PHYTOSTEROL-ENRICHED MARGARINE SUBSTITUTION IN THE MANAGEMENT OF FAMILIAL HYPERCHOLESTEROLAEMIA

Submitted for the degree MSc (Med) Nutrition and Dietetics

By

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Declaration

I, Zarina Hamdulay, hereby declare that the work on which this thesis is based is my original work, unless indicated otherwise, and that neither the whole work or any part of it has been, is being, or is to be submitted for another degree in this or any other University.

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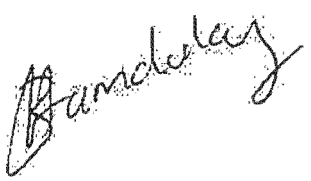

Signature

04 June 2003

Date

ABBREVIATIONS

ABC	: Adenosine triphosphate-binding cassette
ACAT2	: acylCoenzyme A:cholesterol:acyl transferase 2
B100	: Apolipoprotein B100
BA	: Bile acids
BMR	: basal metabolic rate
BSS	: Beta-sitosterol
BSSG	: Beta-sitosterol glycoside
C	: Cholesterol
CE	: Cholesterol esters
CETP	: Cholesterol Ester Transfer Protein
CHD	: Coronary heart disease
CM	: Chylomicrons
EER	: Estimated energy requirements
ER	: Endoplasmic reticulum
FBD	: Familial binding defective apolipoprotein B
FC	: Free Cholesterol
FFA	: Free Fatty Acids
FH	: Familial hypercholesterolaemia
GSH	: Groote Schuur Hospital
HDL	: High density lipoprotein
HMG-CoA	: 3-hydroxy-3methylglutaryl coenzyme A
IFNg	: Interferon gamma
IL4	: Interleukin 4
LDL	: Low density lipoprotein

LDLC : Low density lipoprotein cholesterol

LDLR : Low density lipoprotein receptor

LRP : LDLR related protein

LXR : Liver receptor X

MUFA's : Monounsaturated fatty acids

N 3 fatty acids : Omega 3 fatty acids

P : Phytosterols

PMA : Phorbol 12 myristate 13 acetate

PUFA's : Polyunsaturated fatty acids

RFLP's : Restriction length polymorphisms

SFA's : Saturated fatty acids

SREBPs : Sterol regulatory element-binding proteins

TC : Total cholesterol

TG : Triglycerides

UCT : University of Cape Town

VLDL : Very low density lipoprotein

WB : Whole blood

ABSTRACT

OBJECTIVE:

The primary objective was to substitute phytosterol and regular margarine into the diets of familial hypercholesterolaemia (FH) patients and to investigate whether the phytosterol containing margarine had a cholesterol lowering effect. Another objective was to investigate immune responses to phytosterols.

DESIGN:

A randomized double-blind cross-over study in which subjects with FH consumed 20g of regular or phytosterol margarine for a period of 6 weeks on each margarine, after which a washout period of 4 weeks was followed. There were 5 visits in total, visit 1 being the collection of baseline data, visit 2 the randomisation to either of the regular or phytosterol margarine periods, visit 3 for the cross-over to the alternative margarine form, visit 4 marked the onset of washout period and visit 5, was the last evaluation visit. Margarine use was in addition to current, albeit limited, pharmacological treatment with statins.

SETTING:

University of Cape Town Lipid Clinical Research Facility and Laboratory.

SUBJECTS:

FH subjects were selected on clinical and/or genotypic diagnosis. Of the total of 49 patients recruited, 5 did not meet the inclusion criteria. Forty-four patients were therefore eligible to participate in the study, but 2 patients withdrew from the study before visit 3. Therefore, a total of 42 patients completed the study. At visit 2, 22

patients were issued with the regular margarine and 20 patients were issued with the phytosterol-containing margarine. At visit 3, 20 patients were issued with the regular margarine and 22 were issued with the phytosterol-containing margarine. After consideration of compliance (diet, margarine and medication), and unexplained variances in serum cholesterol values, 12 patients were disqualified from the final analysis. Therefore, only 30 patients were included in the final analysis.

RESULTS:

Thirty subjects were analysed for the study. Compared to the regular margarine, total cholesterol was reduced by 6.4% (p=0.00), LDL-cholesterol by 6.5% (p=0.01) and triglycerides was reduced by 18% (p=0.01), all the above results being statistically significant. Dietary and anthropometric data did not differ significantly between the regular and phytosterol margarine period. The triglyceride concentration at baseline was not statistically significantly different from the triglyceride on phytosterol margarine, while the regular margarine appeared to induce a higher triglyceride concentration. The phytosterols did not alter immune function significantly.

CONCLUSION:

Significant reductions in total cholesterol and low density lipoprotein levels occurred with the phytosterol margarine compared with the regular margarine in FH subjects on partial lipid lowering treatment, without any significant differences in dietary or anthropometric data. High density lipoprotein levels remain unchanged. There was an unexpected response in triglyceride levels. Triglycerides were significantly higher on the regular margarine period.

The effect of the lowering of plasma lipids with the phytosterols is equivalent to doubling the dose of the lipid lowering statins. There was no change in immune function on the regular margarine period.

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2. INTRODUCTION

Familial hypercholesterolaemia (FH) is an autosomal dominant disorder that is caused by mutations in the LDL-receptor. It is usually accompanied by xanthomatosis and accelerated artheroslcerosis. Characteristically LDL-cholesterol levels are elevated, raising the total cholesterol to greater than 7.5 mmol/l (South African Medical Association and Lipid and Artherosclerosis Society of Southern Africa Working Group, 2000).

Only patients with severe dyslipidaemia (total cholesterol greater than 7.5 mmol/l and/or strong family history of heart disease) are selected for clinical evaluation and treatment at the Lipid Clinic at the University of Cape Town (UCT) and Groote Schuur Hospital (GSH), due to limited resources. The clinic sees a large number of indigent patients for whose pharmacalogic management there is an inadequate budget. The result is only partially successful lowering of cholesterol owing to the prescription of low doses of statins. There is also no budget for the addition of bile acid sequestrants, which are even more costly. Nicotinic acid use is also of limited benefit due to the notable side effects. Therefore, there is a need for finding alternative strategies of additional lowering of plasma cholesterol in these very high-risk subjects.

Phytosterols have been shown to have beneficial effects on lowering total and LDL-cholesterol levels (Moghadasian et al., 1999). The supplementation of plant sterols to the lipid management of these patients might therefore be beneficial in further lowering plasma low-density lipoprotein levels.

3. OBJECTIVES

The objectives of this study were to substitute phytosterol and regular margarine into the diets of FH patients and to investigate whether the phytosterol containing

margarine had a cholesterol lowering effect. A comparison of the lipid profiles by a double blind randomized and cross-over design should clearly establish whether the phytosterols have plasma lipid modifying properties.

An additional objective was to investigate whether the margarine had any effect on the immune response of FH patients.

4. LITERATURE REVIEW

4.1.1 PHYTOSTEROLS- SOURCES AND STRUCTURE

Phytosterols are found naturally in all plant products: nuts, seeds, plant oils, corn, rice and other plant sources. They are not synthesized in the human body. Phytosterols are structurally similar to cholesterol, but differ in their nuclear and/or their side chain configuration. The presence of a methyl or an ethyl group in the side chains leads to campesterol and phytosterol respectively (Heineman et al., 1991), see figure 1. Stigmasterol has an unsaturated bond in the side chain.

