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Endothelial dysfunction in familial hypercholesterolaemia

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Research Report

ENDOTHELIAL DYSFUNCTION IN FAMILIAL HYPERCHOLESTEROLAEMIA

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand in fulfillment of the requirements for the Degree of Master of Medicine in the branch of Internal Medicine.

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2004

Declaration

I, Susan Lynn Brown, declare that this research report is my own work. It is being submitted for the degree of Master of Medicine in the branch of Internal Medicine, University of the Witwatersrand, Johannesburg.

It has not been submitted before for any degree or examination at this, or any other, University.

.....Susan..... signature

.....25th..... day of .....May.....2004

Publications and presentations arising from this research report.

High dose atorvastatin therapy is required for significant improvement of endothelial function in heterozygous familial hypercholesterolaemic patients.

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ABSTRACT

Untreated patients with heterozygous familial hypercholesterolaemia (hFH) are at increased risk for atherosclerosis. Surrogate markers to predict atheroma include the presence of endothelial dysfunction (ED) as well as increased levels of inflammatory markers. The aims of this study were to document the presence of ED in untreated hFH patients via measuring flow mediated vasodilation (FMD); to measure their inflammatory markers and to determine if by treatment with high (80 mg/day) and low (20 mg/day) dose atorvastatin, these parameters may be changed. Secondly, to determine whether there is a correlation between the fall in LDL cholesterol, the major atherogenic lipid fraction, and the improvement of ED. At baseline, FMD in 23 untreated hFH patients was significantly reduced (mean ±SD=3.09±0.91%) compared with 10 normocholesterolemic control subjects (8.71±2.41%; $p<0.01$). FMD improved modestly on atorvastatin 20 mg/day to 5.60±1.17% (not significant), and to 8.54±1.11% ($p<0.01$) on 80 mg/day. LDL-C decreased markedly (-42.4%, $p<0.0001$) on 20 mg/day and decreased further (–48.6%, $p<0.05$) on 80 mg/day. FMD improvement, however, did not correlate with reduction in LDL-C with either the 20 mg or 80 mg/day dose of atorvastatin. No significant changes occurred in any of the inflammatory markers measured. ED is therefore present in untreated FH

patients and improves significantly with high dose atorvastatin. There was no correlation between the changes in FMD and LDL-C, suggesting either a LDL-C independent effect of atorvastatin on ED, or that a marked reduction in LDL-C is required in order to normalize ED in FH.

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INTRODUCTION

A raised LDL cholesterol (LDL-C) is one of the major risk factors for the development of atherosclerosis. However, most people that develop atheroma have multiple contributing risk factors such as concomitant hypertension, diabetes, abdominal obesity, and a smoking history. In these subjects the LDL-C values may be only mildly elevated or even within the conventional normal range. In order to determine the relative importance of LDL cholesterol alone, in contributing to the subsequent development of atherosclerosis, the study reported in this research report was performed in a population without other traditional cardiovascular risk factors, but with high serum LDL cholesterol due to a genetic defect – namely heterozygous familial hypercholesterolaemia (FH).

Untreated FH patients develop atherosclerosis at a young age and are thus candidates for primary preventive strategies of pharmacological or mechanical cholesterol lowering. Numerous LDL-C lowering drugs are available, which fall into 3 main classes. Firstly, the HMG CoA reductase inhibitors, or statins, secondly, the fibrates and lastly the bile-acid sequestrants. The statins have an excellent safety profile, have the most potent LDL-C lowering effect, and have been shown to reduce mortality and improve survival (1,2) and are therefore the conventional first line of therapy.

Mechanical methods of cholesterol lowering include plasmapheresis or LDL-apheresis – an effective, although expensive procedure, and is not available at the Johannesburg Hospital.

The target to which LDL should be lowered in order to prevent premature atherosclerosis is not yet determined as large outcome studies with cardiovascular endpoints require a prolonged period of observation and are still in progress. As a consequence, surrogate markers of elevated risk of progression of atherosclerosis have been devised. These may be regarded as guides as to whether the treatment given to a patient will eventually result in a reduction of the risk of progression of atherosclerosis in that individual. Surrogate markers of the risk of progression to eventual atherosclerosis include measures of endothelial dysfuntion, markers of inflammation and coagulation, and determination of the thickness of arterial walls (3,4,5,6).

The endothelium has assumed an increasingly prominent role in the pathogenesis of atherosclerosis. Initially considered a simple barrier between the blood and interstitium, it now appears to be the point of initiation and progression of atherosclerosis due to its significant endocrine, paracrine and autocrine activities (7,8,9). Surface receptors of the endothelium are able to detect alterations in the biochemical constituents of blood and haemodynamic changes and then instruct the surrounding cells and underlying smooth muscle

cells to respond physiologically to alter blood flow, and/or change vascular permeability and/or haemostasis to provide appropriate responses to the detected stimulation. Basal vascular tone is also maintained by a controlled balance between vasodilators and constrictors produced by the endothelium. Unfortunately this process may be altered under pathological stimuli to allow for the development and progression of atheroma. The response of the endothelium to stimulation may be direct – via its production of endothelial adhesion molecules, or indirect, via the production of cytokines, growth factors, vasomodulatory and haemostatically active compounds – that will act on surrounding and distal tissues.

This complex activity of the endothelium may be usefully manipulated in order to determine its state of health. This may be done by artificially presenting it with a stimulus that would provoke an expected in vivo response. Sheer stress is one such stimulus. The latter stimulates opening of calcium-activated potassium channels, that allow for cell depolarization and calcium entry, which in turn activates endothelial nitric oxide synthase (eNOS) (10). The nitric oxide (NO) then produced by the endothelium results in relaxation of the underlying vascular smooth muscle and vasodilation. This response may be acute – in response to immediate intracellular calcium elevations, or over minutes – secondary to shear-stress induced

phosphorylation of eNOS resulting in an increase in its activity, or over hours secondary to changes in eNOS gene transcription (10).

