemia, and that BMI is also the major determinant of leptin levels in FH patients.

In conclusion, in the absence of other causes of insulin resistance, patients with FH have normal fasting insulin levels and, in general, they are not insulin resistant. Insulin resistance appears to play little role in the pathogenesis of accelerated atherosclerosis in FH. Reduction of LDL-cholesterol levels, on the other hand, has been shown to induce regression of coronary atherosclerosis in patients with heterozygous FH [37]. Recent studies have also demonstrated a reduction in coronary events and improved survival with lipid-lowering therapy in subjects with hypercholesterolaemia [38, 39]. The most important therapy for FH patients is therefore reduction of LDL-cholesterol levels.

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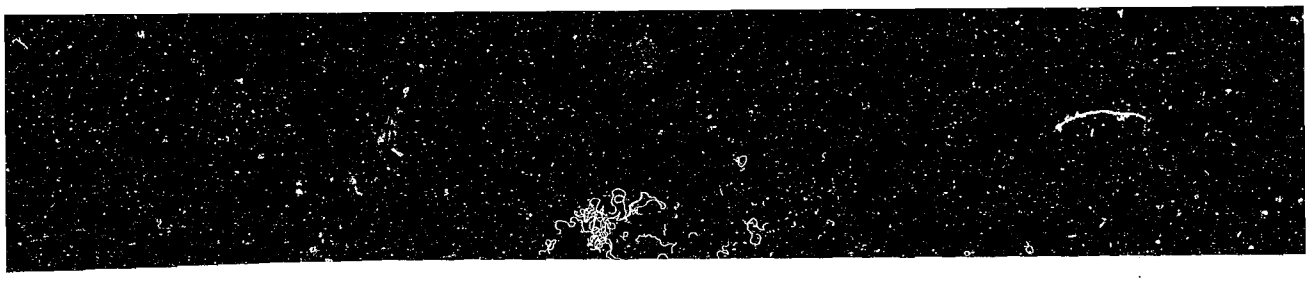

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Paper 6

This study looked at the association between microalbuminuria, another proposed component of the metabolic syndrome, and atherosclerosis in subjects with homozygous FH. The homozygous FH subjects studied did not have microalbuminuria and no association was found between microalbuminuria and overt CAD in these subjects.

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Atherosclerosis 113 (1995) 289–292

ATHEROSCLEROSIS

Short communication

Microalbuminuria is not associated with cardiovascular disease in patients with homozygous familial hypercholesterolaemia

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Abstract

Microalbuminuria is thought to be a predictor of cardiovascular disease (CVD). A high prevalence of microalbuminuria might therefore be expected in patients with homozygous familial hypercholesterolaemia (HFH), as they develop severe premature atherosclerosis. We tested for this in 15 HFH patients (M = 9, F = 6; mean age 19.3 years). In addition, there was no difference in the mean urinary albumin excretion rate (UAER) between those with documented CVD ($n = 8$; UAER = 5.17 μg/min) and those without ($n = 7$; UAER = 3.60 μg/min). There is therefore no association between microalbuminuria and CVD in HFH.

Keywords: Microalbuminuria; Familial hypercholesterolaemia; Coronary artery disease

1. Introduction

Microalbuminuria, defined as urinary albumin excretion rate (UAER) of 20–200 μg/min, is a strong predictor of cardiovascular morbidity and mortality in both type I and type II diabetics [1,2]. Microalbuminuria has also been positively linked to cardiovascular disease (CVD) in hypertensives as well as in normotensive elderly subjects [3,4]. Thus microalbuminuria could reflect the presence of atherosclerotic vascular disease in general [5].

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Patients with homozygous familial hypercholesterolaemia (HFH) develop severe, premature atherosclerosis, the majority dying from coronary artery disease before the age of 30 years [6]. If microalbuminuria is a marker of CVD, one would expect a high prevalence of microalbuminuria in these patients.

2. Patients and methods

Patients with HFH attending the Lipid Clinic at the Johannesburg Hospital were asked to participate in the study. No age restriction was applied, as these patients often develop atherosclerosis very early in life [6]. The diagnosis of HFH was based

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Table 1
Clinical and biochemical characteristics

Rec	Sex	Age (years)	Genotype	BMI (kg/m^2)	Lipogram (mmol/l)				Alb/creat (mg/mmol)	UAER (μg/min)
					TC	TG	LDL-C	HDL-C		
Proven CVD										
1	M	17	Afrik 1/2	23.50	17.59	0.77	16.52	0.72	0.33	3.06
2	M	31	Afrik 1/3	23.10	18.40	0.62	17.24	0.88	0.95	14.30
3	M	35	Afrik 1/1	21.60	17.62	1.37	16.30	0.74	0.71	7.33
4	M	16	Afrik 1/1	20.80	17.28	1.02	16.00	0.65	0.11	1.33
5	F	34	Afrik 2/2	22.70	17.80	1.23	16.30	0.90	0.31	3.89
6	F	16	Afrik 1/1	18.80	17.22	0.68	16.25	0.88	0.70	3.80
7	F	19	Afrik 2/2	24.80	15.40	0.59	14.40	0.71	1.07	6.90
8	M	24	Afrik 1/1	21.30	16.00	0.68	14.60	1.09	0.12	0.81
Mean		24		22.07	17.16	0.87	15.95	0.82	0.54	5.17
(S.D.)		(8.21)		(1.85)	(0.98)	(0.29)	(0.96)	(0.15)	(0.37)	(4.35)
Unproven CVD										
1	F	17	Afrik 1/1	23.50	16.40	1.05	15.44	0.48	1.39	13.38
2	F	9	Afrik 1/?	15.60	17.18	0.86	16.30	0.52	0.14	0.67
3	M	7	Afrik 1/?	15.50	16.82	1.64	15.60	0.44	1.56	2.44
4	M	3	Afrik 2/2	14.80	27.98	0.61	27.00	0.46	1.11	2.22
5	F	31	Afrik 1/2	28.00	13.26	0.32	12.40	0.70	0.32	2.03
6	M	11	FH 664	15.50	17.80	1.00	16.80	0.50	0.31	1.26
7	M 1	7	Afrik 1/3	22.90	15.98	0.92	14.90	0.66	0.32	3.24
Mean		13.57		19.40	17.91	0 91	16.92	0.53	0.73	3.60
(S.D.)		(9.21)		(5.30)	(4.66)	(0.40)	(4.66)	(0.11)	(0.59)	(4.38)
P		<0.05		NS					NS	NS

CVD, cardiovascular disease; BMI, body mass index; TC, total cholesterol; TG, triglycerides; LDL-C, low density lipoprotein-cholesterol; HDL-C, high density lipoprotein-cholesterol; S.D., standard deviation; Alb/creat, albumin/creatinine ratio; UAER, urinary albumin excretion rate.

on the presence of serum total cholesterol levels consistently greater than 12 mmol/l, the appearance of xanthomas in the first decade of life and the documentation in both parents of hypercholesterolaemia or clinical signs indicative of the heterozygous state. The diagnosis was confirmed by DNA analysis of familial hypercholesterolaemia LDL receptor mutations common in South Africa.

The patients were asked to provide 24 h urine collections, which were screened for infection and proteinuria with commercial strips (Combur 9, Boehringer Mannheim) and subsequently stored at −70°C until assayed. All patients were advised to avoid strenuous exercise 24 h prior to and during collection of the urine. None of the patients had evidence of cardiac failure, hypertension, diabetes, renal or liver disease, and none were on lipid lowering medication at the time of the study.

Urinary albumin concentration was measured by means of an immunoturbidimetric method (Boehringer Mannheim, sensitivity 3–400 mg/l), using a BM-Hitachi analyser. Urine creatinine was measured by the Jaffé method. Results in those with proven CVD were compared to those with no, or unknown CVD status using the students *t*-test for normally distributed data or the Wilcoxon test for non-parametric data.

3. Results

Fifteen patients, 9 males and 6 females, agreed to participate in the study (see Table 1). Twelve patients were homozygous or compound heterozygous for FH Afrikaner-1, -2, or -3. These three founder-type mutations together account for more than 80% of FH in Afrikaners [7]. Two subjects fulfilled the clinical criteria for HFH but were heterozygous for the common FH Afrikaner mutations. One patient was homozygous for the FH 664 mutation. The mean age of the patients was 19.3 years (range 3–35); and their body mass index 20.7 kg/m^2 (range 13.2–28.0). Eight of the patients had definite coronary artery disease demonstrated on coronary angiography, or had undergone previous coronary artery bypass surgery.

Microalbuminuria, defined as UAER of greater than 20 μg/min or an albumin creatinine ratio of 3 mg/mmol, was absent in all subjects (see Table 1). There was also no difference in UAER in those patients with CVD compared to those with unproven CVD. The only significant difference was the older age of the former (24.0 years vs. 13.6 years, $P < 0.05$).

4. Discussion

The results of this study show that there is no association between microalbuminuria and CVD in HFH. However, several investigators have reported a definite association between microalbuminuria and cardiovascular risk, both in diabetic and in non-diabetic subjects [1–5]. The reason why microalbuminuria predicts cardiovascular disease in these patients remains unclear. However, if one examines the conditions in which microalbuminuria and CVD coexist, it is apparent that the majority of them are insulin resistant states [8]. Familial hypercholesterolaemia, on the other hand, is associated with normal insulin sensitivity [9,10]. It therefore appears that the so called 'metabolic syndrome' characterised by the cluster of insulin resistance, central obesity, elevation of the serum triglycerides, decreased HDL cholesterol, and hypertension occurring in both diabetic and non-diabetic subjects can affect both the glomerulus and the vasculature leading to CVD and microalbuminuria. However, other causes of CVD, including HFH, in which insulin resistance does not occur, affect only the vasculature leaving the glomerulus intact. Therefore cardiovascular disease is not associated with microalbuminuria in hypercholesterolaemic insulin sensitive states.

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ATHEROSCLEROSIS

Corrigendum

Corrigendum to "Microalbuminuria is not associated with cardiovascular disease in patients with homozygous familial hypercholesterolaemia: Short communication" [Atherosclerosis 113 (1995) 289]☆

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Unfortunately the sentence "Urinary albumin excretion rates (UAER) were normal in all patients." was omitted from the abstract. The abstract should read:

Abstract

Microalbuminuria is thought to be a predictor of cardiovascular disease (CVD). A high prevalence of microalbuminuria might therefore be expected in patients with homozygous familial hypercholesterolaemia (HFH), as they develop severe premature atherosclerosis. We tested for this in 15 HFH patients (M = 9, F = 6; mean age 19.3 years). Urinary albumin excretion rates (UAER) were normal in all patients. In addition, there was no difference in the mean UAER between those with documented CVD ($n = 8$; UAER = 5.17 μg/min) and those without ($n = 7$; UAER = 3.60 μg/min). There is therefore no association between microalbuminuria and CVD in HFH.

Keywords: Microalbuminuria: Familial hypercholesterolaemia: Coronary artery disease

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Paper 7

The aim of this study was to determine whether biochemical risk factors such as lipoprotein(a), fibrinogen, homocysteine and insulin as well as LDL particle size were predictive of carotid artery intima-media thickness, an early marker of atherosclerosis, in FH subjects. The LDL-C level and duration of exposure to elevated LDL-C levels were found to be pivotal determinants of atherosclerosis and were the strongest predictors of carotid intima-media thickness in FH. Other biochemical risk factors measured, including LDL particle size, were of little added predictive value.

Low-Density Lipoprotein Cholesterol Bulk Is the Pivotal Determinant of Atherosclerosis in Familial Hypercholesterolemia

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This study's aim was to determine whether biochemical risk factors such as lipoprotein(a), fibrinogen, homocysteine, and insulin, as well as low-density lipoprotein (LDL) particle size, were predictive of carotid intima-media thickness (IMT), an early marker of atherosclerosis, in subjects with familial hypercholesterolemia (FH). We also determined whether plasma 8-isoprostane, as a marker of in vivo lipid oxidation, correlated with carotid IMT. Twenty-two homozygous and 20 heterozygous subjects with FH were compared with 20 normocholesterolemic controls. On univariate analysis, plasma total and LDL cholesterol, the cholesterol-years score (CYS), lipoprotein(a), and fibrinogen, but not homocysteine or insulin, were positively related, and high-density lipoprotein (HDL) cholesterol was negatively related to carotid IMT. However, on multivariate analysis, only LDL cholesterol and the CYS predicted carotid IMT (multiple $r = 0.82$; $r^2 = 0.68$; $p < 0.0001$). The subjects with FH had large rather than small dense LDL particles, and plasma 8-isoprostane levels were not increased. LDL cholesterol and the CYS, or "cholesterol bulk" are the pivotal determinants of atherosclerosis and are the strongest predictors of carotid IMT in FH. ©1999 by Excerpta Medica, Inc.

(Am J Cardiol 1999;83:1330–1333)

The purpose of this study was to determine whether biochemical risk factors that have been associated with coronary artery disease, namely, lipoprotein(a), fibrinogen, homocysteine, and insulin, as well as low density lipoprotein (LDL) particle size, are predictive of atherosclerosis in subjects with familial hypercholesterolemia (FH). Furthermore, we aimed to determine whether 8-isoprostane levels, as a measure of in vivo lipid oxidation, are increased in patients with FH. We evaluated carotid intima-media complex thickness (IMT) as a marker of atherosclerosis because thickening of the carotid IMT has been shown to be an early step in the atherosclerotic process.[1,2]

METHODS

Study subjects: Twenty-two homozygous and 20 heterozygous patients with FH were recruited from the Lipid Clinic at the Johannesburg Hospital. Twenty healthy normocholesterolemic subjects with no family history of hypercholesterolemia or coronary artery disease served as controls. The diagnosis of homozygous FH was based on the presence of (1) serum LDL cholesterol consistently >12 mmol/L; (2) the appearance of xanthomas in the first decade of life; and (3) documentation in both parents of hypercholesterolemia or clinical features of the heterozygous state. Eighteen of the 22 homozygote patients were confirmed to have mutations in both alleles of the LDL-receptor gene on DNA analysis. The remaining 4 subjects fulfilled all the clinical criteria for the diagnosis of homozygous FH. Heterozygous FH was diagnosed in the presence of primary hypercholesterolemia (total cholesterol >7.5 mmol/L) with an autosomal dominant pattern of inheritance, and with tendon xanthomata or premature coronary artery disease in the patients or their first-degree relatives. Fourteen of the 20 heterozygote patients had proven LDL-receptor gene mutations.