Phytosterol, campesterol and stigmasterol are the most abundant sterols in nature. Sitostanol and campestanol are formed by hydrogenation and are found in trace amounts in nature. Phytosterols are poorly absorbed from the human gut. Only small amounts, (0.3-1.7mg/dl) are found in human serum compared to daily intakes of 160-360mg (Vanhanen et al., 1993). Only 5% of phytosterols and 1% of plant stanols are absorbed (Jones et al., 1997).

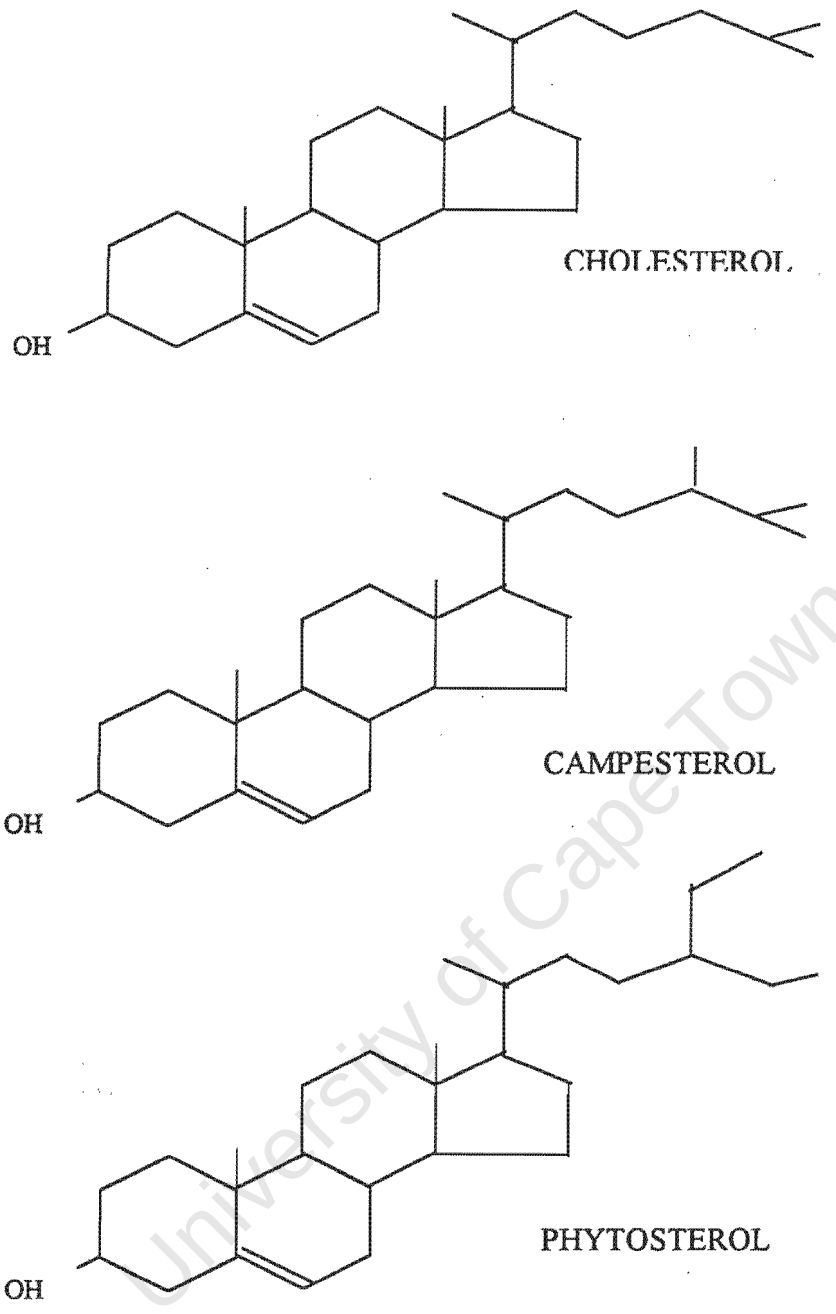


FIGURE 1
Chemical structures of cholesterol, campesterol and sitosterol.Chemical structures of cholesterol, campesterol and phytosterol (Heinaman et al,1999) Plant sterols similar in structure to cholesterol, the presence of ethyl group and methyl group leads to campesterol and sitosterol respectively.

Table 1- Effect of Plant Sterol/Stanol treatment on TC and LDL in Humans

Reference	Study design	Subjects, Age (y) mean ± SD	Treatment	Decrease in LDLC	Decrease in TC
Amundsen et al, 2002	Randomised, double-blind cross-over	38 FH childrem 7-12	1.46g stanol esters 8 weeks	10.2% ($p< 0.05$)	7.4% ($p<0.05$)
Becker et al, 1992	Not indicated	FH children 5-11	Sitosterol pastilles 6g/d for 3 months	18% ($p<0.05$)	17% ($p<0.05$)
Becker et al, 1993	Not indicated	FH children 10-15	Sitostanol 1.5g/day for 7 months	29% ($p< 0.05$)	23% ($p<0.05$)
Denke, 1995	Not indicated	Moderately hypercholesterolaemic 31-70	Sitostanol capsules (3g/day sitostanol for 3 months)	No significant reduction	No significant reduction
Gylling, 1996	Randomized	Hypercholesterolaemic Diabetic men 60 ± 4	Sitostanol-ester margarine (3g/day sitostanol for 7 weeks)	14% ($p< 0.05$)	11% ($p<0.05$)
Gylling, 1997	Randomized double-blind	Postmenopausal women with myocardial infarction 51 ± 5	Sitostanol margarine (3g/day sitostanol for 7 weeks)	20% ($p< 0.05$)	13% ($p< 0.05$)
Hendricks et al, 1999	Double-blind placebo controlled	Normo and mildly hypercholesterolaemic 37 ± 10	Plant-sterol enriched spreads (doses <1g->3g per day of sterol for 3.5 weeks)	7-10% ($p< 0.05$)	5-7% ($p< 0.05$)
Jones et al,	Randomized cross-over double blind	Hyperlipidaemic males 37-61	Sterol/stanol (1.84 g day sterol/stanol for 21days)	7.9 - 12.9%(p,0.05)	10.2-13.4% (p,0.05)
Westrate et al, 1998	Randomized double-blind placebo	Normo and mildly hypercholesterolaemmic	Sterols/sitostanol ester margarine 1.5g-3.3g/day for 3.3 week	14%	8%

4.1.2 CHOLESTEROL LOWERING EFFICACY OF PHYTOSTEROLS

The hypocholesterolaemic effect of phytosterols has been studied as early as 1956 (Peterson et al., 1956). Subsequently several studies have confirmed their findings (Table.1). Moghadasian et al. (1999) reviewed the results of 16 randomised controlled trials in which 590 subjects participated. Various phytosterols and phytostanol mixtures were used to reduce plasma cholesterol levels. Of the clinical trials reviewed, 5 were conducted on normo- and mildly hypercholesterolaemic adult subjects, 7 trials were performed on moderately hypercholesterolaemic adult subjects, 3 trials were done on FH children and two were done on hypercholesterolaemic diabetic adult patients. The ages of the adult patients ranged from 25-60 years old and the children were 5-15 years old. The average intake of phytosterol in these trials was 1-3g per day. The authors found on average, a 10% reduction in total cholesterol and a 13% reduction in LDL cholesterol, with no significant changes in high density (HDL) and triglycerides.