Endothelial cell surface receptors are able to detect ambient intravascular pressures and, on demand, alter the underlying smooth muscle tone to produce either vasodilation or vasoconstriction. This demand may be chemical – via the presence of acetylcholine, or mechanical – via alteration of sheer stress, and the response may be via appropriate production of vasodilatory intermediates - such as NO or vasoconstrictor substances – such as endothelins.

The importance of the endothelium in engineering vasodilation was first described in 1980 in *Nature* by Furchgott and Zawadzki (11) who showed that an intact endothelium was necessary for arterial smooth muscle relaxation and vasodilation of rabbit aortic rings in response to exposure to acetylcholine. The chemical mediator for this reaction was initially labeled endothelium-derived relaxing factor (EDRF) and was only identified as NO in 1987 (12). Of note, was that when rabbit aortic rings were exposed to acetylcholine without an intact endothelium, vasoconstriction occurred.

Nitric oxide generated from L-arginine in endothelial cells via the activity of the enzyme NOS, is important for both the maintenance of vascular tone and

in allowing for vasodilation on demand. It is present therefore, as a result of constitutively active as well as inducible NOS. The demand for vasodilation may be as a result of a number of stimuli including increased shear stress due to increased blood flow, bradykinin, thrombin or acetylcholine. Blocking NO formation, via arginine analogues, such as N-monomethyl-L-arginine (L-NMMA), and direct administration of NO to the endothelium are both useful tools used in research to further clarify the role of NO in the presence of a given stimulus.

Endothelial dysfunction appears to be an early event in the development of atherosclerosis occurring prior to any detectable plaque and this may be quantified using non-invasive and invasive means.

Assessing endothelial function in vivo in humans was first described by Ludmer et al (13) in 1986 who used quantitative coronary angiography to assess the response of the arteries before and after an infusion of acetylcholine and contrasted this with a response to infusion with nitroglycerin – an exogenous source of NO.

As this form of endothelial assessment was invasive, potentially dangerous, time consuming and subjective, the focus shifted to looking at more accessible vessels in the periphery and how to assess them in a more objective

reproducible manner. To this effect, Celermajer et al, published an article in the *Lancet* in 1992 (3) in which he described a non-invasive method of testing endothelial function. Using high-resolution ultrasound to assess the diameter of the superficial brachial and femoral arteries at rest, and during and after stimulation, by reactive hyperaemia as well as sublingual glyceryl trinitrate, the degree of responsiveness of the endothelium could be quantified in a reproducible and relatively non-invasive fashion.

Measurement of brachial artery flow-mediated dilation (FMD) with high resolution ultrasound (14,15) is now established as a non-invasive measure of endothelial function. The theory behind this form of endothelial function assessment, is that reactive hyperaemia, induced by distal forearm occlusion, results in increased flow in the brachial artery. The subsequent sheer-stress induced by the increased flow on the endothelium results in increased NO production and release, inducing vasodilation which may be accurately and reproducably measured by high resolution ultrasound. Parameters that interfere with endothelial function such as elevated LDL cholesterol – may be manipulated to change FMD from abnormal to normal and thus be presumed to act as a marker for reducing the patients risk for the subsequent development of atherosclerosis.

The use of high resolution ultrasound to assess vessel diameter appears to be reliable with a resolution of about 0,1 – 0,2 mm and experienced operators have been shown to have a low coefficient of variation for measurements of arterial wall anatomy and vessel diameter of between 1-3% (3). In blood vessels of between 3-6 mm in diameter the normal vasodilatory response to shear stress is in the order of 10-12% (3). These values correlate well with invasive intra-coronary testing.

As this technique has become widely accepted as a form of assessing endothelial function, the American College of Cardiology put together an international Task Force to formulate guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery which were published by the *American College of Cardiology* in January of 2002 (10). In these guidelines, Corretti el al, detail the required technique for measurement of FMD.

The patient should have fasted for at least 8 to 12 hours, specifically avoiding caffeine, high fat food, vitamin C and high impact exercise. The patient should also not have smoked cigarettes for at least 4-6 hours prior to the study. In premenopausal women, the time of their menstrual cycle should be noted and all subsequent comparisons be performed at the same time. The investigation should be performed in a quiet, temperature controlled room to

minimize environmental sympathetic stimuli and the sonographer should be comfortable and relaxed.

The equipment required to perform an endothelial assessment should include vascular software for two-dimentional imaging, colour and spectral Doppler, an integrated electrocardiogram (ECG) monitor and an ultrasound transducer with a minimum frequency of 7 MH_z arranged in a linear array. These requirements are to ensure that sufficient resolution will be obtained on images to allow for determination of significant changes in vessel diameter. The operator should be experienced and his, or her, inter- and intra-patient value reproducibility should be periodically assessed.

The positioning of the subject must be such that the antecubatal fossa is easily accessible so that the brachial artery can be identified just proximal to the latter and well visualized in the longitudinal plane. A segment of the artery should then be selected which should have surrounding identifiable landmarks – such as associated veins – and have clearly seen anterior and posterior walls. A baseline assessment of vascular diameter may then be taken noting the timing of the ECG.

To start the FMD assessment, a blood pressure cuff is placed either above the antecubital fossa or on the forearm. This cuff should be inflated to supra-

systolic pressures to occlude blood flow for a variable length of time – usually 3-5 minutes. If the cuff is placed below the antecubital fossa, the resulting change is less but, is also less affected by patient movement and interference with changes in the position of the probe.

The distal ischaemia induced by vascular occlusion results in accumulation and release of vasoactive substances. Hence, on release of the cuff, blood flow will increase resulting in an increased sheer-force in the brachial artery. This increased blood flow is the stimulus for endothelial-dependent vasodilation which is the value measured. Vasodilation peaks approximately 60 seconds after release of the cuff, the optimal timing of the FMD-measurement. In order to assess endothelial-independent NO stimulated vasodilation, exogenous NO may be given at least 10 minutes later and its effects measured.