To avoid confounding effects of other established coronary artery disease risk factors, subjects were excluded from the study if they smoked cigarettes, had hypertension or diabetes mellitus. Height and body weight were recorded to calculate the body mass index. Coronary artery disease was diagnosed on the results of coronary angiography; history of a myocardial infarction, coronary angioplasty or coronary artery bypass grafting; or on the basis of a positive stress electrocardiogram.

To avoid the possible effects of long-standing lipid-lowering therapy on the measured parameters, the heterozygote patients with FH recruited for the study had been recently diagnosed, or had been on lipid-lowering therapy for a short period of time (mean 2 years; range 0 to 6). The homozygote FH patients had previously been treated with antioxidant therapy (vitamin E or probucol) and more recently with high-dose statin therapy (simvastatin or atorvastatin 80 to 160 mg/day for a mean of 1 year; range 0 to 2). No

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patient with FH was receiving LDL-apheresis at the time of the study, because this therapy was not available at our institution.

Lipid-lowering medication was discontinued for a 4-week period before blood sampling to avoid the possible effects of lipid-lowering therapy on the measured biochemical parameters. In addition, to account for the variable period of time the patients were on treatment directed at lowering their cholesterol level, a cholesterol-years score (CYS) was calculated using the formulas of Hoeg et al.[3] The CYS is an estimation of the lifetime vascular exposure to hypercholesterolemia and has been correlated with carotid IMT in subjects with FH.[4] The total cholesterol concentration (mmol/L) of each subject at the time of diagnosis was multiplied by the age of the patient at diagnosis. The average cholesterol concentration present after the introduction of lipid-lowering drug therapy was then multiplied by the number of years of treatment. The pre- and post-treatment cholesterol years (mmol/L × years) were then added together for the total CYS.

All subjects gave informed consent to participate in the study, which was approved by the University of the Witwatersrand's Committee for Research on Human Subjects.

Measurement of carotid intima-media thickness: The IMT thickness of the common carotid artery was recorded using a high resolution 5-MHz linear array transducer (Advanced Technologies Laboratory Ultramark 9, Bothel, Washington) using a previous validated technique.[5] Coefficient of variation of interobserver variation was 9.8% (95% confidence interval [CI] ± 0.17 mm; r = 0.79) of observed thickness. Coefficient of variation of intraobserver variation was 6.7% (±0.08 mm; r = 0.84) of observed thickness. Maximum theoretical resolution of 5-MHz wavelength in tissue is 0.15 mm. All ultrasonographic assessments were performed on the same machine by 2 trained technicians.

The distance between the echo arising from the lumen-intima interface and the media-adventitia interface was taken as the measurement of the IMT complex.[6] The same observer (I.I.V.) measured carotid IMT thickness in all subjects. He was blinded to their clinical history, risk factor profile, or previous carotid ultrasound findings.

Laboratory procedures: Blood samples for analysis were drawn in the morning after an overnight fast of ≥10 hours. Samples were immediately centrifuged and the separated serum aliquots were either analyzed immediately or frozen at −70°C until analyzed. Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides were analyzed by enzymatic methods using automated techniques. The LDL cholesterol was calculated using the Friedewald formula.[7] Lipoprotein(a) was quantitated using the Tinaquant method (Boehringer Mannheim, Germany). LDL particle diameters were determined for whole plasma by electrophoresis on nondenaturing 2% to 16% polyacrylamide gradient gels according to the procedure described by Krauss and Burke.[8] An LDL peak particle diameter of <25.5 nm was used to define small dense LDL (pattern B). Plasma levels of free 8-isoprostane were measured by enzyme immunoassay (Cayman Chemical Company, Ann Arbor, Michigan). Plasma samples were purified by solid-phase extraction before analysis and all samples were run in duplicate. This method has been validated by comparison with gas chromatography/mass spectrometry.[9] The intra- and interassay variation of this method was <10%. Citrated plasma was used to measure fibrinogen according to the thrombin time method described by Clauss.[10] Plasma total homocysteine was measured by high performance liquid chromatography using a previously described method.[11] Glucose was measured by the glucose oxidase method and insulin by means of an immunoenzymetric assay (Biosource, Europe S.A., Fleurus, Belgium).

Statistical analysis: Comparisons were performed using 1-way analysis of variance followed by Student's *t* tests with the Bonferroni correction. The Kruskal-Wallis 1-way analysis of variance and the Mann-Whitney U test were used for nonparametric data where appropriate. Fisher's exact test was used for the categorical variables of gender and coronary artery disease. Simple (Pearson's) correlation coefficients between carotid IMT and selected variables were calculated, and a stepwise multiple regression analysis was then used to evaluate the independent association of these variables with carotid IMT. Analyses were performed using GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, Maryland). In all comparisons, $p < 0.05$ was considered significant.

TABLE I Clinical Characteristics and Lipoprotein Concentrations of Patients With Familial Hypercholesterolemia (FH) and Healthy Controls

Characteristic	Controls (n = 20)	Heterozygous FH (n = 20)	Homozygous FH (n = 22)
Age (yrs)	31 ± 4.2	32 ± 12.4	24 ± 10.1*
Men/women	12/8	9/11	11/11
Body mass index (kg/m²)	22.5 ± 1.9	24.6 ± 4.4	23.2 ± 4.9
CYS (mmol/L × yrs)	153 ± 33	316 ± 154	392 ± 184†
Total cholesterol	4.98 ± 0.9 (193 ± 33)	7.98 ± 1.8 (309 ± 68)	15.0 ± 4.4† (581 ± 169)
LDL cholesterol	3.20 ± 0.8 (124 ± 29)	6.29 ± 1.7† (243 ± 67)	13.6 ± 4.4† (526 ± 167)
Triglycerides	1.24 ± 0.6 (110 ± 56)	1.31 ± 0.6 116 ± 57)	1.27 ± 0.6 (112 ± 49)
HDL cholesterol	1.21 ± 0.5 (47 ± 18)	1.09 ± 0.3 (42 ± 13)	0.76 ± 0.2† (30 ± 8)
Lipoprotein(a) (mg/dl)	30 ± 21	38 ± 30	85 ± 71†

Lipid and lipoprotein values are expressed as mmol/L, with mg/dl in parentheses.
Values are mean ± SD.
*p <0.05; †p <0.01 compared with controls.

RESULTS

General characteristics and plasma lipoprotein concentrations of the 3 study groups are shown in Table I. The mean age of the FH homozygotes was less than that of the heterozygotes and control subjects; however, despite this, 8 of the homozygotes (36%) had

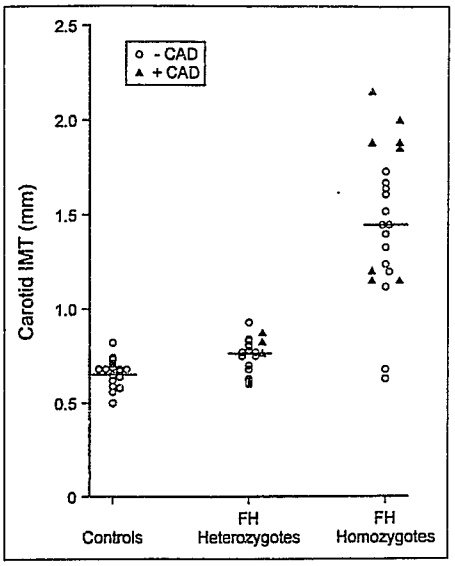

FIGURE 1. Scatter graph showing individual and mean carotid IMT in patients with FH with (+CAD) and without (−CAD) coronary artery disease and in healthy controls.

established coronary artery disease compared with only 3 of the heterozygotes (15%).

Mean carotid IMT was markedly increased in the FH homozygotes (1.45 ± 0.4 mm) compared with the FH heterozygotes (0.76 ± 0.1 mm; p <0.0001), which in turn was increased compared with the control subjects (0.65 ± 0.7; p <0.001) (Figure 1). Carotid IMT was greater in the subjects with FH with coronary artery disease (1.43 ± 0.5 mm) compared with those without (1.01 ± 0.4 mm), but the subjects with coronary artery disease were significantly older (34 ± 9 vs 26 ± 12 years; p = 0.02). As expected, mean fasting plasma total and LDL cholesterol concentrations were higher in the FH homozygotes than in the heterozygotes, which in turn were higher than the controls. The mean CYS was also higher in the patients with FH. HDL cholesterol levels were lower in the FH homozygotes but triglyceride levels were similar in all 3 groups. Lipoprotein(a) levels were higher in the FH homozygous subjects.

Biochemical parameters of the 3 groups are shown in Table II. Fibrinogen levels were higher in the homozygote FH patients. Fibrinogen levels, however, were similar in the 11 patients with FH with coronary artery disease (3.7 ± 0.6 g/L) compared with those without (3.5 ± 0.8 g/L; p = 0.57). Surprisingly, homocysteine levels were lower, rather than higher, in the subjects with FH but not significantly so. There were no differences between the groups in fasting insulin or glucose levels. Insulin levels were also similar in the subjects with FH subjects coronary artery disease (9.5 ± 6 mU/L) compared with those

TABLE II Biochemical Parameters of Patients With Familial Hypercholesterolemia (FH) and Healthy Controls

Biochemical Parameter	Controls (n = 20)	Heterozygous FH (n = 20)	Homozygous FH (n = 22)
Fibrinogen (g/L)	2.9 ± 0.7	3.4 ± 0.5	3.8 ± 0.9*
Homocysteine (μmol/L)	9.3 ± 3.3	8.7 ± 4.4	7.3 ± 2.1
Insulin (mU/L)	9.7 ± 8.5	9.5 ± 5.3	7.4 ± 5.0
Glucose (mmol/L)	4.9 ± 0.5	5.0 ± 0.5	4.8 ± 0.5
LDL particle size (nm)	25.7 ± 0.4	26.0 ± 0.4	26.5 ± 0.4*
8-isoprostane (pg/ml)	75 ± 22	67 ± 30	72 ± 29

Values are mean ± SD.
*p <0.01 compared with controls.

TABLE III Partial Correlations of Clinical and Biochemical Parameters With Carotid Intima-media Thickness in Both the Patients With Familial Hypercholesterolemia (FH) and Control Subjects

Variable	Partial Regression Coefficient	t Value	p Value
Age	0.005	0.91	0.37
Body mass index	−0.006	−0.43	0.67
CYS	0.002	4.80	<0.0001
LDL cholesterol	0.057	5.48	<0.0001
Triglycerides	−0.031	−0.41	0.68
HDL cholesterol	−0.132	−1.14	0.26
Lipoprotein(a)	−0.063	−0.58	0.56
Fibrinogen	0.015	0.26	0.80
Homocysteine	0.002	0.13	0.90
Insulin	0.007	1.04	0.31
Glucose	−0.021	−0.26	0.80
LDL particle size	0.170	1.83	0.07
8-isoprostane	−0.002	−1.12	0.27

Multiple r = 0.82; r^2 = 0.68.

without (8.0 ± 5 mU/L; p = 0.49). Interestingly, the patients with FH had a greater LDL peak particle diameter than the control subjects. None of the FH homozygote subjects and only 2 of the 20 FH heterozygotes (10%) had small, dense LDL particles compared with 20% of the controls. Plasma 8-isoprostane levels were not elevated in the subjects with FH. Isoprostane levels were also not increased in the subjects with FH with coronary artery disease (65 ± 32 pg/ml vs 71 ± 28 pg/ml; p = 0.62).

When simple correlations were performed, carotid IMT was positively related to total cholesterol (r = 0.78; p <0.0001), LDL cholesterol (r = 0.79; p <0.0001), CYS (r = 0.57; p <0.0001), lipoprotein(a) (r = 0.36; p = 0.004), fibrinogen (r = 0.38; p = 0.003), and LDL peak particle size (r = 0.52; p <0.0001), and negatively to HDL cholesterol (r = −0.43; p = 0.0004). However, on stepwise multiple regression analysis, only LDL cholesterol and CYS remained significantly correlated with carotid IMT (Table III). The plasma level of LDL cholesterol and CYS were the strongest predictors of carotid IMT and explained 68% of the variation in carotid IMT.

DISCUSSION

Like others, we found high lipoprotein(a) levels in our patients with FH.[12] Lipoprotein(a) appears to be

an independent risk factor for atherosclerosis[13] and has been positively associated with carotid IMT.[14] We found a correlation between lipoprotein(a) levels and carotid IMT on univariate but not on multivariate analysis, suggesting that lipoprotein(a) is associated with atherosclerosis only in the presence of elevated LDL cholesterol levels, as has been described by others.[13] Levels of homocysteine were not elevated in our subjects with FH despite marked thickening of the carotid IMT. Therefore, although hyperhomocysteinemia is associated with increased carotid IMT,[15] the converse in not necessarily true.