The largest response in LDL levels was seen by Jones et al. (1999) who studied 16 hypercholesterolaemic male subjects aged between 25-60 years in a randomised double blind trial consuming 1.5g of phytosterols for 4 weeks. They reported a 15% reduction in LDL and a 9% reduction in total cholesterol levels. The study reporting the smallest response was by Hendricks et al. (1999). They studied 100 subjects with a mean age of 37 years consuming three different doses (0.83; 1.61 and 3.24g/day) of phytosterols for 3.5 weeks. A reduction of 5-7% in total cholesterol and 7-10% in LDL cholesterol was reported (Hendricks et al., 1999).

In children, the largest response was seen in FH patients aged between 10-15 years consuming 1.5g sitostanol per day, showing a 29% reduction in total cholesterol and 23%in LDL cholesterol respectively (Becker et al., 1993). Another study on FH children compared the effect of diet (composition of diet not reported), sitosterol

(3x2g daily) and bezafibrate (2x200mg/d) on lipid profiles. Bezafibrate had the most pronounced effect on total and LDL values (-18%, -28% respectively, $p<0.05$) compared to sitosterol (-17% on total cholesterol and LDLC $p<0.05$) and diet (-4.5% not statistically significant, -6.6% $p<0.05$) (Becker et al., 1992). The combination of half the dose of bezafibrate (1x200mg) and sitosterol (3x1g/d) was as effective as the highest dose of bezafibrate with reductions of 50% and 40% on TC and LDL respectively persisting for 24 months. Amundsen et al. (2002) also showed a significant and persistent reduction in LDL cholesterol (-10%) and TC (-8%) in FH children using a sterol-enriched margarine.

Vurio et al. (2000) studied the effect of margarine containing sitostanol in families with the North Karelia mutation. Four different groups were studied: 24 children, 4 parents, 16 healthy family members and 12 adults on statin therapy. They showed lowering of total cholesterol and LDLC levels as follows, 14% and 18%; 8% and 7%; 7% and 14% and 14% and 20% in the four groups respectively.

The addition of plant stanols to statin therapy therefore proved to be effective at further lowering cholesterol values in FH patients as well as in moderately hypercholesterolaemic patients. Gylling and Miettenen (1996) showed that there was an additional four percent lowering in LDL cholesterol with phytosterols in moderately hypercholesterolaemic diabetic men who were on Pravastatin and consumed 3g Sitostanol/d for 7 weeks. In another study performed on mildly hypercholesterolaemic postmenopausal women with coronary heart disease (CHD) on Simvastatin, there was an additional 11% reduction in LDL concentrations with the consumption of 3g of sitostanol, for 7 weeks. (Gyling et al., 1997)

Hendricks et al. (1999) looked at the dose response relationship of phytosterols. Subjects were given three phytosterols containing margarines, each with a different

dose of sterol, for 3.5 weeks at each dose. The doses were 0.83, 1.61 and 3.24 g/d. They did not find any significant differences in cholesterol levels between the different doses. They therefore supported the consumption of 1.6g/d for lowering cholesterol because this did not affect plasma carotenoid concentrations (Hendricks et al., 1999). This dose is also confirmed by (Hallikanen et al., 2000). They studied the effect of four different doses (0.8g, 1.6g, 2.4g and 3.2g) of phytostanols added to margarine in patients diets for four weeks. They supported the consumption of 1.6g/day because, although the higher doses produced numerically higher decreases in LDL cholesterol, these decreases were not statistically significant.

Only one study so far has shown no reduction in cholesterol levels with phytostanol therapy (Denke, 1995). In this study, the effect of the dietary supplementation of 3g of phytostanol per day was evaluated in 33 moderately hypercholesterolaemic patients who were consuming an outpatient diet of less than 200 mg of cholesterol daily. This lack of response is attributed by the author to the fact that the patients were on low cholesterol diets. Another possible explanation for Denke's findings could be that the phytostanol was administered in capsules in addition to the dietary fat intake. The sterols in the capsules may not be fully dispersed in the gut, thereby diminishing the reduction of cholesterol absorption.

Jones et al. (2000) found that phytosterols differentially lowered cholesterol. They found a significant reduction in cholesterol in their patients consuming phytosterols esters whereas subjects consuming phytostanols did not have a significant reduction. However, another study reports similar cholesterol lowering properties with phytostanols and phytosterols (Westrate et al., 1998).

From the above randomised and crossover clinical trials it is evident that the consumption of phytosterols/stanols when added to a spread or salad dressing are

effective in reducing plasma total and LDL cholesterol without affecting HDL and triglycerides concentrations.

4.1.3 STEROL ABSORPTION

Cholesterol entering the lumen of the gut is derived from two sources: namely endogenous (bile, shedding of intestinal epithelium) and exogenous sources (diet). In a typical Western diet, about 1/3 of the cholesterol entering the intestine is absorbed. Cholesterol absorption has been well explained and will be briefly summarised. According to Turley et al. (2000) in the intestinal lumen there is a breakdown of dietary triacylglycerols by gastric and pancreatic lipases. Cholesterol ester that may also be present, is hydrolysed by pancreatic esterase. The unesterified cholesterol is then incorporated into mixed micelles and diffusion occurs between these and the enterocyte across the unstirred water layer. The cholesterol then reaches the surface of the enterocyte and inserts in-between the phospholipid molecules in the membrane bilayer. It is not clear whether specialised regions of the cell membrane are preferred or whether specialised proteins are involved in this process. Cholesterol in the cell is ultimately exported in chylomicrons that pass into the lacteals, and ultimately the systemic circulation. In the gut, cholesterol is esterified within the mucosal cell by acylCoA:cholesterol:acyl transferase 2 (ACAT2) and becomes incorporated into the core of chylomicrons by microsomal triacylglycerol transferase protein (MTP). Alternatively, cholesterol may be bound to adenosine triphosphate binding cassette (ABC) transporters for export into the lumen or high-density lipoprotein (HDL). A gene ABCA1, was discovered to be mutated in Tangiers disease (AM Vaughan et al., 2000). This disease results in the inability to produce HDL and includes the finding of lipid-laden cells in the intestine. This new discovery was a breakthrough in lipid metabolism, since it identified the transporter mechanism responsible for removing

excess cholesterol out of the cells into the systemic circulation. The finding of lipid accumulation in the gut indicates that the process may be relevant to the enterocyte.