The importance of ECG timing is that peak systolic vessel diameter is larger than end systolic diameter. The magnitude of this difference is affected by vascular compliance which may in turn be influenced by the presence of underlying atheroma which can occur with advanced age, diabetes and hypertension.

The size of the vessel to be examined is also important. Smaller vessels change more under stimulation than larger vessels. Baseline vessel size, absolute diameter change, and percentage diameter change, should therefore all be noted to accurately determine the significance of any diameter change measured.

Factors that may influence endothelial function include drugs, such as the statins and angiotensin converting enzyme inhibitors (ACE), hormones such as estrogens, as well as concomitant diseases such as hypertension, diabetes and hypercholesterolaemia (6,16). Harmful factors such as hypercholesterolaemia have also been demonstrated to be inducers of inflammation (5,17) resulting in increased thrombogenicity, cellular proliferation and consequent endothelial dysfunction – all of which contribute to eventual atherosclerosis. Conversely, beneficial substances such as statins and ACE inhibitors reduce these factors, improve endothelial function and are associated with a reduction in atherosclerosis.

Elevated LDL cholesterol has been well established as a cause of endothelial dysfunction (3,7,18). This association is more pronounced with oxidized LDL, which is produced under conditions of inflammation. Oxidized LDL causes endothelial dysfunction by a number of mechanisms including increased expression of endothelial adhesion molecules. Circulating soluble

adhesion molecule concentrations may be considered a reflection of endothelial adhesion molecule production, and thus inflammation, and may be considered a predictor of the presence of, or subsequent development of, atherosclerosis (19). Cellular adhesion molecules (CAM) play a role in recruitment of inflammatory cells in the process of inflammation which is involved with the pathogensis of atherosclerosis (5).

Adhesion molecules may be divided into 3 major groups including firstly the selectins (P and E - selectin) which slow rolling leucocytes at the site of inflammation. Secondly the integrins which mediate cell adhesion via interaction with ligands of the immunoglobulin gene superfamily. The latter form the third class of adhesion molecules and they are located on leucocytes and platelets. The immunoglobulin gene super family class of adhesion molecules includes vascular cell adhesion molecule – (VCAM-1), intercellular adhesion molecules – 1 and 2 (ICAM - 1, 2) and platelet-endothelial cell adhesion molecule (PECAM).

Ambient levels of plasma CAMs may be measured at baseline and after some form of intervention to determine whether or not that intervention may have altered the local inflammatory milieu. Another marker of inflammation is highly sensitive hsCRP which is an acute phase reactant produced in the liver in response to pro inflammatory cytokines, such as interleukin 6. Highly

sensitive CRP may therefore be considered as a possible marker of the presence of inflammation but it may also play an active role in the genesis of atherosclerosis. Highly sensitive CRP binds to modified LDL in atherosclerotic plaques thereby activating compliment and facilitating plaque progression. Furthermore, hsCRP impairs endothelial function directly, possibly by decreasing eNOS expression, as demonstrated in cultured endothelial cells (20). Modulation of these markers of inflammation with treatment of elevated LDL-cholesterol may thus be useful as an additional surrogate marker, of the target LDL to aim for with treatment, in order to minimize subsequent development or progression of atherosclerosis.

AIMS OF STUDY

The aims of this study were firstly to demonstrate the expected endothelial dysfunction in untreated FH patients with high cholesterol via measurement of FMD of the brachial artery with high resolution ultrasound as compared to a group of matched healthy controls with normal cholesterol levels. Secondly, to treat the patients with high (80 mg/day) and low (20 mg/day) dose atorvastatin in order to lower serum LDL and to look for an improvement, or normalization, of endothelial function as the lipid levels fall. Thirdly, to determine if there is a correlation between the fall in LDL and change in endothelial function or if statin drug therapy per se may be involved independently of the LDL change in altering endothelial function. Lastly, levels of soluble inflammatory markers (sVCAM-1, sICAM-1, E selectin and hsCRP) were measured at baseline and after treatment, to determine if they are raised off treatment and if they change with the expected statin treatment-induced fall in LDL.

METHODS

Patients

Twenty three patients (12 men, 11 women) aged less than 40 years with heterozygous FH were recruited from the lipid clinic at the Johannesburg Hospital. The diagnosis of heterozygous FH was based on the presence of a family history of hypercholesterolaemia, clinical signs of FH, together with an elevated serum LDL-cholesterol level and confirmation by DNA analysis of FH LDL-receptor mutations common in South Africa (21). Exclusion criteria included known coronary, cerebro- or peripheral vascular disease, hypertension (blood pressure >130/85 mmHg), a history of cigarette smoking within three months of enrolment, and current treatment with vasoactive or antioxidant medication. Lipid lowering drug therapy was discontinued for a minimum of three months prior to the start of the study. All patients were counselled on a standard low-cholesterol, low-saturated fat diet. The diet was reinforced at each visit to avoid the confounding effect of diet on lipid levels and endothelial dysfunction during the study. Carotid intima-media thickness and the presence or absence of carotid plaques was also noted in these patients.

In addition, 10 healthy normocholesterolemic control subjects (4 men, 6 women) with no established risk factors for coronary artery disease were studied. These controls were age and weight-matched to the FH patients.

None of the female patients or controls were on oral contraceptives or hormone replacement therapy at the time of the study; two of the male patients were taking aspirin (81 mg/day). 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.