Our patients with FH had large buoyant LDL particles as has been previously reported.[16,17] Patients with FH also have normal or only mildly elevated triglyceride levels, normal postprandial lipemia,[18] and have no other features of the metabolic syndrome despite accelerated atherosclerosis. Measurement of LDL particle size in patients with FH therefore appears to be of little added benefit in predicting risk for atherosclerosis.

The role of measuring LDL susceptibility to oxidation ex vivo, although reported to be increased in some studies of patients with coronary artery disease, has also been questioned.[19] We have previously shown that LDL isolated from FH patients is more resistant to oxidation.[20] This is in keeping with the larger LDL particle size found in FH patients.[16,17] However, although initially more resistant, once lipid peroxidation has been initiated, these larger, more cholesterol-enriched LDL particles may generate more total oxidized lipid within the arterial wall resulting in plaque formation. However, there was no difference in the plasma levels of 8-isoprostane in the patients with FH compared with normal controls. In addition, no correlation was found between the plasma levels of 8-isoprostane and carotid IMT. Therefore, at least in subjects with FH, one cannot use susceptibility to oxidation or presently available plasma oxidation assays to assess the extent, or severity, of atherosclerosis.

In subjects with FH, the LDL cholesterol level and duration of exposure to the elevated LDL cholesterol levels, that is, the LDL "bulk," appears to be the most important determinant of atherosclerosis. Traditional risk factors such as hypertension, smoking, and diabetes mellitus increase the risk for developing coronary artery disease, particularly in men,[21] but the other biochemical risk factors measured appear to be of little added predictive value. Similarly, the role of lipid oxidation in the pathogenesis of atherosclerosis remains uncertain. Therapy should therefore be aimed primarily at reducing LDL cholesterol levels. Reduction of LDL cholesterol has been shown to induce regression of coronary atherosclerosis in patients with heterozygous FH.[22] In addition, recent prospective primary and secondary prevention studies have demonstrated a marked reduction in coronary events as well as reduced mortality with reduction in LDL cholesterol levels.[23,24] In these studies LDL cholesterol was reduced by 25% to 35% and clinical benefit was independent of the baseline LDL cholesterol level. The most important therapy for patients with FH is therefore reduction of LDL cholesterol even if LDL cholesterol goals are not achieved.

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Papers 8 and 9

Previously it was thought that subjects with homozygous FH responded poorly, if at all, to lipid-lowering drug therapy. In the following two studies the efficacy and safety of high dose statin therapy was evaluated in patients with homozygous FH. High dose statin therapy was found to be at least partially effective in reducing LDL-C levels in these subjects.

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Expanded-dose simvastatin is effective in homozygous familial hypercholesterolaemia

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Abstract

Patients with homozygous familial hypercholesterolaemia (HFH) have abnormalities in both low-density lipoprotein (LDL) receptor alleles, resulting in severe hypercholesterolaemia and premature coronary heart disease. Limited treatment options are available and the response to drug therapy has been poor. In the present paper, we have evaluated the efficacy and safety of simvastatin at doses beyond the current maximal dose of 40 mg/day in patients with HFH. After a 4 week placebo diet run-in period, 12 patients with well-characterized HFH were randomized to simvastatin 80 mg/day administered in three divided doses ($n = 8$; group 1) or 40 mg once daily ($n = 4$; group 2). After 9 weeks, the dose in group 1 was increased to 160 mg/day while the dose in group 2 was kept at 40 mg/day, but with the drug given in three divided doses and treatment continued for an additional 9 weeks. All 12 patients completed the study and there were no serious or unexpected adverse effects. LDL-cholesterol concentrations fell by 14% at the 40 mg/day dose, but were reduced further at the higher doses (25% at the 80 mg/day and by 31% at the 160 mg/day dosage, $P < 0.0001$). Excretion of urinary mevalonic acid, as an index of in vivo cholesterol biosynthesis, was reduced but did not correlate with reduction in LDL-cholesterol in the individual patients. The magnitude of response to therapy was not predicted by the LDL-receptor gene defect as patients with the same LDL-receptor mutations responded differently to the same dose of simvastatin therapy. The ability of expanded doses of simvastatin (80 or 160 mg/day) to reduce LDL-cholesterol levels in patients with HFH, even if receptor negative, suggests that at these doses, the drug reduces LDL production. Simvastatin therapy, at doses of 80 or 160 mg/day, should therefore be considered in all patients with HFH, either as an adjunct to apheresis, or as monotherapy for those patients who do not have access to apheresis or other such treatment modalities. © 1997 Elsevier Science Ireland Ltd.

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1. Introduction

The low-density lipoprotein receptor (LDLR) is responsible for the cellular binding and subsequent uptake and degradation of low-density lipoprotein (LDL) and remnant lipoproteins from the blood and plays a key role in regulating the plasma concentrations of LDL [1]. Familial hypercholesterolaemia (FH) is an inherited disorder caused by mutations in the LDLR gene which results in either the failure to synthesize receptors from the mutant gene or in the production of

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receptors which fail to bind or internalize LDL normally [2]. The LDL receptor deficiency results in an impaired clearance of LDL from plasma and the resultant hypercholesterolaemia predisposes these patients, if untreated, to premature atherosclerosis, particularly coronary artery disease. The HMG CoA reductase inhibitors, or statins, act primarily in the liver to inhibit the rate limiting enzyme in cholesterol synthesis, which in turn leads to a decrease in the intracellular pool of cholesterol and results in an increased expression of LDL receptors and a concurrent increase in the catabolism of LDL and other apolipoprotein-B containing lipoproteins [3]. Patients with heterozygous FH respond remarkably well to statin therapy due to their ability to upregulate the remaining normal LDLR allele and thus, the number of functional LDL receptors. In contrast, patients with homozygous FH (HFH), in whom both LDLR alleles are abnormal, respond poorly, if at all, to conventional doses of statin therapy, because they produce only a small quantity of functional receptors (receptor defective) or no receptors at all (receptor negative) [2]. HFH remains a very difficult condition to treat and the majority of patients with this disorder die from accelerated atherosclerosis before 30 years of age. Recent advances in therapy have, however, improved the prognosis for patients with HFH. Regular plasma exchange or LDL-apheresis has been shown to reduce the integrated mean LDL-cholesterol levels by 40–50% and can delay the progression of coronary artery disease and improve survival [4]. Apheresis is expensive, concentrated in a few highly specialized centres and requires 4–5 h every week or 2 weeks to be effective. Liver transplantation is the most definitive treatment currently available for HFH but carries a significant risk of morbidity and mortality, including that associated with the long term use of immunosuppressants [5]. More recently, gene therapy has been attempted, with little success, to restore function of the defective LDLR gene [6]. In view of the lack of convenient and safe treatment modalities to lower LDL-cholesterol in patients with HFH, we have evaluated the LDL-cholesterol lowering efficacy and short term safety of expanded doses of simvastatin (80 and 160 mg/day) which are beyond the current recommended dosage range in patients with HFH. The rationale for the study was that higher doses of simvastatin would result in more profound inhibition of cholesterol synthesis leading to decreased hepatic production of apo-B containing lipoproteins.

2. Patients and methods

2.1. Patients

Patients with HFH attending the Lipid Clinic at the Johannesburg Hospital were asked to participate in the study. The diagnosis of HFH was based on the presence of (i) serum LDL-cholesterol levels consistently greater than 12 mmol/l; (ii) the appearance of xanthomas in the first decade of life; (iii) documentation in both parents of hypercholesterolaemia or clinical signs indicative of the heterozygous state; and (iv) confirmation by DNA analysis of the LDL receptor gene. Familial defective apo-B100 due to a glutamine to arginine substitution at codon 3500 of apo B was excluded by DNA analysis in all subjects. Participants had to be at least 13 years of age and weigh more than 40 kg. Patients were excluded from the study if they had previously undergone portacaval shunting or ileal bypass surgery, had hepatic or renal dysfunction, or had known hypersensitivity to HMG CoA reductase inhibitors. None of the patients were receiving LDL-apheresis as this therapy is currently not available at our institution. In all patients, secondary causes of hypercholesterolaemia such as hypothyroidism, diabetes mellitus and nephrosis were excluded. All lipid lowering therapy, other than probucol, was stopped at least 2 weeks prior to the onset of the diet run-in period. Patients on stable doses of probucol for at least 3 months were included as the drug has minimal effect on LDL-cholesterol levels and no known interactions with simvastatin [7]. Furthermore, being highly fat soluble, an adequate washout period of 1 year or more would have been necessary.

After detailed explanation, all recruited patients gave their written informed consent to the study, which was approved by the University of the Witwatersrand's Committee for Research on Human Subjects.

2.2. Trial design

The study was a single-centre, randomized, double-blind, parallel dose escalation study. After a 4 week placebo diet run-in period, patients were randomized to simvastatin 80 mg/day administered in three divided doses (group 1), or to simvastatin 40 mg once daily, given in the evening (group 2). After 9 weeks, the dose in group 1 was increased to 160 mg/day administered in three divided doses and treatment was continued for an additional 9 weeks. Multiple daily dosing of expanded dose simvastatin was selected in an attempt to obtain continuous inhibition of cholesterol synthesis in the liver. To determine whether this hypothesis was valid, group 2 continued on a dose of 40 mg simvastatin/day but with the drug administered in three divided doses for the additional 9 week period.

All patients were counselled to follow the American Heart Association Step 1 diet. Dietary compliance was assessed at regular intervals and the diet was reinforced

at each visit to avoid the confounding effect of diet on lipid levels during the study. Patients were seen at 3 weekly intervals and blood was drawn for biochemical and haematological safety tests. Venous blood samples for lipid and lipoprotein analyses were drawn in the morning after an overnight fast of at least 12 h and were obtained at randomization and at the end of each 9 week treatment period. Urine specimens (24-h) were collected at the same time for measurement of urinary mevalonic acid (MVA) and urinary free cortisol levels. Serum gonadotropin and steroid hormone levels were also measured.

2.3. Analytical methods

Samples for lipid and apolipoprotein measurements were collected in EDTA (1 mg/ml), the plasma separated within 2 h and transported at 4°C via overnight courier to a central laboratory (Clinical Research, Brussels, Belgium). Total-cholesterol, HDL-cholesterol and triglycerides were analyzed by enzymatic methods on a Hitachi 747 (Boehringer Mannheim Diagnostics, Germany), as previously described [8]. The laboratory (LSP-148/INT-320) was certified and maintained Centre for Disease Control—National Heart, Lung and Blood Institute (CDC-NHLBI) Part III standardization for all lipid parameters [9]. HDL was isolated using heparin-2 molar manganese chloride [10]. The LDL-cholesterol was calculated using the Friedewald formula [11]. At the onset of the study and following each dose escalation period (weeks 9 and 18), ultracentrifugation was carried out at density (d) 1.006 kg/l to isolate VLDL ($d < 1.006$) and LDL plus HDL ($d > 1.006$) [12]. Apolipoprotein B was measured by immunonephelometry (BNA-100, Behring Diagnostics, Marburg, Germany) using WHO traceable standards [13]. Apolipoprotein E was measured by ELISA using a monoclonal antibody which bound to all three isoforms [14]. Lipoprotein(a) (Lp(a)) was quantitated by competitive ELISA [15]. Urinary MVA levels were measured by radioenzymatic methods as previously described [16]. To allow for potentially incomplete 24-h urinary collections, urine creatinine levels were also measured and urinary MVA was expressed per gram of creatinine. Biochemical and haematological safety tests were also performed at the central laboratory using standard methods. The laboratory maintained full accreditation by the College of American Pathologists (CAP #55146-01). Plasma drug levels were obtained by analyzing hydroxymethylglutaryl coenzyme A reductase inhibitor activity, expressed as equivalents of the hydroxyacid form of simvastatin (ng eq/ml), at the Department of Drug Metabolism, Merck, West Point, Pennsylvania [17].

2.4. Data analysis

The parameters of interest were total cholesterol, triglycerides, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol, apolipoprotein B, apolipoprotein E and Lp(a). Baseline was defined as the pre-dose measurement of each parameter observed at randomization. Percent change from baseline in each parameter was calculated at weeks 9 and 18 for each group. The sequence and time effect in the study was assumed to be negligible in the analysis. The t-test was the test statistic. Data are presented as mean ± S.E. A P value of < 0.05 was considered to be statistically significant.

3. Results

A total of 12 patients, seven males and five females, were recruited for the study. Nine patients were homozygotes or compound heterozygotes for FH Afrikaner-1, -2 or -3. These three founder-type LDLR mutations together account for more than 80% of FH in Afrikaners [18]. The FH Afrikaner-1 and -3 mutations are functional class 2B mutations which have 10–20% of residual LDLR activity (receptor defective) [19,20]. The FH Afrikaner-2 mutation is a functional class two and five mutation with < 2% of LDLR activity (receptor negative) [21]. One subject was heterozygous for the Afrikaner-1 mutation, but in addition had an exon nine mutation (Asp407Lys). The remaining two subjects were true homozygotes for an exon 16 mutation (Ser765Cys) and a promoter mutation (P-174), respectively. These mutations are newly described and have not yet been characterized at a functional level. Promoter mutations are, however, null mutations as no LDLR mRNA or LDLR protein is produced [22].

All 12 subjects completed the study (Table 1). The mean age of the patients was 26 years (range 15–39) and their body mass index 23.1 kg/m^2 (range 17.2–30.7). The mean LDL-cholesterol level was 14.3 (1.0) mmol/l. Five patients had previously undergone coronary artery bypass surgery. One patient had undergone aortic valve replacement for supravalvular aortic stenosis. Only one of the patients smoked cigarettes. Four patients were on probucol and remained on their established dose of this drug for the duration of the study. Two subjects were on β-blockers and one subject was on captopril, amiodarone and furosemide. The majority of subjects were taking aspirin which they continued to take for the duration of the study.