Recently Berge et al. (2000) discovered two new transporter genes, namely ABCG5 and ABCG8. These genes were located by the authors in an attempt to look for mutations that cause sitosterolaemia. ABCG5 and ABCG8 are oppositely orientated genes and encode new members of the ABC transporter family. Seven different mutations, six in ABCG8 and one in ABCG5, were located. The authors suggest that ABCG5 and ABCG8 are ABC half transporters that dimerise to form a functional protein. Both of these genes were expressed in the liver and intestine and were up-regulated by high cholesterol diets fed to mice. They co-operate to limit intestinal absorption and promote biliary excretion of sterols. Although the transporters preferentially move phytosterols, they also transport cholesterol since cholesterol uptake is also greatly increased in sitosterolaemia. These protein complexes protect against sterol over-accumulation but mutations in them cause sitosterolaemia. The authors also suggest the possibility of minor defects in these proteins or their regulation that could explain the different responses of healthy individuals to high cholesterol diets.

Sitosterolaemia, also known more correctly as phytosterolaemia to encompass the broad range of plant sterols, is a rare inherited lipid storage disease. There is an accumulation of phytosterols and 5 alpha saturated stanols in plasma and tissues leading to xanthomatosis and premature artherosclerosis (Salen et al., 1992) Patients with homozygous sitosterolaemia usually have xanthomata (cutaneous and tendinous) during childhood and can develop vascular disease in teenage years. Cholesterol levels are also usually increased, but only moderately and certainly will not be as high as in homozygous FH.

4.1.4 CHOLESTEROL AND PLANT STEROL ABSORPTION – SUMMARY

1. Ingested phytosterols (P) and cholesterol enter the stomach from the diet.
2. Ingested sterols enter the epithelial cells probably by passive diffusion from the emulsified lipids crossing the unstirred water layer.
3. Inside the enterocyte, the proteins ABCG5 and ABCG8 unite to form a transport protein that preferentially pump out phytosterols and some cholesterol into the gut lumen, thereby decreasing sterol absorption. Only a small amount of phytosterols is therefore absorbed.
4. Absorbed sterols are packaged for transport into chylomicrons for transport through the liver.
5. Chylomicrons enter the lymphatic system. In interstitial fluid, they pick up apo C and apo E from HDL. They then enter the circulation. Lipoprotein lipase is responsible for their degradation, where triglyacylglycerols in the chylomicrons are degraded to leave more dense particles rich in cholesterol esters. These are recognized by the liver by the apo E on the surface of the particle.
6. In the liver, phytosterols are secreted into the bile by ABCG5 and ABCG8, while cholesterol is also delivered to the bile for excretion.
7. It may also leave the liver in VLDL to be transported to peripheral tissues by lipoproteins (VLDL, IDL and LDL). Apo B100 acts as enzyme activators and receptor ligands. The action of lipoprotein lipase releases free fatty acids and glycerol which is used as an energy source by tissues or stored for fuel in fat.
8. Phytosterols and cholesterol may also be converted into bile acids.
9. The return of cholesterol from peripheral cells is mediated by ABCA1 and HDL. Newly synthesized HDL may receive cholesterol in the ciculation to form mature HDL. The mature HDL particles transfers cholesterol back to

chylomicron remnants or IDL which may be cleared by hepatic receptors. This process in known as reverse cholesterol transport.

Abbreviations for figure

ABCG5- adenosine triphospate binding protein
ABCG8- adenosine triphosphate binding protein
BA - bile acids
B100 - apoprotein B100
CE - cholesterol esters
CETP - cholesterol ester transfer protein
FFA - free fatty acids
HDL - high density lipoprotein
LCAT - Lecithin cholesterol acyltransferase
LDLR - low density lipoprotein receptor
LRP - LDLR related protein
LPL – lipoprotein lipase
P - phytosterols
C - cholesterol
CM - chylomicrons

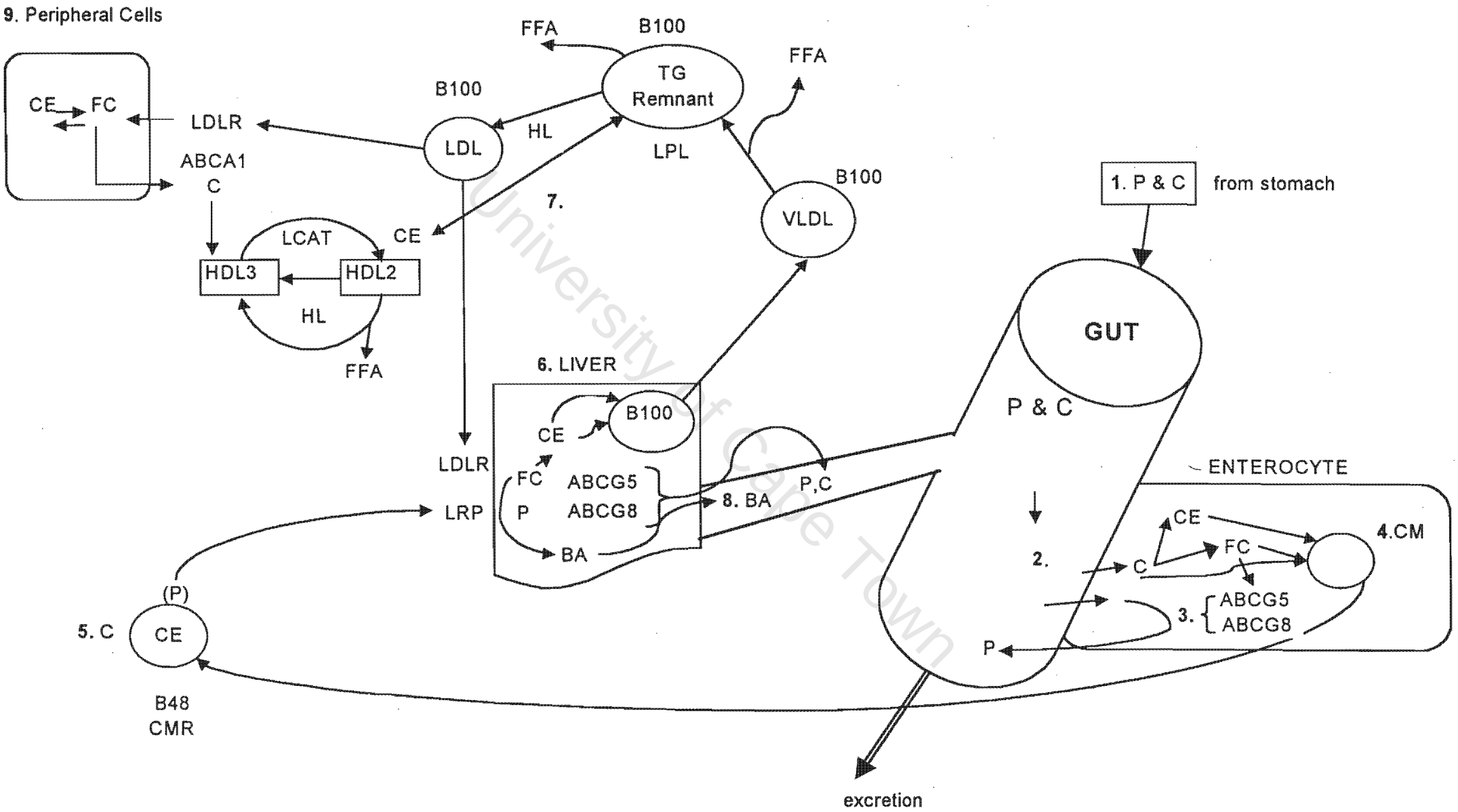


FIGURE 2 - CHOLESTEROL AND PLANT STEROL ABSORPTION
(Allayee et al, 2000; Dodson and Barnett , 1999; Campbell and Smith, 2000)

4.1.5. CHOLESTEROL REGULATION

A regulatory system exists to maintain a constant level of free cholesterol within the cell by controlling the intracellular and extracellular sources of cholesterol. This involves 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-controlling enzyme in cholesterol biosynthesis. Enrichment of the cell with cholesterol suppresses the activity of this enzyme so that cholesterol synthesis is turned off in the cell. The excess cholesterol is a substrate for ACAT, a cholesterol esterifying enzyme, so that free excess cholesterol is stored as cholesterol esters. Excessive cholesterol also turns off synthesis of the LDL receptor, thereby preventing further entry of LDL into the cell and protecting cells against the accumulation of cholesterol through this cholesterol rich lipoprotein (Goldstein, Hobbs and Brown, 1995).