Study Design

At baseline, after a 10 hour overnight fast, FMD was measured in the patients and control subjects. Thereafter blood samples were taken for analysis of lipids, circulating endothelium-related adhesion molecules and hsCRP, the carotid arteries were also visualized . Patients were then randomized into two groups. Group 1 (n=12) was treated with atorvastatin (Pfizer SA) 20 mg/day for three months followed by 80 mg/day for the next three months. Group 2 (n=11) was given 20 mg/day atorvastatin for one month, 40 mg/day for one month, and 80 mg/day for one month, followed by 20 mg/day for the next three months. This cross-over design was used to ensure that the changes observed were related to the dose of atorvastatin that was being taken and to exclude the possible carry-over effect of high dose atorvastatin. The reason for up titration of the atorvastatin doses in Group 2 rather than initiating with 80 mg for 3 months and then changing to 20 mg for three months was due to concerns with starting with what was considered the maximum dose of the

drug may not be medically correct. Compliance with study medication was assessed with pill counts at the end of each treatment period. Measurements of FMD, lipids, adhesion molecules and hs CRP were repeated at three months and six months in Group 1, and monthly for three months and at six months in Group 2. Safety parameters (muscle and liver enzymes) were also tested at these times. Carotid artery intima-media thickness (IMT) was measured in the patients and controls as a quantitative preclinical marker (20).

Measurement of FMD

The technique for measuring FMD has been described previously (6,10,14,15). Briefly, patients were measured supine in a clinical laboratory maintained at 21 to 23°C. To avoid the possible confounding effect of estrogen on FMD, female participants were measured in the luteal phase of the menstrual cycle. The brachial artery was identified above the left elbow and was measured to ensure a baseline diameter between 2.5 – 5 mm. Once the artery was identified, a B-mode scan using a high resolution 7.5 MHz linear array transducer (Hewlett Packard, Palo Alto, California) was performed to obtain baseline measurements at four separate times at the same point in the artery in end-diastole at the onset of the R wave. Pulsed wave Doppler arterial flow measurement was also taken from the centre of the artery. A distal pneumatic cuff was then inflated to 250 mmHg for 4.5 min. B-mode scanning was repeated at 30s prior to, and 60s post, cuff inflation.

The last scan was measured at 10 min post cuff deflation. Nitroglycerine (0.4 mg) was given sub-lingually after the procedure to ensure that endothelial independent dilation was normal. FMD data are expressed as the percent change from baseline diameter measurement of the artery (normal range in our laboratory: 6-12%). All FMD measurements were done by the same technician who was blinded as to the subjects' drug therapy or previous FMD measurements, with an intra-observer variation of <5%.

Biochemical Analysis

Fasting blood samples were collected following the FMD measurements. The samples were centrifuged and the separated serum aliquots were stored at -70°C until analyzed. Quantitative determinations of circulating endothelium-related adhesion molecules: soluble intercellular adhesion molecule –1 (sICAM-1), soluble vascular cell adhesion molecule –1 (sVCAM-1) and soluble E-selectin (sE-Selectin) were performed by enzyme linked immunosorbent assay (ELISA) using kits supplied by R & D Systems, Minneapolis, Minnesota. Inter-assay coefficients of variation (CV) were 10.8% for sICAM-1 (normal range: 115-306 ng/ml), 6.7% for sVCAM-1 (normal range: 395-714 ng/ml) and 8.1% for sE-Selectin (normal range: 29.1 – 63.4 ng/ml). Highly sensitive CRP was measured by monoclonal antibody agglutination using the N Latex CRP mono kit (DADE Behring, Marburg, Germany). The upper limit of the normal range in healthy adults is 5.0 mg/l

and the intra-assay CV was 4.4%. Enzymatic, colorimetric methods were used to measure total cholesterol, high density lipoprotein (HDL) – cholesterol and triglycerides, employing an Hitachi autoanalyzer and reagents supplied by Boerhringer Mannheim, Mannheim, Germany. Intra-assay CV for each of these assays was <5%. LDL-C values were calculated according to the formula of Friedewald et al (22).

Measurement of carotid IMT

The carotid arteries of patients and controls were evaluated once at the start of the study with high-resolution B-mode ultrasonography using a previously validated technique (23). 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. Carotid IMT was defined as the average of 5 measurements randomly selected between 10 and 30 mm proximal to the carotid bifurcation. The same observer performed all the measurements and was blinded as to the subjects' drug therapy or previous carotid ultrasound findings. Using this technique, the intra-observer variation was 5.7%.

Statistics

Statistical analyses were performed using GB-STAT (Dynamic Microsystems, Inc, Silver Spring, Maryland, USA), with a value of $p<0.05$ considered significant. Comparisons were made by one-way analysis of variance and repeated measures of analysis of covariance, the Students' t-test for paired samples and the Wilcoxon Signed Rank Test for unpaired samples. Results are expressed as mean ± SEM for parametric data, or as median and range for data that were non-parametrically distributed.

RESULTS

Baseline characteristics of patients in Groups 1 and 2 and control subjects are detailed in Table 1. All groups were matched for age and BMI. Mean FMD was reduced significantly at baseline in both patient groups compared with control subjects ($p<0.05$). As expected, mean total cholesterol and LDL-C levels were significantly higher in both groups of untreated patients than in the control subjects ($p<0.0001$). Carotid IMT was greater in the FH patients than in the controls but this was not significant. Since results of these and other variables in Groups 1 and 2 were the same at baseline (Table 2), and the improvement in each group was similar after treatment with different doses of atorvastatin and was not affected by the duration of therapy, the two groups were combined for further analysis. In untreated patients, FMD was significantly reduced (mean ±SD=3.09±0.91%) compared with 10 normocholesterolaemic controls (8.71±2.41%; $p<0.01$).

Changes in FMD and LDL-C following atorvastatin treatment are shown in Figure 1 and Table 3. Mean FMD improved significantly from baseline only with 80 mg/day ($p<0.01$) but not with atorvastatin 20mg/day (5.60±1.17%). A dose of 20 mg/day produced a marked decrease from baseline (-42.4%) in the mean LDL-cholesterol level ($p<0.0001$), with a further small (6.2%), but

significant ($p<0.05$) reduction with 80 mg/day. ANOVA showed no correlation between LDL-C reduction and improvement in FMD at either the 20 mg or 80 mg/day dose of atorvastatin.