Compliance with simvastatin therapy, as assessed by tablet counts was > 90% in all but one subject. Overall compliance during the first and second 9 week periods was 94 and 96%, respectively.

Table 1
Patient charachteristics

Patient number	Age (years)	Sex	BMI (kg.m^{-2})	FH genotype	Baseline lipid levels (mmol/l)				Other
					Total-C	TG	LDL-C	HDL-C	
1[a]	22	M	27.0	Afrik 1/3	15.4	0.89	14.3	0.67	
2	16	M	17.2	Exon 16/16	12.6	0.60	11.5	0.88	
3	20	F	21.4	Afrik 1/1	13.8	0.82	12.6	0.88	
4[a]	19	M	21.9	Afrik 1/2	12.5	0.75	11.7	0.47	CABG age 18
5	26	F	21.8	Afrik 1/2	21.6	1.62	19.8	1.14	CABG age 9 and 17
6	38	F	30.7	Afrik 1/Exon 9	11.1	1.50	9.4	1.04	Coronary angioplasty age 21. CABG age 24
7	15	M	18.6	Afrik 1/1	18.5	1.61	17.2	0.62	CABG age 35
8	39	M	21.3	Afrik 1/1	20.7	2.00	19.1	0.67	CABG age 30. coronary angioiplasty age 33
9	27	F	23.5	Afrik 1/1	12.1	0.82	11.2	0.57	Removal intracranial xanthomas age 16. 22 and 27
10[a]	29	M	21.7	Promoter mutation	15.6	1.36	14.1	0.83	
11[a]	26	F	26.8	Afrik 1/2	19.8	0.92	18.3	1.01	
12	33	M	25.0	Afrik 1/3	14.5	1.70	12.8	0.85	AVR age 30

AVR, aortic valve replacement; CABG, coronary artery bypass graft.
[a] On probucol.

In group 1, LDL-cholesterol levels were reduced from 14.8 (1.3) mmol/l by 25% to 10.9 (1) mmol/l at the 80 mg/day dose and by 31% to 10.1 (1) mmol/l at the 160 mg/day dose of simvastatin. $P < 0.0001$ (Table 2). Despite this good overall response to high dose simvastatin therapy, the range of individual responses varied widely from an 8% increase to a 58% reduction in LDL-cholesterol levels at the 160 mg/day dosage (Fig. 1). Interestingly, the receptor negative patient, homozygous for a promoter mutation, responded remarkably well to high dose simvastatin therapy with a 46% reduction in LDL-cholesterol at the 160 mg/day dosage.

In group 2, treated with 40 mg simvastatin/day, there was only a modest reduction in LDL-cholesterol. Interestingly, there was no difference in LDL-cholesterol reduction when simvastatin was given as a single daily dose (13.8%) or given in three divided doses (13.2%). Mean baseline and on treatment plasma lipid, lipoprotein and apolipoprotein concentrations in the two groups are shown in Table 2. Reductions in total-cholesterol and apo-B paralleled those in LDL-cholesterol in both groups. Triglyceride levels decreased by 18% at the 80 mg/day and by 24% at the 160 mg/day dose of simvastatin ($P < 0.01$). There was a similar reduction in VLDL-cholesterol at the higher doses of simvastatin. Baseline apolipoprotein E levels were elevated in our HFH patients as reported by others [23]. There was a marked 50% reduction in apolipoprotein E levels at both the 80 and 160 mg/day dose of simvastatin. $P < 0.0001$. HDL-cholesterol levels increased modestly in both groups. Lp(a) levels also increased slightly in both groups on simvastatin therapy.

In group 1, 24-h urinary MVA levels decreased by 47% from 6.24 (1.3) to 2.78 (0.5) μmol/g creatinine at the 80 mg/day dose and by 39% to 3.32 (0.9) μmol/g creatinine at the 160 mg/day dose. In group 2, urinary MVA levels decreased modestly from 6.55 (1) to 5.39 (0.8) μmol/g creatinine with 40 mg simvastatin administered once daily and to 3.75 (0.5) μmol/g creatinine when simvastatin was given in three divided doses. There was, however, no correlation between reduction in LDL-cholesterol and urinary MVA levels in any of the groups (Fig. 2). No serious adverse clinical or biochemical effects occurred during the study. In particular, no patient developed myositis or hepatitis and none had clinically significant elevations in transaminases or creatine kinase levels (Table 3). Minor gastrointestinal disturbance occurred in two subjects but was short-lived and did not necessitate discontinuation of therapy. One subject complained of headache on the 40 mg/day dose of simvastatin. No subjects complained of headache on the 80 or 160 mg/day dose. No subject had sleep disturbance. There were no changes in adrenocortical or sex-hormone concentrations during the study. A single untimed, trough plasma HMG CoA reductase inhibitor level was obtained in each patient after 9 weeks of treatment with each dose. Mean plasma trough levels were 4.05, 3.35, 10 and 14.9 ng eq/ml at the 40 h, 40 tid, 80 tid and 160 mg tid doses of simvastatin, respectively. These levels are comparable to the active inhibitor levels obtained 10 h after a single evening dose of simvastatin 40, 80 and 160 mg/day [24].

Table 2
Plasma lipid, lipoprotein and apolipoprotein concentrations at baseline and during treatment with 40, 80 and 160 mg/day simvastatin (mean ± S.E.)

	Total-C (mmol/l)	TG (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)	VLDL-C (mmol/l)	Apo-B (mg/dl)	Lp(a) (mg/dl)	Apo-E (mg/dl)
Group 1 (n = 8)								
Baseline	16.2 (1.3)	1.41 (0.16)	0.84 (0.06)	14.8 (1.3)	0.64 (0.07)	415 (32)	91 (23)	15.3 (1.1)
80 mg/day: tid dosing	12.3 (1.0)**	1.11 (0.15)	0.89 (0.07)	10.9 (1.0)***	0.51 (0.06)	340 (26)	101 (24)	5.9 (1.0)***
160 mg/day: tid dosing	11.4 (1.0)**	1.03 (0.10)**	0.86 (0.06)	10.1 (1.0)***	0.47 (0.04)**	323 (27)*	111 (27)	7.5 (0.3)***
Group 2 (n = 4)								
Baseline	14.5 (1.8)	0.83 (0.03)	0.73 (0.13)	13.4 (1.7)	0.38(0.02)	406 (62)	44 (22)	13.1 (1.0)
40 mg/day	12.8 (1.8)*	0.81 (0.13)	0.80 (0.14)	11.7 (1.7)*	0.36 (0.04)	347 (43)*	53 (28)	6.0 (0.6)**
40 mg/day: tid dosing	12.7 (1.2)	0.78 (0.10)	0.85 (0.10)	11.5 (1.1)	0.35 (0.04)	359 (28)	49 (25)	7.6 (0.3)**

* $P<0.05$; ** $P<0.01$; *** $P<0.0001$.

4. Discussion

This study shows that doses of simvastatin beyond the recommended maximum dose of 40 mg/day are effective in lowering LDL-cholesterol in subjects with HFH. At a dose of 160 mg/day there was a 31% decrease in LDL-cholesterol. The LDL-cholesterol lowering effect appears to be dose dependent and is not determined by the frequency of administration of simvastatin. Reduction of LDL-cholesterol can induce regression of coronary atherosclerosis in patients with familial hypercholesterolaemia [25]. In addition, recent primary and secondary prevention studies have demonstrated a marked reduction in cardiovascular events as well as reduced mortality with reduction of LDL-cholesterol levels [26,27]. Clinical benefit was independent of the baseline cholesterol level. Therefore, although LDL-cholesterol levels were not reduced to therapeutic targets [28,29], the reduction in LDL-cholesterol achieved in our HFH patients should, if maintained for a sustained period of time, reduce their risk for atherosclerosis.

The main mechanism of action of the statins appears to be inhibition of cellular cholesterol biosynthesis leading to enhanced receptor mediated clearance of LDL [3]. Upregulation of hepatic LDL receptors also causes increased removal of LDL precursors, namely VLDL and remnants, from the circulation, thereby reducing the pools of these lipoproteins available for conversion to LDL. The marked reduction in apolipoprotein E, one of the major protein constituents of remnant particles, achieved with simvastatin in our HFH patients supports this view. The majority of our HFH subjects were receptor defective rather than receptor negative and thus may have been able to upregulate their LDL

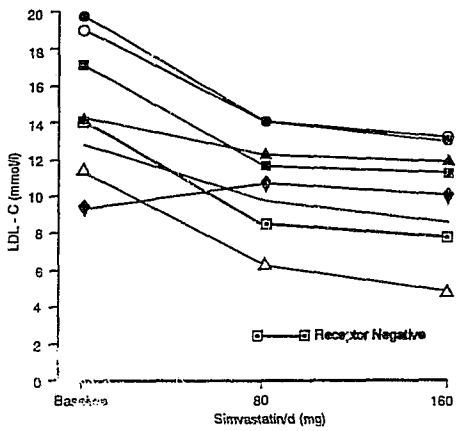

Fig. 1. Individual patients responses to 80 and 160 mg/day simvastatin in group 1.

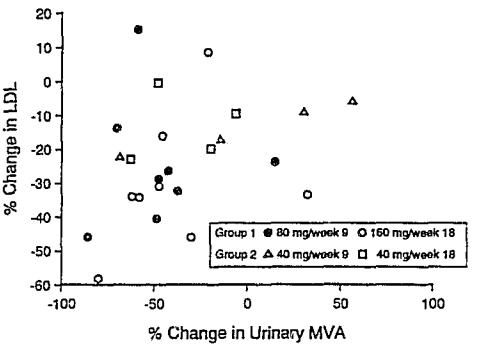

Fig. 2. Correlation between percent change in LDL-cholesterol and percent change in 24-h urinary MVA levels in both groups of HFH patients following simvastatin therapy.

Table 3
Hepatic transaminase, creatine kinase and hormone levels at baseline and during treatment with 40, 80 and 160 mg/day simvastatin (mean ± S.E.)

	ALT (mU/ml)	AST (mU/ml)	CPK (mU/ml)	FSH (mU/ml)	LH (mU/ml)	Testosterone (ng/ml)	Cortisol (μg/dl)	Urinary cortisol (μg/μgCr)
Group 1								
Baseline	15 (4)	17 (2)	67 (13)	6 (1)	7 (3)	5.0 (0.6)	25 (3)	5.1 (3.2)
80 mg/day; tid dosing	13 (2)	16 (2)	70 (12)	5 (1)	5 (1)	5.2 (1.0)	21 (3)**	2.6 (0.6)
160 mg/day; tid dosing	17 (2)	17 (2)	66 (6)	9 (2)	16 (11)	5.0 (1.0)	21 (3)*	3.4 (0.8)
Group 2								
Baseline	29 (23)	23 (12)	33 (11)	5 (1)	4 (1)	5.0 (–)	24 (4)	2.3 (0.2)
40 mg/day	9 (3)	12 (1)	40 (17)	4 (2)	9 (6)	4.0 (–)	20 (4)	2.0 (0.5)
40 mg/day; tid dosing	12 (5)	12 (1)	47 (17)	5 (2)	7 (2)	4.0 (–)	19 (4)	1.2 (0.1)**

ALT, alanine transaminase; AST, aspartate transaminase; CPK, creatine kinase; FSH, follicle stimulating hormone; LH, luteinising hormone.
* $P < 0.05$; ** $P < 0.01$.

receptors on the higher dose of simvastatin. If this is the only mechanism of action of the statins, receptor negative homozygotes should be completely refractory to this therapy. However, simvastatin reduced serum cholesterol by 30% in a HFH individual with no functional receptors [30]. In addition, one of our subjects, who was homozygous for a promoter mutation, also responded remarkably well to simvastatin therapy. An effect of the drug, other than the stimulation of LDL receptors, has to be invoked to explain this finding. In both heterozygous and homozygous FH patients, apo-B production is increased [31,32]. Intrahepatic cholesterol availability appears to be a key regulator of hepatic apo-B secretion [33]. Statins, by inhibiting hepatic cholesterol synthesis, may limit cholesterol availability for apo-B lipoprotein formation, thereby decreasing the rate of entry of apo-B lipoproteins into the serum. Reduction in apo-B synthesis by statin therapy has been demonstrated in patients with both heterozygous familial hypercholesterolaemia and combined hyperlipidaemia [34,35]. The marked inhibition of cholesterol synthesis accompanied by a greater reduction in apo-B secretion could also explain the triglyceride lowering effect of high dose simvastatin shown in our study. This has also been demonstrated with the new HMG CoA reductase inhibitor atorvastatin [36]. However, if this was the main mechanism of action of statin therapy there should have been a good correlation between reduction in urinary MVA levels, as an index of in vivo cholesterol biosynthesis and reduction in apo-B lipoprotein. Although there was a reduction in urinary MVA, the reduction did not correlate with reduction in LDL-cholesterol in the individual patients. There is a wide coefficient of variation for the measurement of urinary MVA in individual patients and our findings are limited by the relatively small number of patients. However, these findings are in agreement with previous studies of patients with heterozygous FH treated with simvastatin at doses of 20, 40 and 80 mg/day [37].

Both mechanisms may therefore play a role, the relative importance of each depending on the nature of the LDL receptor defect. In HFH subjects who totally lack LDL receptors decreased apo-B lipoprotein production is the most likely mechanism. In receptor defective HFH subjects who are able to produce some functional receptors, both decreased hepatic apo-B lipoprotein production and enhanced LDL receptor clearance could be involved. Of interest is that patients in our study with the same LDLR mutations had different baseline LDL-cholesterol levels and responded differently to the same dose of simvastatin therapy. This implies that serum cholesterol levels and response to therapy is not only determined by the LDLR gene defect, but that other mechanisms must be involved. Inherited differences in sterol-mediated regulation of HMG CoA reductase activity [38] or variability in the efficiency of either drug absorption or hepatic metabolism are potential factors which warrant further study.