The liver receptor X (LXR) has been implicated in cholesterol homeostasis (Patel et al., 1998). The action of LXR is to detect high concentrations of cholesterol and to then increase the expression of genes that limit its accumulation; it increases the synthesis of bile acids, increases cholesterol efflux from peripheral tissues by inducing ABCA1 and ABCG1 expression and by limiting cholesterol absorption in the intestine by activating ABCA1 and perhaps ABCG5 and ABCG8. However, more studies are necessary to determine the structural and functional properties of the ABC proteins and whether they work together or in separate pathways of cholesterol metabolism.

Regulation of cholesterol at cellular level is explained by Simons et al. (2000). Membrane bound transcription factors called sterol regulatory element-binding proteins (SREBPs) cycle between the endoplasmic reticulum (ER) and the Golgi. They activate genes involved in cholesterol uptake and synthesis. They also explain

how the small cholesterol pool senses the bulk pool peripherally in a mechanism involving lipid rafts.

4.1.6 IMPACT OF PHYTOSTEROLS ON STEROL METABOLISM

Phytosterols lowers serum cholesterol by reducing the absorption of dietary cholesterol in the gut. Phytosterols seem to compete with cholesterol for the incorporation into mixed micelles. The reduced availability of cholesterol to the enterocyte results in a lesser amount of cholesterol being incorporated into chylomicrons. Therefore, less intestinally derived cholesterol enters the liver through chylomicron remnants. This results in the upregulation of the low-density lipoprotein cholesterol (LDL) receptor, which results in increased hepatic LDL uptake thereby lowering serum LDL cholesterol levels. (Miettinen et al., 1990 and Law M 2000)

Free stanols and phytosterols have limited solubility, and they are therefore difficult to incorporate into margarines at high enough concentrations to be of any effect. Esterifying the phytostanols and phytosterols with long chain fatty acids, increases their lipid solubility. These esters of phytosterols and phytostanols can then be incorporated into nutriceutical foodstuffs (Law, 2000).

4.1.7 FACTORS AFFECTING ABSORPTION RATE OF PHYTOSTEROLS

Micellar solubility affects the absorption rate of phytosterols, the discrimination between absorbable and non-absorbable sterols occur during their uptake into intestinal mucosa (Vanhanen et al., 1993).

A slower rate of transfer of phytosterols to the intracellular site than that of cholesterol may also explain the lower absorption of phytosterols (Allayee et al., 2000). There could also be reduced sterol absorption due to mucosal esterification (Nguyen, 1999).

4.1.8 PLANT STANOL AND STEROL SPREADS

A spread containing plant stanols, called Benecol® was released in several European countries by Raisio in 1999. A similar margarine with added phytosterols was released by Unilever in 2000 called Flora Pro-Activ®. These margarines are expensive. In the UK the cost is £2 per 250g and in South Africa it costs between R14 - R17 per 250g (June 2002). These spreads could, however, be viewed as foodstuffs that are deliberately consumed for health benefits; i.e. they are nutriceuticals or functional foods. The high cost may therefore be compensated for by the health benefits, which will also be examined in this study.

4.1.9 SAFETY OF PHYTOSTEROLS

Studies have been done to evaluate the safety of phytosterols in humans and rats. These studies included evaluations of oestrogenecity, reproduction, and faecal concentrations of bile acids. It was found that phytosterols do not bind to the rat oestrogen receptor and did not have transcriptional activity to the human oestrogen receptor (Baker et al., 1999). There was no indication of oestrogenecity from the uterotrophic assay. Furthermore, there was also no evidence to suggest changes in reproduction parameters (Waalkens-Berendsen et al., 1999). High intakes of phytosterols in healthy males and females do increase the neutral sterols in the faeces, but did not increase the formation of bile acids or sterol metabolites (Westrate et al., 1999). In a 90-day study to evaluate the oral toxicity of phytosterols on rats, there was no evidence to suggest that phytosterols were toxic (Hepburn et al., 1998).

There has been some concern that phytosterols may affect the absorption of fat-soluble vitamins. Reductions in beta-tocopherol and carotenoid concentrations were shown (Gylling, 1996). It should be remembered that these lipid soluble substances

are found in lipoproteins and that changes of their concentration may merely reflect changes in lipoprotein concentrations. However, Hendricks et al. (1999). found that with consumptions of 1.6g per day of phytosterols there was no effect on fat-soluble vitamins. Doses of 0.84g and 3.24g showed reductions in plasma (alpha and beta) carotene concentrations.

The use of plant sterols would not be safe in patients with phytosterolaemia, due to the accumulation of phytosterols and saturated stanols in this condition. However, in heterozygote (carriers) of the condition the plasma phytosterols increases to levels found in healthy individuals when consuming phytosterols in the diet (Stalenhoef, 2001).

Law, Thompson and Wald (1994), reviewed studies to see whether there were any hazards associated with reductions in cholesterol levels. They examined published data on mortality from causes other than ischaemic heart disease obtained from 10 of the largest cohort studies, 2 international studies and 28 randomised trials. They looked at circulatory diseases other than ischaemic heart disease, cancer, accidents, suicide and other diseases. The only cause of death associated with low cholesterol levels was haemorrhagic stroke.

Cholesterol levels less than 5mmol/l were associated with excess mortality. This affects 6% of the population in the Western World. The authors conclude that in people with low cholesterol levels the benefits of the low risk of ischaemic heart disease far outweigh the potential risk for stroke (Law, Thompson and Wald, 1994).

FAMILIAL HYPERCHOLESTEROLAEMIA

4.1.10 DEFINITION

FH is an autosomal dominant disorder caused by mutations in the low-density lipoprotein receptor that render the molecule dysfunctional (Goldstein, Hobbs and Brown, 1995). FH is usually accompanied by xanthomatosis and accelerated artherosclerosis (Gevers, 1986). Heterozygous FH is one of the most common inherited metabolic diseases: on average it has a worldwide prevalence of about 1in 500 (Goldstein, Hobbs and Brown, 1995). Such individuals express half the normal receptors on the surface of the cells, so that the plasma LDL concentration is approximately double that of normal individuals.