No significant differences were found in concentrations of circulating endothelium-related adhesion molecules or hs CRP between patients and controls at baseline or with different doses of atorvastatin (Table 3).

Safety analysis.

Liver enzymes were noted to be significantly elevated in one patient in Group 2 that had received 80 mg for one month. After reduction of the dose, as per protocol, to 20 mg/day, the results returned to normal levels within 3 months. Symptomatically the patient had noticed a headache but no clinically significant manifestations of hepatic dysfunction were noted on examination. No other adverse effects were reported and all patients completed the study.

DISCUSSION

The finding that untreated heterozygous FH patients with high LDL-C, but without clinical manifestation of atherosclerotic disease, have ED is in agreement with previous studies (3,4). The demonstration of a significant improvement in ED after lowering LDL-C is also consistent with the current literature (24,25,26). Cholesterol reduction has been shown to improve ED as rapidly as one hour after LDL-apheresis (25) and within two weeks of statin therapy (27) emphasizing the important effect that elevated LDL-C has on endothelial function. However, like others (28), no relationship was found between the improvement in ED and reduction in LDL-C. The results of this study show that most LDL-C reduction was achieved on low dose atorvastatin (20 mg/day), whereas high dose therapy (80 mg/day) produced a small, albeit significant, further reduction in LDL-C. Endothelial dysfunction, on the other hand, improved significantly only on high dose atorvastatin.

In so far as attributing this improvement to a reduction in the level of LDL-C, one endothelial effect of statin therapy is to directly influence bioavailable NO levels via modification of endothelial NO synthase (eNOS), which may be adversely reduced by ambient levels of LDL-C (29,30). An important regulator of eNOS and NO availability is the protein caveolin, which in the

resting state suppresses NO activity. Endothelial activation increases cytosolic calcium, thus promoting a reversible dissociation from caveolin and binding to calmodulin that activates eNOS (31,32). A critical level of LDL-C may have to be reached before the beneficial effect of statin therapy on eNOS can be achieved, and this might explain why only high dose atorvastatin was effective in improving ED in these FH patients. Similarly, the direct effect of LDL-C on increasing endothelial superoxic (O_2)-production, thereby lowering ambient NO may also have a threshold effect (33). Furthermore, studies in which ED improved as a result of lowering LDL-C without the use of statins via LDL apheresis (25) or dietary manipulation (34) (in rabbits) also underscores the importance of the LDL-C level in contributing to ED, and confirms that reduction in LDL-C produces an improvement in ED. This study supports the suggestion of a threshold effect noted by Shecter et al (24), who demonstrated that ED improved significantly at a LDL-C level of ≤100 mg/dl (2.6 mmol/l) compared with LDL-C of >100 mg/dl, in accordance with the latest National Cholesterol Education Program (NECP) guideline target for cholesterol management (35).

Another indication of a critical threshold of LDL-C reduction is suggested by the finding of carotid IMT regression in FH subjects after treatment with atorvastatin 80 mg/day and a mean reduction in LDL-C of 45% compared with 40 mg/day simvastatin with an LDL-C reduction of 41%, in which

progression of carotid IMT was still demonstrated (23). Extrapolating a change in carotid IMT to a change in cardiovascular disease risk seems feasible in view of previously documented correlations (36,37). Moreover, if ED precedes carotid IMT, it might be considered an even earlier surrogate marker for atherosclerosis, and indicate a threshold for intervention to reduce LDL-C so as to lessen the patient's ultimate risk for cardiovascular disease.

The second theory offered in explanation of the findings is that of LDL-C independent effects of statins on endothelial function. Much work on various statins has been done in this field (9,17,20,32,38), and various mechanisms have been identified and include direct modulation of eNOs levels and activity; modulation of the renin-angiotensin system; decreased production and expression of endothelin - 1 (ET-1) and reduction of pro-inflammatory molecules such as superoxide anion. The resultant reduction in a pro-inflammatory milieu may explain the observed reduction in inflammatory cells in atherosclerotic plaque as a consequence of reduced cytokine, CRP and adhesion-molecules expression. In order to address some of these pleotrophic effects of statins, markers of endothelial activation (sVCAM-1, sICAM-1 and sE-Selectin), and inflammation (hsCRP) were measured. However, these markers were unchanged, despite an improvement in ED (table 3).

A further LDL-C independent statin effect is by increasing levels of bioavailable eNO via a direct effect on constitutive NO synthase (39). Statin-induced increased mRNA expression and mRNA stability results in up-regulation of eNOS activity independent of LDL-C modulation and may further explain why high dose atorvastatin therapy was required for significant improvement in endothelial function. Similarly, other metabolites of the mevalonate pathway which are reduced by statins may also play a role in increasing bioavailable eNO, further reducing the activation of coagulation and inflammation cascades (16,29) (fig 2). Statins, by inhibiting L-mevalonic acid synthesis, prevent the synthesis of other important isoprenoid intermediates and effectively reduce the activity of small guanosine triphosphate (GTP) binding proteins Ras/Rho, thereby improving vascular function (20,32,40) (Fig 2).

Rho and Ras are small proteins that are inactive in the cytoplasm bound to guanosine diphosphate (GDP) but are activated by being translocated to the cell membrane and associated with GTP, this translocation being facilitated by prenylated proteins. If no protein prenylation occurs due to statin-inhibition there is a consequent reduction in the expression of adhesion complexes with decreased inflammation.

Limitations of the study

Certain limitations of the study warrant consideration. The results were obtained in a relatively small number of FH patients. However, repeated measurements were performed and the sample size was sufficient to detect a statistically significant improvement in FMD (10). The possible variability in ED due to the effect of estrogen on FMD in premenopausal women was also minimized by testing them in the luteal phase of the menstrual cycle. Since ED deteriorates with age (6), all subjects entered into the study were less than 40 years of age. As far as possible, other confounding effects such as cigarette smoking and hypertension that might have contributed to ED (6) were also excluded.