Although there was a marked reduction in LDL-cholesterol levels on expanded dose simvastatin therapy, Lp(a) levels were not reduced and in fact, increased modestly. This is in keeping with previous studies which have shown no change or an increase in Lp(a) levels with statin therapy [39,40]. Therefore, the LDL receptor probably plays little role in mediating Lp(a) catabolism in humans [41].

Our observation that even HFH subjects who are receptor negative may respond to expanded doses of simvastatin has important therapeutic implications for the treatment of these patients. Expanded dose simvastatin therapy should therefore be considered in all patients with HFH, either as an adjunct to apheresis, or as monotherapy for those patients who do not have access to apheresis or other such treatment modalities. Long term follow-up studies are however necessary in order to determine the safety of expanded doses and whether the LDL-cholesterol reduction achieved in the present short term study will be maintained during extended treatment.

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INHIBITION OF CHOLESTEROL SYNTHESIS BY ATORVASTATIN IN HOMOZYGOUS FAMILIAL HYPERCHOLESTEROLAEMIA

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Abstract

Patients with homozygous familial hypercholesterolaemia (HoFH) have markedly elevated low density lipoprotein (LDL) cholesterol levels that are refractory to standard doses of lipid lowering drug therapy. In the present study we evaluated the effect of atorvastatin on steady state concentrations of plasma lipids and mevalonic acid (MVA), as well as on 24-hour urinary excretion of MVA in patients with well characterized HoFH. Thirty five HoFH patients (18 males; 17 females) received 40mg and then 80mg atorvastatin/day. The dose of atorvastatin was increased further to 120mg/day in 20 subjects and to 160mg/day in 13 subjects who had not achieved LDL cholesterol goal, or in whom the dose of atorvastatin had not exceeded 2.5 mg/kg body weight/day. LDL cholesterol levels were reduced by 17% at the 40mg/day and by 28% at the 80mg/day dosage ($p< 0.01$). Reduction in LDL cholesterol in the 5 receptor negative patients was similar to that achieved in the 30 patients with residual LDL receptor activity. Plasma MVA and 24-hour urinary excretion of MVA, as markers of *in vivo* cholesterol synthesis, were elevated at baseline and decreased markedly with treatment. Urinary MVA excretion decreased by 57% at the 40mg/day dose and by 63% at the 80mg/day dosage ($p<0.01$). There was a correlation between reduction in LDL cholesterol and reduction in urinary MVA excretion; those patients with the highest basal levels of MVA excretion and thus the highest rates of cholesterol synthesis having the greatest reduction in LDL cholesterol ($r=0.38$; $p=0.02$). Increasing the dose of atorvastatin to 120 and

160mg/day did not result in any further reduction in LDL cholesterol or urinary MVA excretion suggesting a plateau effect with no further inhibition of cholesterol synthesis at doses of atorvastatin greater than 80mg/day.

Key words: atorvastatin; cholesterol synthesis; mevalonic acid; homozygous familial hypercholesterolaemia

Homozygous familial hypercholesterolaemia (HoFH) is an autosomal dominantly inherited disorder characterized by markedly elevated levels of plasma low density lipoprotein (LDL) cholesterol, tendon xanthomas, and severe premature coronary artery disease.[1] If untreated, the majority of patients with this disorder die from accelerated atherosclerosis before the age of 30 years.[2] HoFH has hitherto been regarded as refractory to lipid-lowering drugs necessitating the use of radical forms of therapy such as apheresis, portacaval shunting and liver transplantation. High dose HMG CoA reductase inhibitor or statin therapy has recently been shown to be partially effective in lowering LDL cholesterol levels in this patient group.[3,4] However, the mechanism(s) by which high dose statins reduce LDL cholesterol levels in HoFH requires further elucidation.

Mevalonic acid (MVA), an intermediate in cholesterol synthesis and the immediate product of HMG CoA reductase is present in plasma and urine.[5] Previous studies have shown that the concentrations of mevalonate in plasma and urine parallel the rates of hepatic and whole body cholesterol synthesis in both animals and man.[6,7] There is also a very good correlation between plasma MVA and the rate of incorporation of deuterium into plasma free cholesterol in humans.[8] Plasma MVA concentrations exhibit diurnal variations, whereas the 24-hour urinary MVA excretion reflects the integrated plasma concentrations and thus provides a more practical way of measuring the influence of dietary or pharmaceutical manipulations on cholesterol synthesis than conventional sterol balance techniques.[9]

In the present study we evaluated the effects of high doses of atorvastatin (40-160mg/day) on steady state concentrations of plasma lipids and lipoproteins, plasma MVA and the 24-hour urinary excretion of MVA in patients with well characterized HoFH.

Methods

Patients

Patients with HoFH attending the lipid clinics at the University of the Witwatersrand and University of Cape Town participated in the study. The diagnosis of HoFH was based on the presence of an untreated serum LDL cholesterol consistently greater than 12 mmol/l; the appearance of xanthomas in the first decade of life; documentation in both parents of hypercholesterolaemia or clinical features of heterozygous FH; and confirmation by DNA analysis for LDL receptor gene mutations. Familial defective apo-B100 due to a glutamine to arginine substitution at codon 3500 of the apo-B gene was excluded by DNA analysis in all subjects. Patients were excluded from the study if they had hepatic or renal dysfunction, or had known hypersensitivity to HMG CoA reductase inhibitors. No patient was receiving LDL-apheresis at the time of the study. All recruited patients gave their written informed consent to the study which was approved by the Research Ethics Committees of both Universities.

Trial design

The study was a double-centre, dose escalation study. After an untreated baseline fasting lipid profile, patients received atorvastatin at doses of 40, 80, 120 and 160mg/day with a minimum period of 4 weeks of therapy between dosage escalation. Initially, all patients received 40 and then 80mg atorvastatin/day. In patients who did not achieve an LDL cholesterol goal of ≤3 mmol/L the dose of atorvastatin was increased further to 120mg and subsequently to 160mg/day with the limitation that in all patients the maximum atorvastatin dose did not exceed 2.5mg/kg body weight/day. All patients were counselled on a standard low-cholesterol, low-saturated fat diet and the diet was reinforced at each visit to avoid the confounding effect of diet on lipid levels during the study. Venous blood samples for lipoprotein analysis were drawn in the morning after an overnight fast of at least 10 hours. Samples were obtained at baseline and then at the time of each dose escalation. Interim biochemical safety tests were also regularly performed.

DNA analysis

DNA screening for three locally prevalent founder-related Afrikaner LDL receptor (LDLR) mutations, D206E (Afrik-1), V408M (Afrik-2) and D154N (Afrik-3), was performed in a single reaction by a multiplex amplification refractory mutation system - polymerase chain reaction.[10] The DNA samples were also screened for mutation P664L (FH-Gujerat) previously identified in South African Indians.[11] After screening for familial defective apolipoprotein B100, subjects

negative for these mutations underwent a more extensive search by heteroduplex and/or single-strand conformational polymorphism analysis.[10,12]

Measurement of mevalonic acid

Venous blood samples as well as 24 hour urine specimens were collected for measurement of the plasma and urine concentrations of MVA. Three separate 24-hour urine samples were collected before the initiation of atorvastatin therapy and then one after at least 4 weeks on each dose of atorvastatin. Urine and plasma samples were stored at -70^{0}C and, after thawing, were analyzed by radioenzymatic methods as previously described.[13]

Other measurements

Total cholesterol, HDL cholesterol and triglycerides were analyzed by enzymatic methods using automated techniques. The LDL cholesterol was calculated using the Friedewald formula.[14] Biochemical safety tests (alanine transaminase, aspartate transaminase and creatine kinase) were also performed at regular intervals throughout the study. Trough (predose) plasma atorvastatin concentrations were measured by high-performance liquid chromatography tandem mass spectrometry immediately prior to each atorvastatin dose escalation.[15] The effect of increasing atorvastatin dosage on plasma atorvastatin concentrations was expressed as the % change in concentration calculated as follows:

% change = [(trough concentration after dose increase - trough concentration before dose increase)/ trough concentration before dose increase] x 100.

Statistical Analysis

Comparisons were performed using one-way analysis of variance (ANOVA) for repeated measures followed by student Newman-Keuls method for post hoc comparisons using Sigmastat Statistical Software for Windows (Jandel Corp., CA). The relationship between the changes in MVA and LDL cholesterol was explored by univariate regression analysis using JMP Statistical Software Package Version 3.1 (SAS Institute Inc.). In all comparisons, $p< 0.05$ was considered significant. Results are expressed as mean ± standard error of the mean (SEM).

Results

A total of 35 HoFH patients, 18 males and 17 females, participated in the study **(Table 1)**. Twenty seven subjects were confirmed to have LDLR mutations in both LDL alleles. The majority were genetically compound heterozygotes, having inherited two different LDLR mutations rather than true homozygotes. Twenty three of these patients were homozygote or compound heterozygote for the FH Afrik-1, -2 or -3 mutations. These three founder LDLR mutations together account for more than 80% of FH in Afrikaners.[16] The FH Afrik-1 and -3 mutations are functional class 2B mutations which have 10-20% of residual LDLR activity (receptor defective).[17,18] The FH Afrik-2 mutation has <2% of LDLR

activity (receptor negative).[19] LDLR mutations in the remaining 8 subjects have not been fully characterized, but these patients fulfilled all the clinical criteria for HoFH. The mean age of the patients was 22 years (range 2-39) and their body mass index was 22.6 kg/m^2 (range 13.9-35.1). The mean LDL cholesterol was 15.0±0.6 mmol/L. Thirteen patients had previously undergone coronary angioplasty, aortic root surgery or coronary artery bypass grafting. None of the patients smoked cigarettes.

In the 35 patients, LDL cholesterol levels were reduced by 17% (from 15.0±0.6 to 12.4±0.6 mmol/L) on the 40mg/day dose of atorvastatin and by 28% (to 10.9±0.5 mmol/L) on the 80mg/day dose ($p< 0.01$; **Table 2**). Interestingly, the 5 subjects who were considered receptor negative had a 14% and 28% reduction in their LDL cholesterol levels which was similar to the 18% and 27% achieved in the 30 receptor defective subjects on the 40mg/day and 80mg/day dosage of atorvastatin. Triglyceride levels decreased by 9% on the 40mg/day dose and by 18% on the 80mg/day dose ($p<0.01$). There was no significant change in HDL cholesterol levels.

Plasma MVA levels were elevated at baseline and decreased by 56% and 63% on the 40mg/day and 80mg/day dosage of atorvastatin age ($p<0.01$). Reduction in the 24-hour urinary excretion of MVA was similar with a 57% and 63% reduction ($p<0.01$; **Table 2**). There was a strong correlation between the baseline 24-hour urinary excretion of MVA and the reduction in urinary MVA excretion in response to treatment with atorvastatin ($r=0.92$; $p< 0.01$; **Figure 1**). There was also a significant correlation between the magnitude of reduction in

LDL cholesterol and the reduction in urinary MVA excretion (r=0.38; p=0.02; **Figure 2**). There was no correlation between baseline LDL cholesterol levels and baseline urinary excretion of MVA however.

The dose of atorvastatin was increased to 120mg/day in 20 of the 35 patients but this increase in dose did not yield any further reduction in LDL cholesterol despite a 107% increase in plasma atorvastatin concentration. A further increase in dose to 160mg of atorvastatin/day was made in 13 of the patients but this also did not result in any further reduction in LDL cholesterol levels despite a further 84% increase in plasma atorvastatin concentration (**Table 3**). In addition, there was no further reduction in plasma MVA or urinary excretion of MVA on these higher doses of atorvastatin (**Figure 3**). Transaminase and creatine kinase levels increased modestly on the higher doses of atorvastatin but the medication was extremely well tolerated with no adverse clinical or biochemical effects occurring during the study.

Discussion

This study shows that atorvastatin is effective in lowering LDL cholesterol in HoFH. At a dose of 80mg/day there was a 28% reduction in LDL cholesterol. There was however no further reduction in LDL cholesterol at higher doses of atorvastatin, suggesting a "plateau" effect.

The main mechanism of action of statins is inhibition of cellular cholesterol biosynthesis leading to a reduction in VLDL synthesis and upregulation of LDL receptors thus increasing the rate of removal of both LDL and LDL precursors

from the blood.[20] The majority of our HoFH patients were receptor defective rather than receptor negative and thus may have been able to increase LDLR activity in response to treatment with atorvastatin. However, atorvastatin also reduced LDL cholesterol similarly in the 5 homozygotes with no functional LDL receptors; these results are consistent with an inhibition of VLDL (and LDL) synthesis.

The mean basal rate of urinary MVA excretion was significantly increased in our HoFH patients and was about twice that of normocholesterolemic controls.[21] This is in keeping with sterol balance techniques which have found a twofold increase in whole body cholesterol synthesis in HoFH.[22,23]

Cholesterol appears to play a key role in regulating apo-B lipoprotein secretion by the liver[24] and a strong correlation between the rate of cholesterol synthesis and apo-B lipoprotein production has been demonstrated in normal subjects.[25] HoFH subjects who have increased cholesterol biosynthesis also have increased apo-B lipoprotein production.[26] Atorvastatin, by markedly inhibiting cellular cholesterol synthesis may limit cholesterol availability for apo-B lipoprotein formation thus decreasing the rate of production of LDL.[4]

Our results demonstrate a positive correlation between the reduction in LDL cholesterol and urinary excretion of MVA implying that the reduction in cholesterol synthesis by atorvastatin also leads to an accompanying reduction in the production of LDL and its precursors. Interestingly, those HoFH subjects with the highest basal levels of MVA (and thus the highest rate of cholesterol synthesis) showed the greatest reduction in LDL cholesterol in response to

treatment with atorvastatin. This implies that the greater the inhibition of HMG CoA reductase the greater the effect on apo-B lipoprotein secretion.