4.1.11 FOUNDER EFFECT

There is a high prevalence of FH in a few areas of the world. Lebanon has a prevalence of heterozygous FH estimated at 1 in 171. In the Quebec Province of Canada, the prevalence is 1 in 270 amongst French Canadians (Goldstein, Hobbs and Brown, 1995). In South Africa, there is a high prevalence amongst the Afrikaners with a heterozygote frequency of 1 in 72 (Steyn et al., 1996). There are three founder –related gene mutations (FH Afrikaner –1,-2, -3) that affect the LDL receptor and are responsible for 90% of FH in South African Afrikaners. The FH-1 mutation result in some residual LDL binding activity. Patients with this type of mutation have lower plasma cholesterol levels than those with the FH-2 mutation, which completely abolishes LDL receptor activity. The FH-1 and FH-3 patients tend to be less affected by coronary heart disease and xanthomata than the FH-2 patients (Graadt van Roggen et al., 1995).

Furthermore, in South Africa Ashkenazy Jews and Indians are believed to have prevalances of FH of about 1 in 100 (Goldstein, Hobbs and Brown, 1995).

The Coloured population of mixed ancestry, is believed to have a prevalence of FH of about 1 in 500 but it is possible that founder genes could have increased the prevalence of FH in this population (Loubser et al., 1999).

4.1.12 DIAGNOSIS

The clinical diagnosis of FH is relatively easy to make. It is characterized by an elevated LDLC level which raises the total cholesterol to greater than 7.5mmol/l and is accompanied by xanthomata-usually in the Achilles tendon and/or a family history of hypercholesterolaemia or premature ischaemic heart disease. This phenotype is also found in familial binding defective apolipoprotein B100 (FDB), in which the clearance of LDL is impaired because the ligand, apo B, has a mutation that renders binding to the LDL receptor ineffective (South African Medical Association and Lipid and Atherosclerosis Society of Southern Africa Working, 2000). Genetic tests are therefore advocated to exclude FDB100 for which only 3 mutations are known and tested for at the lipid clinic.

4.1.13 MORTALITY RISK

The average age of death for heterozygous FH is 43 years (Slack, 1969).

Miettinen and Gylling (1988) found a 27% mortality in FH in 96 patients followed up over a 15 year period. Forty- four percent of the men and 10% of the women died from coronary heart disease and 4% of non-coronary causes. Adverse prognostic factors included male gender, previous myocardial infarction and smoking. Sijbrands et al. (2001), investigated mortality over two centuries in a large pedigree of (untreated) FH in the Netherlands in the 19th century. They found a total mortality of 70 out of 250 people analyzed for 6950 person years. They found that mortality did not increase in the 19th and early 20th century. Mortality rose after 1915, reached its

peak between 1935 and 1964 and decreased thereafter. They also found differences between two branches of the pedigree. They attributed the variances in time and between the two branches to environmental factors (Sijbrands et al., 2001).

4.1.14 PATHOPHYSIOLOGY OF FH

The LDLR is synthesized in the rough endoplasmic reticulum. The LDL receptor is transported to the Golgi apparatus for maturation involving glycosylation. From here, it goes to the cell surface where it migrates to specialised regions called clathrin coated pits (basket like invaginations). Clustering of the LDL receptors occurs in these pits. The receptor binds to two known ligands: ApoB and apoE. Endocytosis of LDL particles bound to receptors in the clathrin coated pits results in uptake into the cell after an interaction with adaptor proteins. Acidification of the endosome lets the receptor dissociate from the ligand and hereafter the LDL receptor (LDLR) recycles back to the cell surface. The LDL is transported from the endosome to the lysosome where it is degraded. The cholesterol released by the catabolism of LDL is used to synthesize cellular membranes in all cells but in specialized cells is also used for lipoproteins, corticosteroids, and bile acids as well as biliary cholesterol. The expression of several genes is regulated by membrane cholesterol (Goldstein, Hobbs and Brown, 1995).

There are five different classes of LDLR mutations (Goldstein, Hobbs and Brown et al, 1995). Class 1 mutations do not produce immunoprecipitable protein (null alleles). Class 2 mutations encode proteins that are blocked completely (2a) or partially (2b) in transport and maturation between the ER and the Golgi apparatus. Class 3 mutation describes proteins that are synthesized and transported to the cell surface but do not bind LDL. Class 4 mutations encode proteins that move to the cell surface and bind LDL, but do not cluster in clathrin coated pits and therefore do not

internalise LDL or do not permit internalization owing to defective cytoplasmic tails. Class 5 mutations internalize the LDL, but do not discharge the ligand in the endosome and are thus rapidly degraded and fail to recycle to the cell surface. More than 600 mutations have been recorded in the LDLR. The functional impact of these mutations is often described by the capacity of maximally up-regulated LDLR in fibroblast cultures. Binding or uptake is compared to normal cells. Receptor negative mutations in homozygotes result in less than 2% of mutations while greater binding capacity may be described as defective LDLR status. These may be arbitrarily classified into high and low binding. Afrikaner 1 and Afrikaner 3 mutations have defective function as class 2b mutations whereas Afrikaner 2 mutations are null mutations due to class five status (Graadt van Roggen et al., 1995).

While there have been no controlled trials to evaluate the impact of lipid modifying treatment on mortality in FH, there is ample documentation of reduced cardiovascular morbidity and mortality in primary and secondary prevention of mild to moderate hypercholesterolaemia.

(Scandinavian Simvastatin Survival Study Group (1994), Sacks et al. (1996), Lipid Research Clinics Program (1984), West of Scotland Coronary Study Group (1998).

4.1.15 TREATMENT OF FH

The primary aim of treatment is to prevent the development of complications of artherosclerotic disease, i.e. to lessen the occurrence of coronary artery disease either as angina or as myocardial infarctions (Berkow and Beers, 1997).

According to the South African Guidelines for the management if dyslipidaemias (2000), the aim of treatment is to reduce LDL cholesterol concentrations to less than 3 mmol/l or at least to lower LDL by 45% in very severe hyperlipidaemia. (SA Medical

Association and Lipid and Atherosclerosis. Society Working Group). The management of dyslipidaemia requires a thorough clinical evaluation and risk assessment. A formal diagnosis of FH predicts a very high risk of ischaemic heart disease.

The National Cholesterol and Education Expert Panel in the USA on the detection, evaluation and treatment of high blood cholesterol (Adult Treatment Panel or ATP) in adults periodically produces updates in clinical guidelines as warranted by advances in science. Three reports have so far been released, with the ATP 111 being the most recent (NCEP, 2000). It emphasizes primary prevention in people with multiple risk factors or at extremely high risk by virtue of disorders such as FH and elevated triglycerides. It also encourages the use of plant stanols and sterols and viscous fibres as therapeutic dietary options for lowering cholesterol. Its target LDLC is 2.5 mmol/l.

The Lipid Clinic at UCT and GSH places emphasis on a thorough clinical assessment and further attempts to prove FH by performing genetic tests for founder mutations to confirm the diagnosis of a patient as LDLR mutation or FDB_{100}. The diet advised for FH is similar to the NCEP guidelines, but the cholesterol recommendation is 100mg versus 200 mg/day. Cholesterol intake is limited with FH patients due to higher levels of total and LDLC levels and since dietary cholesterol absorption is affected by many factors, it is preferable to limit the cholesterol intake. The diet also includes the use of omega 3 fatty acids.