The reason for separating the study population initially into two groups, each of which received alternate low then high dose atorvastatin therapy or vice versa, was to exclude any carry-over effect of the high dose therapy. The fact that benefit was shown only on the high dose of atorvastatin suggests that ED improvement was dynamic, of relatively rapid onset (1 month) and of short duration (3 months).

CONCLUSIONS

In conclusion, ED was present in untreated heterozygous FH patients and improved following atorvastatin therapy. Most LDL-C reduction occurred with a low dose (20 mg/day) and there was a further small decrease with high dose (80 mg/day) therapy. ED, however, improved significantly only on the high dose, suggesting either an effect of atorvastatin on ED that is unrelated to LDL-C lowering, or that a further critical reduction of LDL-C is required to normalize ED in FH.

Unfortunately the lack of change in measures of inflammation (adhesion molecules and hsCRP) in this study could not be implicated to explain the findings, and other pleotrophic effects of statins may have been involved. These include direct modulation of NO levels, direct effects on inflammation, inhibition of hypercoagulability with a reduction in platelet aggregation, and adhesion, and liberation of substances that may contribute to ED.

Since ED is a predictor of subsequent cardiovascular disease, these results have important clinical implications. The findings of this study emphasize the importance of aggressive lipid-lowering in FH patients to prevent, or even reverse, the progression of atherosclerosis.

TABLE 1 Baseline characteristics of patients with familial hypercholesterolaemia (group 1 & 2) and control subjects.

Variable	FH Subjects		Control Subjects (n=10)
	Group 1(n=12)	Group 2(n=11)	
Gender (M/F)	7 / 5	5 / 6	4 / 6
Age (yrs)	34.5±3.0	35.0±3.1	37.6±2.2
BMI (kg/m^2)	24.3±1.2	25.4±1.1	24.0±1.6
FMD (%)	3.68±1.11$^{\$}$	2.55±1.45$^{\$}$	8.71±2.41
Cholesterol (mmol/l)	10.48±0.51*	9.85±0.54*	5.17±0.34
Triglyceride (mmol/l)	1.32±0.18	1.76±0.32	1.26±0.19
HDL-C (mmol/l)	1.38±0.10	1.16±0.08	1.54±0.15
LDL-C (mmol/l)	8.59±0.51*	7.87±0.49*	3.04±0.28
Carotid IMT (mm)	0.66±0.14	0.67±0.16	0.56±0.06

Data are expressed as mean ± SEM

$^{\$}$ $p<0.05$; * $p<0.0001$

BMI = body mass index; HDL-C = high density lipoprotein cholesterol; LDL-C = low density lipoprotein cholesterol; FMD = flow mediated dilation; IMT = intima-media thickness

TABLE 2 Brachial artery diameter and FMD change at baseline and on treatment in Group 1 and 2 analysed separately.

		Group 1	Group 2	P values
Baseline	Resting diameter FMD induced diameter % change	3.72(0.20) 3.86(0.21) 3.68(1.11)	3.63(0.15) 3.71(0.13) 2.55(1.45)	0.72 0.58 0.54
Atorvastatin 20 mg	Resting diameter FMD induced diameter % change	3.67(0.19) 3.87(0.21) 5.46(1.74)	3.66(0.15) 3.87(0.19) 5.75(1.64)	0.99 0.99 0.91
Atorvastatin 80 mg	Resting diameter FMD induced diameter % change	3.60(0.21) 3.90(0.22) 8.82(1.82)	3.68(0.15) 3.99(0.18) 8.24(1.32	0.76 0.77 0.80

Data is expressed as mean ± SEM

FMD = flow mediated dilation

Diameters measured in mm

TABLE 3 Changes in flow mediated dilation, LDL-cholesterol, circulating endothelium-related adhesion molecules and hsCRP following atorvastatin therapy – group 1 and 2 combined (n=23). Values are mean ± SEM or median (ranges).

	Baseline	Atorvastatin 20 mg/day	Atorvastatin 80 mg/day
FMD (%)	3.09±0.91	5.60±1.17	8.54±1.11**
LDL-C (mmol/l)	8.24±0.35	4.69±0.23***	4.17±0.24*
sICAM-1 (ng/ml)	241(149-363)	233(179-290)	244(199-293)
sVCAM-1 (ng/ml)	513(379-677)	503(412-748)	511(414-713)
sE-Selectin (ng/ml)	60(22-100)	55(27-105)	53(28-102)
hs CRP (mg/l)#	0.7 (0.3-7.2) (n=16)	0.9(0.3-4.7) (n=18)	0.7(0.3-7.9) (n=17)

* $p<0.05$ comparing with 20 mg/day value.