We observed a statistically significant correlation between the decrease in LDL cholesterol and the reduction in urinary MVA in HoFH; this is contrary to previous studies in heterozygous FH subjects [27,28] and a small number of HoFH patients [3] in which no correlation was found. We interpret this to indicate that the mechanisms by which statins lower LDL cholesterol in FH patients depends on the nature and severity of the LDL receptor defect. The major mechanism in heterozygous FH subjects and in homozygotes with residual LDL activity is probably upregulation of LDL receptors with increased clearance of LDL. In FH homozygotes with severely dysfunctional or absent LDL receptors, inhibition of cholesterol synthesis and apo-B lipoprotein production is the predominant mechanism of action and in this case a correlation between inhibition of cholesterol synthesis and LDL cholesterol reduction would be expected to be seen.

Another important finding in this study was the plateau effect in response to increasing doses of atorvastatin. Although well tolerated, there was no further reduction in LDL cholesterol, plasma MVA or urinary MVA excretion above a dose of 80mg atorvastatin/day suggesting maximum suppression of cholesterol synthesis at this dose. A similar threshold effect has been demonstrated with simvastatin in subjects with heterozygous FH.[29] There is therefore probably a limit to the LDL cholesterol lowering capacity of statins. Further reduction of LDL cholesterol would therefore require addition of a drug with a different mechanism

of action such as a microsomal transfer protein (MTP) inhibitor [30] or ACAT inhibitor [31], or the concomitant use of an alternative form of therapy such as LDL apheresis.

Recent prospective primary and secondary prevention studies have demonstrated a marked reduction in coronary events as well as reduced mortality in hypercholesterolemic subjects with reduction in LDL cholesterol levels of 25 to 35%.[32,33] Clinical benefit in these studies was independent of baseline LDL cholesterol level. Therefore, although LDL cholesterol was not reduced to within acceptable limits, the reduction of 28% in LDL cholesterol by atorvastatin should be of benefit to HoFH subjects and, if maintained in the long-term, may improve their survival. Therapy with potent statins such as atorvastatin, should therefore be considered in all HoFH subjects, either as an adjunct to apheresis, or as an alternative for those subjects who do not have access to apheresis.

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Figure 1

Correlation between baseline 24-hour urinary excretion of mevalonic acid (MVA) and reduction in urinary MVA excretion with atorvastatin 40mg/day (●) and 80mg/day (○) in patients with homozygous familial hypercholesterolaemia.

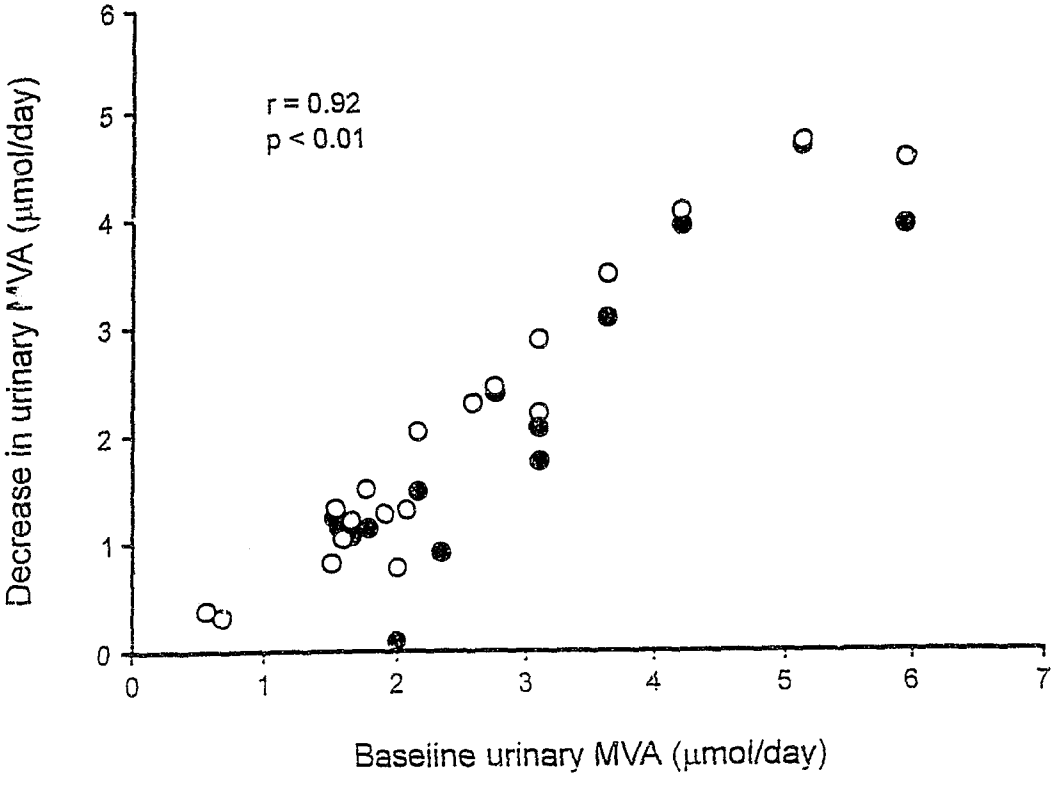

Figure 2

Correlation between changes in LDL cholesterol and 24-hour urinary excretion of mevalonic acid (MVA) with atorvastatin 40mg/day (●) and 80mg/day (○) in patients with homozygous familial hypercholesterolaemia.

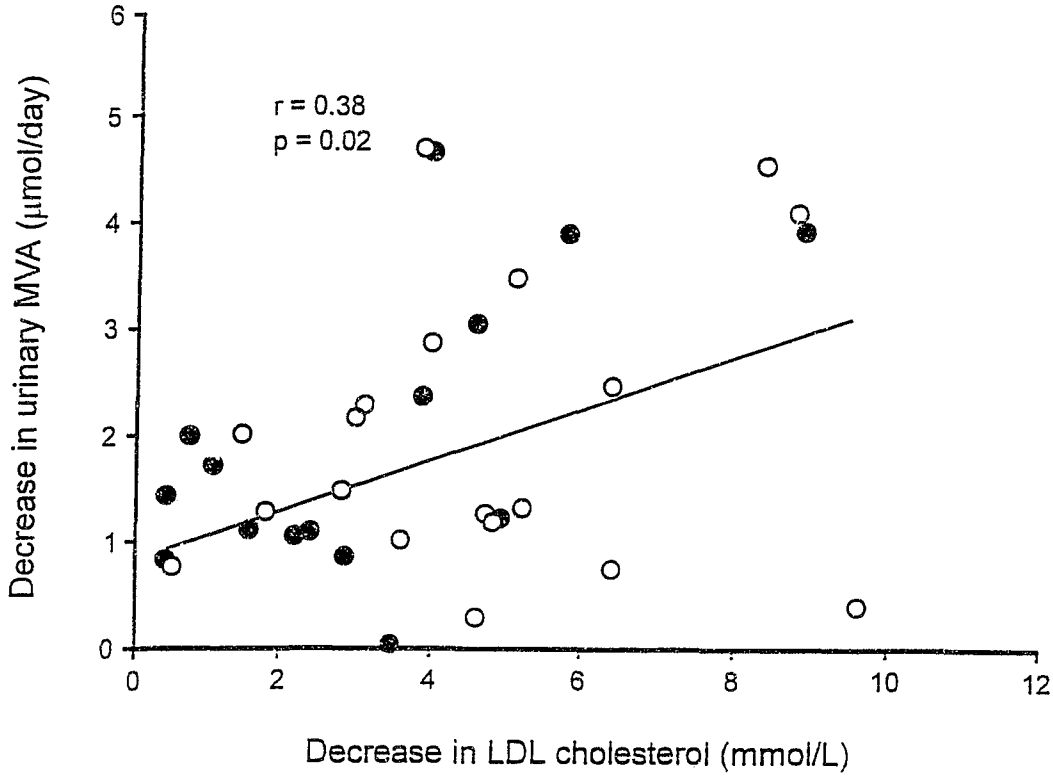

Figure 3

The effect of atorvastatin on (a) LDL cholesterol levels and (b) 24-hour urinary excretion of mevalonic acid (MVA) in patients with homozygous familial hypercholesterolaemia (n=9).

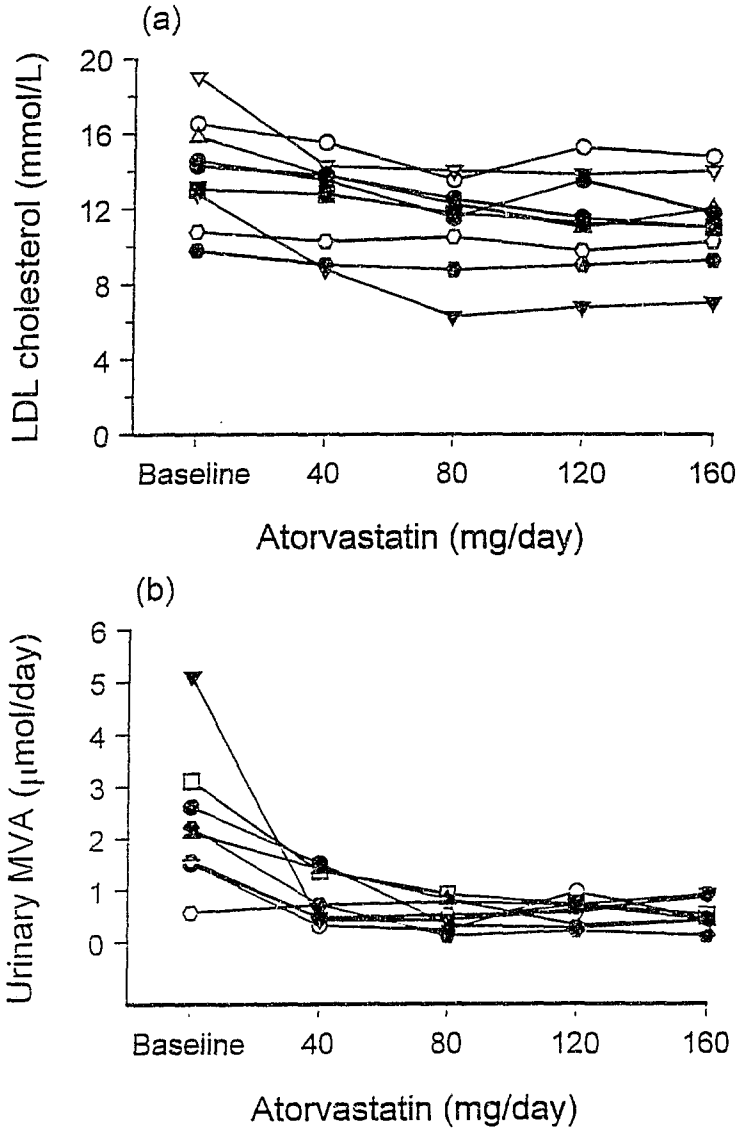

Table 1

PATIENT CHARACTERISTICS

Patient number	Sex (M/F)	Age (years)	BMI (kg/m2)	FH Genotype	Baseline lipid levels (mmol/L) Total cholesterol	Triglyceride	LDL cholesterol	HDL cholesterol
1	F	12	21.1	Afrik 1/ ?	14.8	1.6	13.4	0.6
2	M	15	15.0	FH 664/ FH 664	16.6	1.8	15.0	0.8
3	M	6	13.9	Afrik 2/2	26.5	1.2	25.4	0.5
4	F	21	20.4	FH664/ FH664	18.9	1.0	17.3	1.1
5	M	29	21.8	?	19.4	2.8	17.3	0.7
6	M	26	23.5	Afrik 1/1	11.7	2.3	9.7	1.0
7	F	20	35.1	Afrik 1/1	15.6	1.4	14.6	0.3
8	F	27	26.6	Afrik 1/2	20.6	1.3	19.1	0.8
9*	M	34	25.7	Afrik 1/3	14.3	1.8	12.8	0.6
10*	M	33	28.1	Afrik 1/2	13.2	4.1	10.7	0.6
11*	M	20	22.9	Afrik 1/2	14.1	0.9	13.1	0.5
12*	M	39	22.6	Afrik 1/1	18.5	2.9	16.5	0.6
13*	F	39	30.4	Afrik 1/ ?	12.2	1.6	10.2	1.2
14	M	10	16.6	Afrik 1/ ?	15.5	1.5	14.4	0.4
15	F	11	19.8	Afrik 1/1	18.1	2.3	16.5	0.5
16	M	27	26.7	Afrik 3/3	10.9	0.3	9.7	1.1
17*	F	23	26.6	Afrik 2/2	21.9	0.6	20.6	0.9
18	M	15	19.5	Afrik 1/1	15.8	0.9	14.8	0.5
19*	F	37	25.8	Afrik 2/2	21.3	1.4	19.8	0.8
20*	F	20	21.5	Afrik 1/1	14.8	1.1	13.6	0.7
21	F	2	16.9	Afrik 1/2	16.9	2.9	15.2	0.4
22*	F	27	20.8	Afrik 1/2	20.7	1.7	18.9	0.9
23	F	35	27.9	Afrik 1/2	17.8	1.1	15.9	1.4
24	M	23	26.0	Afrik 1/3	15.8	1.7	14.3	0.5
25	M	11	17.3	?	11.5	1.3	9.95	0.9
26	M	17	17.7	?	12.6	1.2	11.4	0.6
27	M	10	14.8	Afrik 1/1	19.1	1.3	17.4	1.1
28	M	24	27.8	Afrik 1/1	15.8	1.1	14.8	0.5
29*	F	25	26.0	Afrik 1/1	11.6	0.7	10.0	1.3
30	M	24	19.8	Afrik 1/ ?	18.8	1.7	17.1	0.9
31	F	25	31.2	Afrik 3/3	15.6	0.6	14.6	0.7
32	F	25	21.2	CT2/CT2	16.3	0.7	15.1	0.9
33*	F	13	16.5	CT2/CT2	21.0	1.1	19.7	0.8
34*	M	27	25.6	Afrik 1/ ?	15.2	4.3	12.6	0.6
35*	F	13	18.6	Afrik 1/1	15.8	0.6	14.5	1.0