4.1.16 DIETARY MODIFICATION

Dietary modification is the first line of treatment for hypercholesterolaemia irrespective of whether the diagnosis is ascribed to genetic or environmental factors (ADSA Position Statement, 2000). The recommended diet advises a reduction in total

fat, especially saturated fat, substituting saturated fat for monounsaturated and polyunsaturated fat, reducing total dietary cholesterol, and increasing dietary fibre, especially soluble fibre.

Many studies have been done to evaluate the effect of low cholesterol and fat diets on lowering plasma cholesterol levels. Yo Poth et al. (1999) evaluated 37 intervention studies on free-living subjects. They compared the effects of the Step 1 and Step 2 diets on total cholesterol, LDL-C, triglycerides and TC: HDL-C ratios. The Step 1 diets showed a lowering of 10%, 12%, 8%, and 10%, whereas the Step 2 diets showed a lowering of 13%, 16%, 8% and 7% respectively.

Dietary studies in FH patients are, however, limited. Makonno et al. (1990) gave heterozygous FH patients a cholesterol-free diet for 11 days without medication. They found a 14.2% reduction in total cholesterol and a 17.5% reduction in LDL cholesterol. Gaddi et al. (1991) fed a standard hypocholesterolaemic diet to FH patients for 4 weeks and showed no significant changes in lipid levels. Next, they fed the same diet for 4 weeks, but they substituted animal protein with soy protein and showed a reduction of 21% in total cholesterol and 26% in LDL cholesterol. Therefore, it seems that subjects with mildly to moderately high cholesterol levels due to secondary factors respond well to a standard low cholesterol diet whereas those with FH respond to diets very low in cholesterol. An analysis of the first 100 genotyped FH subjects at the Lipid Clinic revealed a response of LDLC of approximately 1.5mmol/L by dietary counseling (personal communication-Professor AD Marais, 20 September 2001).

4.1.17 EFFECTS OF DIETARY FACTORS ON PLASMA CHOLESTEROL

The effects of dietary factors will be discussed briefly under the following headings: Dietary cholesterol, dietary fat, carbohydrate, fibre and other dietary factors.

DIETARY CHOLESTEROL

The effect of dietary cholesterol on plasma cholesterol levels varies in individuals with a range of responses between hyper- and hypo-responders. The response may be influenced by bile acid synthesis, the rate of absorption in the intestine, cholesterol turnover, unknown genes conferring varied response in hyper- and hyporesponders and apo E phenotypes. (Gylling and Miettinen, 1992).

The liver is the main organ responsible for regulating plasma LDLC. It is the major site for the production of VLDL, which is the precursor of LDL as well as being the organ mainly responsible for the bulk of receptor mediated clearance of LDL from the circulation (Turley, 1999). Excess cholesterol entering a regulatory pool of cholesterol, within the hepatocyte, is converted to cholesterol esters through the action of ACAT, excess formation of CE will in turn drive VLDL secretion (Turley, 1999). Therefore the increase in dietary cholesterol will result in an increase in LDLC through increased VLDL secretion and resultant increase in LDLC and down regulation of LDLR activity.

Yu-Poth et al.(1999) found that dietary cholesterol significantly affected TC, LDLC and possibly triacylglyerols, but not HDLC. Clifton et al. (1990) found an increase in HDL cholesterol. It was found that hypercholesterolaemic individuals were sensitive to dietary cholesterol whereas normocholesterolaemic individuals were not. Within the hypercholesterolaemic group, there were individuals that had a significant reduction in TC and LDLC with a reduction in dietary cholesterol and a low fat diet and those who did not have a significant difference in LDLC and TC (Clifton et al.,

1990). Katan et al., (1986) also showed modest differences in the responsiveness of serum cholesterol to dietary cholesterol and attributed the responses to chance flactuations.

DIETARY FAT

Saturated fatty acids (SFAs) are known to increase plasma cholesterol levels. The SFAs known to increase cholesterol are lauric, myristic and palmitic acids, whereas stearic acid does not have a plasma cholesterol raising effect (Denke and Grundy, 1992).

In free-living populations a mean reduction of TC and LDLC of 5% were found on a diet with less than 30% energy coming from total fat and less than 7% from saturated fat (Hunninghake et al., 1993). Similar results were also reported. Howell et al. (1998) evaluated 224 published studies between 1966 and 1994 reporting quantitative data on changes in dietary cholesterol and fat and corresponding changes in serum TC, LDLC, HDL, VLDL and TG. Predictions indicated that compliance with standard dietary recommendations (less than 30% of energy from total fat, less than 10% from saturated fat and less than 300mg from cholesterol), will reduce TC and LDLC by approximately 5%. However under well-controlled conditions, greater reductions in TC and LDLC have been observed (Yo Poth., et al., 1999). The TRANSFAIR study which measured intakes of total fat and individual fatty acids in 14 European countries, showed that the intake of saturated fat is higher that the recommended limit of 10% (Hulshof et al., 1999).

Monounsaturated fatty acids (MUFAs) do not have any effect on plasma cholesterol levels, except if they replace saturated fat in the diet, were it is known to reduce cholesterol levels (ADSA Position Statement, 2000). The Lyon Diet Heart Study found that patients after having survived a first myocardial infarction and then

assigned to an alpha-linolenic acid rich diet, had significant reductions in recurrence, other cardiac events and total mortality (Loergeril et al., 1994).

Polyunsaturated fatty acids (PUFAs) seem to have a cholesterol lowering effect over and above that of replacing SFAs in the diet but decrease HDL levels (Katan et al., 1992). n-3 fatty acids does not affect TC, LDLC or HDL in normotriglyceridaemic subjects, but does increase LDLC in hypercholesterolaemic subjects (Adsa Position Statement, 2000). Secondary prevention trials with n-3 fatty acids show that with intakes of 1-2g/day lowers lowers cardiovascular risk in patients with a previous myocardial infarction (Singh et al., 1997; GISSI-Prevenzione Investigators, 1999). n-6 fatty acids have been shown to have protective effects (Pietenen et al., 1997 ; Hu et al., 1997).

CARBOHYDRATE

A high carbohydrate diet may increase triglyceride levels (Baggio et al, 1988; Nelson, Schimdt and Kelley, 1995), but this effect may be counteracted by high fibre diet (Rivellesse and Mafetone, 1995) and therefore high fibre carbohydrates are advised.

FIBRE

Wheat bran does not have a cholesterol lowering effect. However, soluble fibre does have a cholesterol lowering effect (Truswell, 1995). The soluble fibre affects LDL, but not HDL or TG.

The major mechanism by which soluble fibre decreases serum cholesterol is probably due to the decreased reabsorption of cholesterol in the small intestine and consequent increased excretion of bile acids. The consequent reduced bile acid pool results in

more cholesterol conversion to bile acids and the upregulation of LDLR results in a decrease of serum cholesterol (Baghurst et al., 1996).

A meta-analysis of 10 trials using oatmeal or oat bran found that 3g of soluble fibre from oats, independently reduced cholesterol levels by 0.13mmol in individuals with average cholesterol levels of 5.9mmol, that is 2.2% reduction. Larger reductions were seen in people with higher cholesterol levles (Ripsin CM et al., 1992).