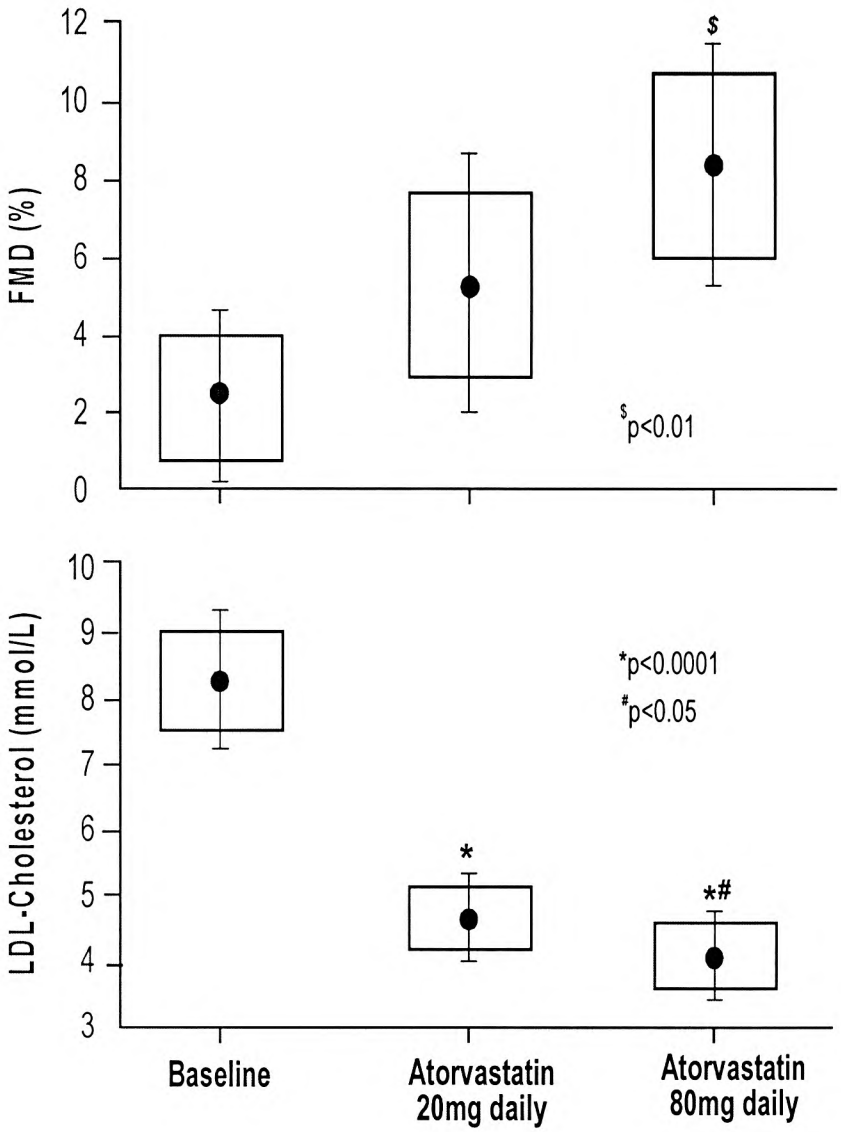
** $p<0.01$ comparing with baseline value.

*** $p<0.0001$ comparing with baseline value.

Values below the detection limit of the assay (0.2 mg/l) were excluded.

Abbreviation: FMD, flow mediated dilation; LDL-C, low density lipoprotein-cholesterol; sICAM-1, soluble intercellular adhesion molecule –1; sVCAM-1, soluble vascular adhesion molecule –1; sE-Selectin, soluble E-Selectin; hs CRP, highly sensitive C-reactive protein.

FIGURE 1


FMD (%)
12
10
8
6
4
2
0
$
$p<0.01
LDL-Cholesterol (mmol/L)
10
9
8
7
6
5
4
3
*p<0.0001
#p<0.05
*
*#
Baseline
Atorvastatin 20mg daily
Atorvastatin 80mg daily

LEGEND

Figure 1. Box and whisker plots showing median and 75% interquartile range of changes in FMD and LDL-cholesterol in patients with familial hypercholesterolaemia following treatment with atorvastatin.