FH = familial hypercholesterolaemia
* = established coronary artery disease
? = LDL receptor gene mutation yet to be fully characterized

Table 2

Effect of atorvastatin on lipids, enzymes and mevalonic acid (MVA) levels in 35 patients with homozygous familial hypercholesterolaemia (mean±SEM)

Parameter	Atorvastatin dose (mg/day) 0	40	80
Total cholesterol (mmol/L)	16.5±0.6	13.8±0.6*	12.3±0.6*#
Triglyceride (mmol/L)	1.6±0.2	1.3±0.1	1.2±0.1*
HDL cholesterol (mmol/L)	0.8±0.1	0.8±0.0	0.9±0.1
LDL cholesterol (mmol/L)	15.0±0.6	12.4±0.6*	10.9±0.5*#
Alanine transaminase (IU/L)	18.4±1.5	25.8±2.7^	27.7±2.5*
Aspartate transaminase (IU/L)	20.7±1.1	27.1±2.6^	26.2±1.7^
Creatine kinase (IU/L)	92.7±11.1	118.4±15.2	100.1±10.4
Plasma MVA (pmol/mL)	55.0±7.1	21.8±2.4*	16.1±2.8*
Urine MVA (umol/day)	2.3±0.3	0.8±0.1*	0.6±0.1*

* significantly different from baseline p< 0.01
^ significantly different from baseline p<0.05
# significantly different from Atorvastatin 40mg/day p< 0.01

For MVA measurements n=25

Table 3

Effect of atorvastatin on lipids, enzymes and mevalonic acid (MVA) levels in the FH homozygotes in whom the dose was increased to 160mg atorvastatin/day (n=13) (mean±SEM)

Parameter	Atorvastatin dose (mg/day) 0	40	80	120	160
Total cholesterol (mmol/L)	15.6±0.7	13.7±0.5*	12.4±0.5*#	12.8±0.7*	12.6±0.6*
Triglyceride (mmol/L)	1.7±0.4	1.3±0.3	1.3±0.2	1.0±0.2	0.9±0.2^
HDL cholesterol (mmol/L)	0.7±0.1	0.8±0.1	0.7±0.1	0.8±0.1	0.8±0.1
LDL cholesterol (mmol/L)	14.1±0.7	12.3±0.6*	11.0±0.6*#	11.5±0.7*	11.4±0.7*
Alanine transaminase (IU/L)	21.5±2.6	24.9±3.0	25.8±3.4	31.3±4.4	33.3±5.8
Aspartate transaminase (IU/L)	18.7±1.4	22.1±2.2	21.9±2.3	25.4±2.6	27.7±2.9
Creatine kinase (IU/L)	81.4±9.6	116.8±23.1	88.7±13.0	116.6±22.4	147.1±41.8
Plasma MVA (pmol/mL)	45.3±5.5	19.1±3.4^	13.1±3.8^	12.9±1.8^	18.1±3.5^
Urinary MVA (umol/day)	2.3±0.4	0.6±0.2^	0.5±0.1^	0.5±0.1^	0.5±0.1^

* significantly different from baseline $p < 0.01$
^ significantly differerit from baseline $p<0.05$
# significantly different from Atorvastatin 40mg/day $p < 0.01$

For MVA measurements n=9

Paper 10

In this final paper the efficacy of antioxidant compared to lipid-lowering therapy on delaying progression of atherosclerosis was evaluated. Over a 4-year period, fifteen patients with homozygous familial hypercholesterolaemia received vitamin E (1000 mg dl-alpha-tocopherol acetate daily) for 24 months and then high-dose statin therapy for a further 26 months (initially simvastatin 80-160 mg daily for 8 months and then atorvastatin 80 mg daily for 18 months). Plasma lipid levels did not change with vitamin E therapy and the rate of progression of carotid intima-media thickness, used as an *in vivo* assessment of atherosclerosis, was rapid (0.19 mm/year; range 0.02-0.39; $p < 0.0001$). High-dose statin therapy reduced low density lipoprotein cholesterol levels by 28% (range -12 to -51) and caused regression in carotid intima-media thickness in all but one subject (-0.19 mm/year; range +0.03 to -0.80; $p = 0.0004$). Therapy in subjects with severe hypercholesterolaemia should therefore be primarily aimed at reducing low density lipoprotein cholesterol levels.

Efficacy of Vitamin E Compared to Either Simvastatin or Atorvastatin in Preventing the Progression of Atherosclerosis in Homozygous Familial Hypercholesterolemia

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The oxidative modification of low density lipoprotein (LDL) is thought to play a pivotal role in the pathogenesis of atherosclerosis and antioxidant therapy has been advocated for the treatment and prevention of atherosclerosis.[1,2] Over a 4-year period we compared the efficacy of antioxidant (vitamin E) to lipid-lowering (statin) therapy in patients with homozygous familial hypercholesterolemia (HFH) as these patents are known to suffer from severe, accelerated atherosclerosis.[3] We evaluated carotid intima-media complex thickness (I-MT) as a marker of atherosclerosis as thickening of the carotid intima-media has been shown to be an early step in the atherosclerotic process.[4,5]

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Patients with HFH attending the Lipid Clinic at the Johannesburg Hospital participated in the study. The diagnosis of HFH was based on the presence of a) an untreated serum LDL cholesterol consistently > 10.3 mmol/L (400 mg/dl); b) the appearance of xanthomas in the first decade of life; c) documentation in both parents of hypercholesterolemia or clinical features of the heterozygous state; and d) confirmation by DNA analysis for LDL receptor gene mutations. No patient was receiving LDL-apheresis because this therapy was not available at our institution. All recruited patients gave their written informed consent to the study, which was approved by the University of the Witwatersrand's committee for research on human subjects.

We treated the HFH patients initially with high dose vitamin E (1000mg dl-alpha-tocopherol acetate) daily. Over a 2-year period we observed no benefit from vitamin E on xanthoma regression and, in fact, showed progression of

coronary atherosclerosis in 2 subjects who underwent repeat coronary angiography.[7] Vitamin E therapy was therefore discontinued and replaced with high-dose statin therapy which had been shown to be partly effective in reducing LDL cholesterol in these patients.[8] Initially, patients received simvastatin 80 - 160mg/day and subsequently atorvastatin 80mg/day. All patients were kept on a standard low-cholesterol, low-saturated fat diet for the duration of the study.

The carotid arteries were evaluated with high-resolution B-mode ultrasonography using a previously validated technique.[9] All subjects were examined in the supine position. Both common carotid arteries were scanned longitudinally to visualize the intima-media complex of the far wall of the artery. The distance between the echo arising from the lumen-intima interface and the media-adventitia interface was taken as a measure of the intima-media complex.[10] The carotid I-MT was defined as the average of five measurements randomly selected between 10 and 30mm proximal to the carotid bifurcation. Using this technique, coefficient of variation of interobserver variation was 8.3% ([95% confidence interval ±0.13mm]; r=0.84) of observed thickness. Coefficient of variation of intraobserver variation was 5.7% ([±0.08mm]; r=0.87) of observed thickness. Carotid I-MT was recorded in all patients at baseline and then following vitamin E and statin therapy. The same observer (M.V.) measured carotid I-MT thickness in all patients. He was blinded as to the clinical history, risk factor profile, drug therapy or previous carotid ultrasound findings.

DNA screening for three founder-related Afrikaner mutations, D206E (Afrik 1), V408M (Afrik 2) and D154N (Afrik 3), was performed in a single

reaction by a multiplex amplification refractory mutation system - polymerase chain reaction, as previously described.[11] The DNA samples were also screened for mutation P664L (FH-Gujerat) previously identified in South African Indians.[12] After screening for familial defective apolipoprotein B100, subjects negative for these mutations underwent a more extensive search by heteroduplex and/or single-strand conformational polymorphism analysis.[11,13]

Total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were analyzed by enzymatic methods using automated techniques. The LDL cholesterol was calculated using the Friedewald formula.[14] Lipoprotein(a) was quantitated using the Tinaquant method (Boehringer Mannheim). Biochemical (alanine transaminase, aspartate transaminase and creatine kinase) and haematological safety tests were also performed at regular intervals throughout the study.

Statistical comparisons were performed using one-way analysis of variance for repeated measures followed by Student's *t* tests with the Bonferroni correction where appropriate. Simple (Pearson's) correlation coefficients between carotid I-MT and selected variables were calculated, and a stepwise multiple regression analysis was then used to evaluate the independent association of these variables with carotid I-MT. In all comparisons, $p < 0.05$ was considered significant.

• • •

A total of 15 HFH patients, 9 males and 6 females, participated in the study **(Table I)**. Ten patients were homozygote or compound heterozygote for

FH Afrik 1, 2 or 3. These 3 founder mutations together account for more than 80% of FH in Afrikaners and have been confirmed to cause defective LDL-receptor function at the cellular level.[15] Two subjects were homozygous for the FH-Gujerat mutation.[12] LDL-receptor mutations in the remaining 3 subjects are yet to be fully characterized, but these 3 patients fulfilled all the clinical criteria for HFH. The mean age of the patients was 17 years (range 5-36) and their body mass index was 22.1 kg/m^2 (range 13.9-35.1). The mean LDL cholesterol was 16.3 ± 2.4 mmol/L (631 ± 94 mg/dl). Three patients had previously undergone coronary artery bypass surgery. None of the patients were hypertensive and only one patient smoked cigarettes. All of the patients were taking aspirin which they continued to take for the duration of the study.

At the start of the study the mean carotid I-MT was 1.28 mm ([1.04-1.52]; range 0.57-2.05) compared to 0.49 mm ([0.41-0.57]; range 0.37-0.68) in a group of 40 age matched controls with no evidence of vascular disease; $p<0.01$.[9] Baseline carotid I-MT correlated with age ($r=0.61$; $p<0.02$), but not with body mass index, total, LDL or HDL cholesterol, triglyceride, or lipoprotein(a) levels.

Patients were treated with vitamin E alone for a mean of 24 months (range 7-41). There was no significant change in total, HDL or LDL cholesterol during this period, although triglyceride levels increased modestly. The rate of progression of carotid I-MT during vitamin E therapy was 0.19 mm/year ([0.13-0.24]; range 0.02-0.39). This was substantially faster than the rate of progression observed in a group of 140 normal controls (0.005 mm/year; $p<0.01$).[9]

After withdrawal of vitamin E therapy, patients were treated on high dose simvastatin or atorvastatin for a period of 26 months (range 18-35). During this period there was a significant reduction in total cholesterol (-26%; range -10 to -42) and LDL cholesterol (-28%; range -12 to -51) levels, but no change in HDL cholesterol or triglyceride levels **(Table II)**. There was no further progression in carotid I-MT during this period. In fact there was regression in carotid I-MT in all but one subject (-0.19 mm/year; [-0.08 to -0.30]; range +0.03 to -0.80) **(Figure I)**.

• • •

Our study demonstrates that HFH patients have markedly increased carotid I-MT despite the absence of other established coronary artery disease risk factors such as systemic hypertension and smoking. This confirms the dominant effect that hypercholesterolemia has in determining carotid I-MT. During the period of vitamin E therapy there was no significant change in LDL cholesterol levels and the rate of progression of carotid I-MT was rapid. In contrast, during therapy with high-dose statin therapy, which resulted in a mean reduction of 28% in LDL cholesterol, no further progression occurred and there was regression in nearly all patients.

In conclusion, reduction in LDL cholesterol by high-dose simvastatin or atorvastatin is more effective than vitamin E in delaying progression of atherosclerosis in HFH patients with severe hypercholesterolemia.

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Figure I

Progression of Carotid I-MT in Homozygous Familial Hypercholesterolemia (HFH)

Carotid I-MT in the 15 individual HFH patients at the onset of the study and following vitamin E therapy for 24 months (range 7-41) and then high-dose simvastatin or atorvastatin for a further 26 months (range 18-35).