$ $p<0.01$ Baseline vs 80 mg/day

* $p<0.0001$ Baseline vs 20 mg/day and 80 mg/day

# $p<0.05$ 20 mg vs 80 mg/day

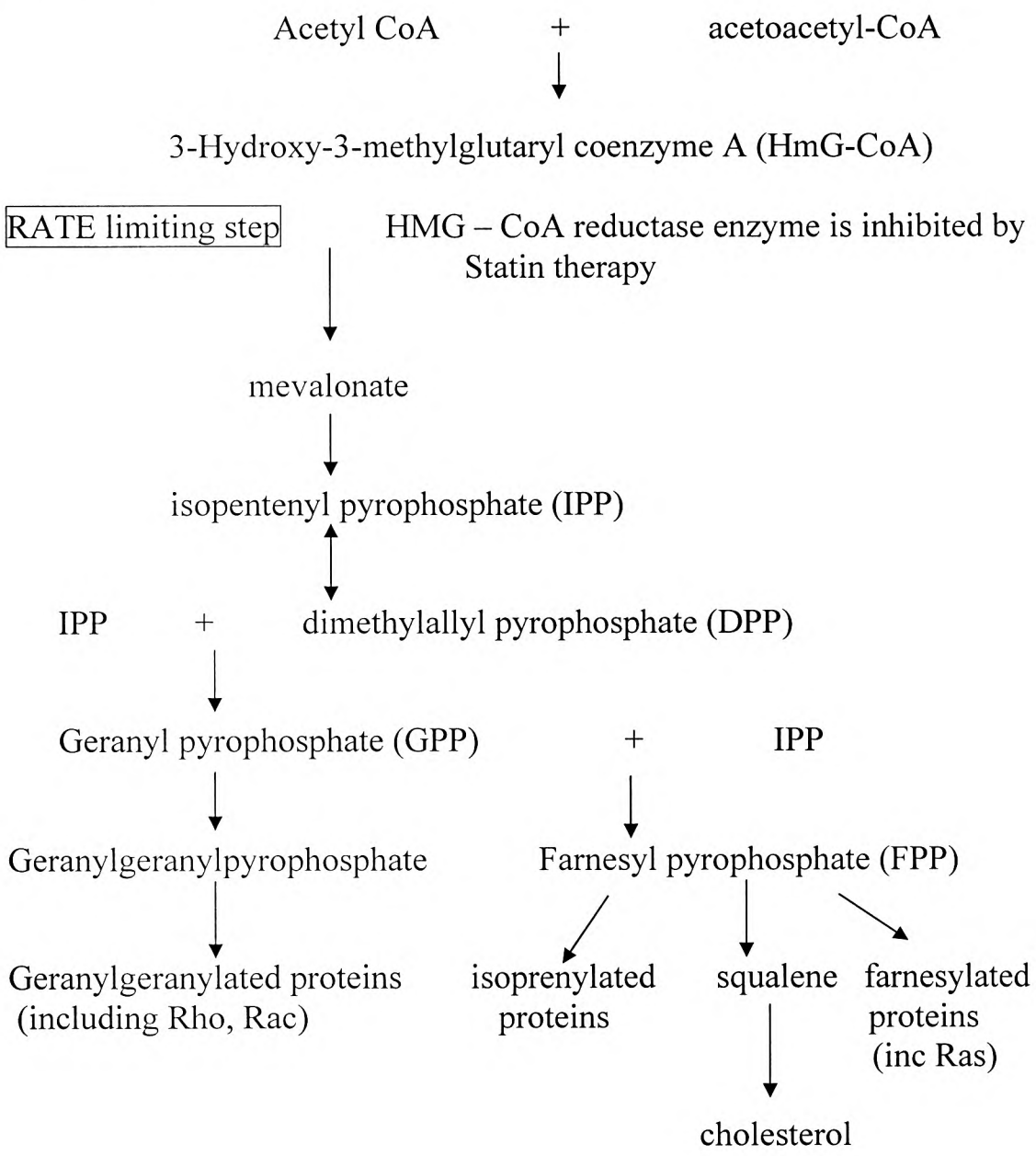
FMD = flow mediated dilation

- Median Value

□ 25% - 75%

I minimum-maximum values

FIGURE 2

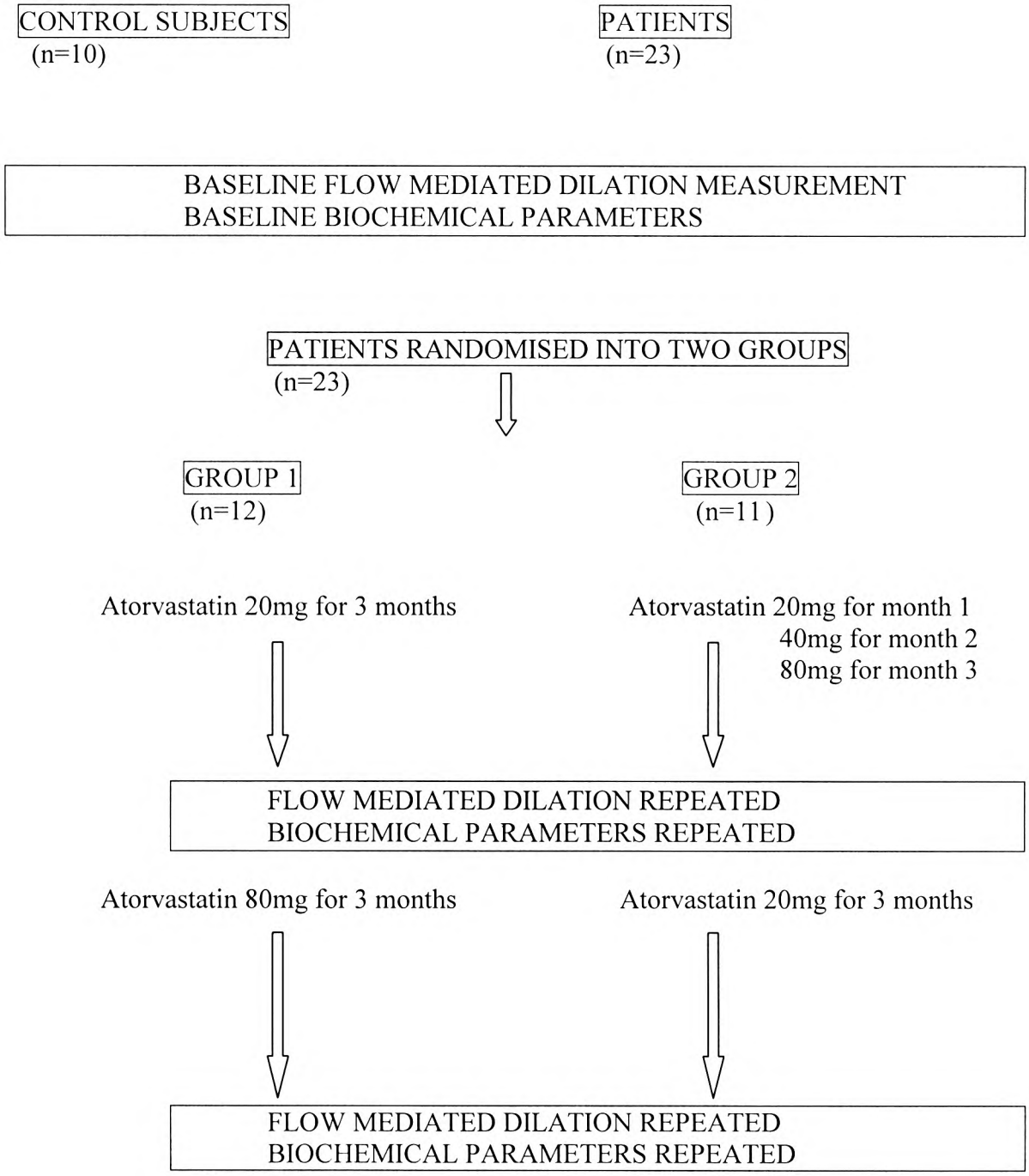


Modified from ref 20,32

LEGEND

Figure 2. A diagrammatic representation of the pathway of cholesterol and isoprenoid synthesis. Inhibition of HMC-CoA reductase reduces not only cholesterol production but all substances downstream including the isoprenoid intermediaries.

TRIAL DESIGN



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COPY OF ETHICS CLEARANCE CERTICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

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

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M980309

PROJECT Endothelial Dysfunctional In Familial Hypercholesterolaemia

INVESTIGATORS Dr SL Brown

DEPARTMENT Dept of Community Health, Dept of Community Health

DATE CONSIDERED 980327

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 980513 **CHAIRMAN**.....(Professor P E Cleaton-Jones)

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

c c Supervisor:
Dept of ,

Works2\Iain0015\HumEth97.wdb\M 980309
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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.