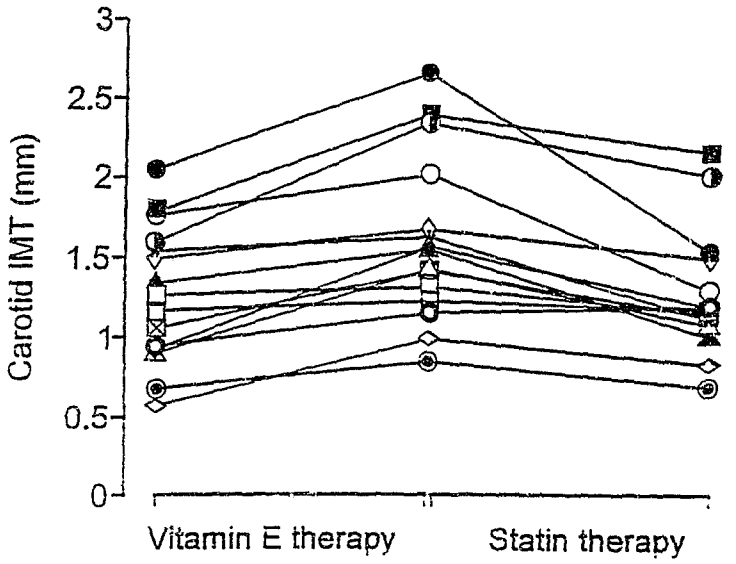

Table 1

PATIENT CHARACTERISTICS

Patient number	Sex (M/F)	Age (years)	FH Genotype	Baseline lipid levels				Lipoprotein(a) (mg/dl)	Carotid IMT (mm)
				Total cholesterol	Triglyceride	LDL cholesterol	HDL cholesterol		
1	M	5	Afrik 2/2	22.3	0.6	21.5	0.5	38	0.97
2	M	6	Afrik 1/?	14.3	0.8	13.4	0.5	21	0.57
3	F	8	Afrik 1/?	16.5	1.7	15.2	0.6	88	0.68
4	F	9	Afrik 1/1	18.0	0.2	17.4	0.5	5	0.93
5	M	12	FH-Gujerat	18.6	0.7	18.1	0.4	88	1.15
6	M	13	Afrik 1/1	18.0	0.6	17.3	0.4	99	0.95
7	F	16	FH-Gujerat	16.6	1.4	15.2	0.8	33	2.05
8	M	16	Afrik 1/2	18.6	1.3	17.4	0.5	44	1.80
9	F	16	Afrik 1/1	17.5	1.5	15.6	1.2	25	1.50
10	F	17	Afrik 1/1	15.8	0.9	15.0	0.3	53	1.52
11	M	19	Afrik 1/3	18.1	0.5	17.3	0.6	70	1.06
12	M	25	Afrik 3/3	12.2	0.8	11.2	1.0	13	1.28
13	M	27	?	16.2	0.8	15.0	0.8	237	1.39
14	F	32	Afrik 1/2	16.7	0.1	15.9	0.8	44	1.76
15	M	36	Afrik 1/1	21.0	1.4	19.0	0.7	120	1.60

Lipid and lipoprotein values are expressed in mmol/L, with mg/dl in parentheses
FH=familial hypercholesterolemia; CABG = coronary artery bypass graft
? = LDL receptor gene mutation yet to be fully characterized

Table 2

Fasting lipid profiles in 15 patients with homozygous familial hypercholesterolemia at baseline and on antioxidant or statin therapy

	Baseline	Antioxidant therapy	Statin therapy
Total cholesterol (mmol/l)	17.4 (2.5)	16.9 (3.4)	12.4 (2.3)**
Triglyceride (mmol/l)	0.9 (0.5)	1.5 (0.7)*	1.2 (0.4)
HDL cholesterol (mmol/l)	0.6 (0.2)	0.7 (0.3)	0.7 (0.4)
LDL cholesterol (mmol/l)	16.3 (2.4)	15.5 (3.4)	11.1 (2.4)**

* $p < 0.05$ compared to baseline and statin therapy
** $p < 0.01$ compared to baseline and antioxidant therapy

The American Journal of Cardiology

William C. Roberts, M.D.

Editor in Chief

July 5, 1999

Frederick J. Raal, M.D.
Department of Medicine
University of the Witwatersrand
7 York Road, Parktown
Johannesburg 2193
South Africa

MS# 90400
Title: Efficacy of Vitamin E Compared to Either Simvastatin or Atorvastatin in Preventing the Progresion of Atherosclerosis in Homozygous Familial Hypercholesterolemia

Dear Dr. Raal:

Your manuscript is accepted and scheduled for publication in December 1999. Thanks for the changes.

Sincerely,

William C. Roberts, MD

Baylor University
Medical Center

Wadley Tower No. 457

3600 Gaston Avenue

Dallas, TX 75246

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Conclusion

This series of studies in FH subjects show that LDL-C level and duration of exposure to the elevated LDL-C levels, that is the LDL quantity or 'LDL bulk', are important determinants of the extent and severity of atherosclerosis. Qualitative differences in LDL such as LDL particle size and susceptibility of LDL to oxidation appear to be of less importance.

FH patients have large, buoyant LDL particles, which are less susceptible to lipid oxidation than smaller denser LDL particles. In the absence of other causes of insulin resistance, patients with FH have normal fasting insulin and triglyceride levels, normal postprandial lipaemia,[1] and do not have microalbuminuria. They therefore usually show no features of the metabolic syndrome despite severe, accelerated atherosclerosis. However components of the metabolic syndrome such as central obesity may add to the atherogenic potential of hypercholesterolaemia due to raised LDL-C concentrations. In this regard, abdominal obesity and hyperinsulinaemia have been shown to act synergistically to increase the risk for CAD among males with FH, as they are potentially exposed over time to both the quantitative atherogenicity and qualitative alterations of apo-B containing lipoproteins.[2]

Similarly, the role of lipid oxidation in the pathogenesis of atherosclerosis in FH remains uncertain. LDL isolated from FH patients is more resistant to oxidation and antioxidant therapy appears to be of little or no benefit in preventing progression of atherosclerosis in these hypercholesterolaemic subjects. These findings challenge, but do not disprove, the oxidative hypothesis of atherosclerosis.[3] LDL particles do not

undergo a significant degree of oxidation within the circulation because of the presence of abundant antioxidant defences.[4] However, LDL particles are continuously entering and leaving the arterial wall; some of these particles becoming trapped in the interstitial matrix, and because of prolonged resident time, may be subjected to sustained oxidative stress. Thus, although initially more resistant, once lipid peroxidation has been initiated, a larger, more-cholesterol-enriched LDL, such as that typically found in FH patients, may generate more total oxidised lipid within the arterial wall with pathologic consequences.

Lack of effect of vitamin E on the progression of atherosclerosis in FH subjects also suggests a 'threshold' LDL-C level at which currently available antioxidants are unable to protect LDL sufficiently against oxidation. Support for this hypothesis can be found in animal studies.[5,6,7] During conditions of marked hypercholesterolaemia neither probucol nor vitamin E were effective in preventing the progression of atherosclerosis in hyperlipidaemic animals whereas with mild degrees of hypercholesterolaemia both were effective [5]. Hypercholesterolaemia may therefore outweigh the therapeutic effectiveness of antioxidants.[6] This does not exclude the possibility that antioxidant therapy might be additive to, or possibly even synergistic with, treatment with cholesterol-lowering agents.[8] However, particularly in severely hypercholesterolaemic subjects, more conclusive proof of a protective effect of antioxidants from large prospective studies presently in progress [9] is needed before antioxidant therapy can be advocated for the treatment and prevention of atherosclerosis.

In subjects with FH, quantitative rather than qualitative differences in LDL are associated with accelerated atherosclerosis. Therapy in FH should therefore be aimed primarily at reducing LDL-C levels. Reduction of LDL-C has been shown to induce regression of coronary atherosclerosis in patients with heterozygous FH.[10] In addition, recent prospective primary and secondary prevention studies in hypercholesterolaemic subjects have demonstrated a marked reduction in coronary events as well as reduced mortality with reduction in LDL-C levels.[11,12] In these studies LDL-C was reduced by 25-35% and clinical benefit was independent of baseline LDL-C level. A recent analysis of mortality trends over time suggests that the prognosis of FH patients is improving now that more effective treatment is available to lower LDL-C levels.[13] The most important therapy for patients with FH is therefore reduction of LDL-C even if conventional therapeutic goals are not achieved.

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Appendix

Ethics approval from the Committee for Research on Human Subjects, University of the Witwatersrand, for the clinical studies performed in this thesis.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 (Registry)

CLEARANCE CERTIFICATE PROTOCOL NO: 14/3/90

PROJECT: The effect of .-tocopherol (Vitamin E) on xanthomas in homozygous familial hypercholesterolaemia.

INVESTIGATORS: Dr F J Raal

DEPARTMENT: Medicine, Johannesburg Hospital

DATE CONSIDERED: 29 June 1990

RECOMMENDATION OF THE COMMITTEE: APPROVED

Date: 8 July 1990 Chairman:.....
Prof P E Cleaton-Jones

* Guidelines for written "Informed Consent" attached where applicable.

DECLARATION OF INVESTIGATOR/S

To be completed in duplicate and ONE copy returned to Miss S M Boshoff at Room 10-002, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorised to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

Date: 18/7/90 Signature:.....

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Raal

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 960526

PROJECT Carotid intima-media complex thickness in familial hyperchloresterolaemia

INVESTIGATORS Dr FJ Raal

DEPARTMENT Medicine,
Johannesburg Hospital

DATE CONSIDERED 960531

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 960605

CHAIRMAN. .....(Professor P E Cleaton-Jones)

c c Supervisor: Professor B Jaffe
Dept of Medicine, Johannesburg Hospital

==========

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE.....11/6/96.....SIGNATURE .....

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Raal

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 950822

PROJECT	A single centre, double blind, parallel two period dose escalation study to evaluate the efficacy and safety of high dose simvastatin (80 and 160mg) in patients with homozygous familial hypercholesterolaemia
INVESTIGATORS	Dr F J Raal
DEPARTMENT	Medicine, Johannesburg Hospital
DATE CONSIDERED	950825

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 951002

CHAIRMAN. .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr F J Raal
Dept of Medicine, Johannesburg Hospital

==========

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE....5/10/1995.....SIGNATURE .....

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Raal

CLEARANCE CERTIFICATE PROTOCOL NUMBER M 950821

PROJECT Open label, treatment protocol for the use of atorvastatin (CI-981) in the treatment of patients with severe hypercholesterolemia (protocol 981-80)

INVESTIGATORS Dr F J Raal

DEPARTMENT Medicine, Johannesburg Hospital

DATE CONSIDERED 950825

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 950929

CHAIRMAN. .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr F J Raal
Dept of Medicine, Johannesburg Hospital

==========

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and ONE COPY returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE....3/10/95.....SIGNATURE .....

Department of Medicine

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

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Fax No. (011) 643-8777

4 March, 1997.

Mrs. Pam Kissane
Secretary: Committee for Research on Human Subjects (Medical)
Division of Deputy Registrar Research
10th Floor, Room 10004
Senate House

FURTHER AMENDMENT TO CRHS PROTOCOL NO: M950821
'Open-label treatment protocol for the use of atorvastatin (CI-981) in the treatment of patients with severe hypercholesterolaemia (Protocol 981-80)'

Dear Mrs. Kissane,

In November 1996 I asked for approval of an amendment to Human Ethics protocol M950821 which would allow for more patients to be eligible for atorvastatin (CI-981). This amendment was acceptable to the Chairman of the CRHS.

I am now asking for approval of a further amendment - **ADDENDUM A** . This amendment will allow us to increase the dose of atorvastatin to a maximum dose of 160mg/day (not to exceed 2.5 mg/kg body weight).
Although most of our patients with familial hypercholesterolaemia have responded remarkably well to atorvastatin administered at 80mg/day with no adverse effects, many of our patients have not yet achieved satisfactory plasma cholesterol levels. The Pharmaceutical company, Parke-Davis, has therefore kindly agreed to allow us to increase the dose of medication in these patients. The increased dose of atorvastatin should lower the plasma cholesterol levels of our patients even further. The patients will be carefully monitored for any adverse effects of the higher dose of medication and the medication will be immediately stopped if any adverse effects are noted. All patients will also be asked to sign another consent form as the previous consent form indicated a maximum dose of 80mg atorvastatin/day.

A copy of the amendment is attached - **Addendum A.** I trust that this amendment meets with the approval of the Committee.

Yours sincerely,

DR. F.J.RAAL
FCP(SA), MRCP(UK), FRCPC
Consultant Physician

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division
of Deputy Registrar (Research)

M E M O R A N D U M

TO: Dr F J Raal
Department of Medicine
Medical School

FROM: Mrs Pam Kissane
Secretary: Committee for Research on Human Subjects (Medical)
Tel: 716-3556 Fax: 339-5708

DATE: 7 April 1997

REF: R14/49

Amendment to Protocol M950821: Open-label treatment protocol for the use of atorvastatin (CI-981) in the treatment of patients with severe hypercholesterolaemia (Protocol 981-80)

Addendum A of the above protocol dated 25 February 1997, has been approved by the Chairman of the Committee for Research on Human Subjects (Medical).

Thank you for keeping us informed.

Kissane

/pk

Letters of confirmation from co-authors to include articles as part of this thesis submission to the University of the Witwatersrand.

Department of Chemical Pathology

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26 July 1999

To Whom it May Concern:

I, Antonio Jose Areias, ID number 631112503306, do hereby confirm that Dr. Frederick J. Raal made a substantial contribution to the article entitled:

> *Areias AJ, Richardson J and Raal FJ. "Rapid method for measuring copper induced LDL oxidation" published in Eur J Lab Med 1995, 1:87-89.*

And agree that he include this article as part of Ph.D. submission to the University of the Witwatersrand.

Should you require any information please contact me.

Thank You

AJ Areias
MSc (Med)

POLYCLINIC
LITO
PARALIMNI

Dr Michael Zouvanis
Med (Ger) FCP (SA) M Med (Wits)
Specialist in Internal Medicine
Specialist Endocrinologist

29 July 1999

I, Dr Michael Zouvanis, confirm that Dr Frederick J Raal made a substantial contribution to the article entitled:

Zouvanis M, Raal FJ, Joffe BI and Seftel HC.
'Microalbuminuria is not associated with cardiovascular disease in patients with homozygous familial hepercholesterolaemia' published in Atherosclerosis 1995; 113:289-292, and agree that he can include the article as part of his PhD submission to the University of the Witwatersrand.

Yours sincerely

Dr Michael Zouvanis

8 SOTIRAS AVE. P O Box 251, 5312 PARALIMNI, CYPRUS, TEL 03 825050 (6 LINES), FAX 03 825060