ALCOHOL

A moderate intake of alcohol may protect against CHD, but high intakes increase risk. (Klatsky et al 1977 Marmot, 1991).

There is a positive association with alcohol and HDL-C, which is beneficial in prevention of CHD, but there are indications that alcohol increased TG levels and the production of VLDL-TG. The effect of alcohol on TG levels is dependant on underlying factors such as weight and diabetes and those with an underlying hyperlipidaemia.

TRANS-FATTY ACIDS

These fatty acids increase LDLC, although to a lesser extent than SFAs. It also decreases HDLC (Mensink and Katan, 1992).

Oomen et al. (2001) positively associated intake of trans fatty acids with a ten year risk of coronary heart disease. They studied 667 men from the Zutphen Elderly Study who were free from coronary heart disease at baseline and followed them up for 10 years. After 10 years of follow up there were 98 cases of fatal and non-fatal coronary heart disease. It was found that a 2% energy intake from trans fatty acids would increase the chance of developing coronary heart disease (Oomen et al., 2001).

OTHER DIETARY FACTORS

SOYA

A meta-analysis of 38 studies examining the effect of soya on cholesterol lowering, reported an average reduction in LDL cholesterol of 12.9% associated with an intake of 47g or protein/ day. For a 25g intake, the estimated reduction in LDL cholesterol would be 0.23mmol/l (Anderson, 1995).

The UK Joint Health Claims Initiative (JHCI) recently approved a claim that soya protein reduces blood cholesterol which can now be communicated to the public in the UK. The scientific dossier to support the soya claim was reviewed by JHCI's committee of seven scientists. The claim states that "The inclusion of at least 25g of soya protein per day, as part of a diet low in saturated fat, can help reduce blood cholesterol levels" (Joint Health Claims Iniative, 2002).

LECITHIN

Lecithin is not recommended because of controversial results regarding the effect of lecithin on lipoprotein levels, poor study designs and data analysis errors (ADSA Position Statement., 2000).

GARLIC

According to the ADSA Position Statement (2000), there is insufficient evidence to recommend garlic use for lipid lowering benefits, although there is some data to indicate positive results with supplementation over a few months. More research will have to be done before recommending garlic supplementation, but garlic can be included as part of a varied diet adhering to general prudent guidelines.

4.1.18 LIPID MODIFYING TREATMENT

According to the South African Guidelines on Coronary prevention, lipid modifying drugs should be instituted in patients with a 10 year risk of 20% or greater than 20% projected to the age of 60 years (Pyorala et al., 1994). Young adults with FH or a related monogenic dyslipidaemia or the presence of CHD especially a previous MI, are thus candidates for drug treatment. Drug treatment should not be instituted without prior lifestyle modification. Drugs used in the treatment of hyperlipidaemia can be classified into bile acid sequestrants, statins (HMGCoAR), nicotinic acid, fibrates and probucol.

HMG-CoA reductase inhibitors (statins) are usually instituted as the first line treatment of pure hypercholesterolaemia. Statins are highly effective in reducing LDL cholesterol. They also lower triglycerides and increase HDL (Berkow et al., 1997).

Side effectrs with statins tend to be mild. Careful monitoring of transaminase levels, to creatinine kinase and muscle pain and stiffness should be done. Occassionally patients complain of gastrointestinal problems such as diarrhoea or constipation, headache, insomnia and other vague symptoms (South African Medical Association and Lipid and Atherosclerosis Society of Southern Africa Working Group, 2000).

The CURVES study was the first study to directly compare the efficacy of the different HMG-CoA reductase inhibitors. The study found that different doses of Atorvastatin (10, 20 and 40 mg) produced greater reductions in LDL cholesterol, than the milligram equivalent doses of Simvastatin, Pravastatin, Lovastatin and Fluvastatin (Jones et al., 1998). The doubling of Atorvastatin from 10mg to 20mg and 40mg resulted in lowering of LDL by 38%, 46% and 51%. This is roughly in accordance with the 6% rule (Knopp, 1999).

Atorvastatin (10 mg) produced the LDL reductions greater or equal to the different doses of Simvastatin (10, 20, 40mg), Pravastatin (10, 20, 40mg) , Lovastatin (20 and 40 mg) and Fluvastatin (20 and 40mg).

The statins lower LDLC by up-regulation of LDLR and enhanced clearance of this lipoprotein. There is evidence for reduced production as well as to effect reductions of LDLC in homozygous FH where LDLR cannot play a role, or play only a minor role. The partial inhibition of de novo synthesis of cholesterol results in a decreased pool of FC in the cell and consequently both HMGcoAR and LDLR are up regulated. This increases clearance of LDL and thus decreases LDLC but the response becomes limited by an increased mass of HMGCoAR and maximal LDLR (Goldstein, Hobbs and Brown, 1995). .

Bile acid sequestrants (BAS) are not usually recommended as first line drugs, except in children with FH. They are usually used in conjunction with statins in severe hypercholesterolaemia. BAS decrease recycling of BA, leading to an increased conversion of FC to BA in the hepatocyte. The decrease in FC sets up the up-regulation of HMGCoA as well as increased expression of LDLR. The chief drawbacks for BAS are the high incidence of gastrointestinal effects and their expense (South African Medical Association and Lipid and Atherosclerosis Society of Southern Africa Working Group, 2000).

Nicotinic acid is also beneficial in reducing LDL levels and increasing HDL, but usually in high doses that are frequently accompanied by flushing, gout, hyperglycaemia and diabetes. Hepatic and ophthalmic toxicity have been described. Its action is believed to be due to decreased flux of fatty acids from adipocytes to hepatocytes, limiting the substrate for VLDL production (South African Medical Association and Lipid and Atherosclerosis Society of Southern Africa Working Group, 2000).

The Lipid Clinic at Groote Schuur Hospital uses HMG-coA reductase inhibitors after modifying the patient's diet and other lifestyle factors. However, the clinic is not able to achieve target LDL levels with the limited amounts of HMG-coA reductase inhibitors as the use is restricted to maximal dose of 20mg of Atorvastatin. Assuming that a person with FH has a LDL concentration of 6mmol/L after lifestyle modification, it is obvious that the lowering of LDLC by 50% is required to required to reach target while 20mg of atorvastatin on average lowers the LDLC by 42%. For this reason, dietary measures including nutriceuticals, have a great appeal to improve control of dyslipidaemias.

4.1.19 IMMUNE SYSTEM AND PHYTOSTEROLS

The immune system functions to clear the body of foreign substances (or antigens), such as viruses, bacteria, and cells and from blood or tissue. Normally, when antigens interact with the cells of the immune system, they are cleared from the body without an adverse reaction. Three types of cells respond to the antigens presented: B lymphocytes, T lymphocytes and macrophages. The lymphocytes arise from the bone marrow and are the basis for the functioning of the two branches of the immune system, namely the humoral pathway and the cell mediated pathway. The cell mediated defence mechanism and the antibody mediated defence mechanism comprise the specific immune response whereas the monocyte-macrophage phagocytic system represents the non-specific immune response.

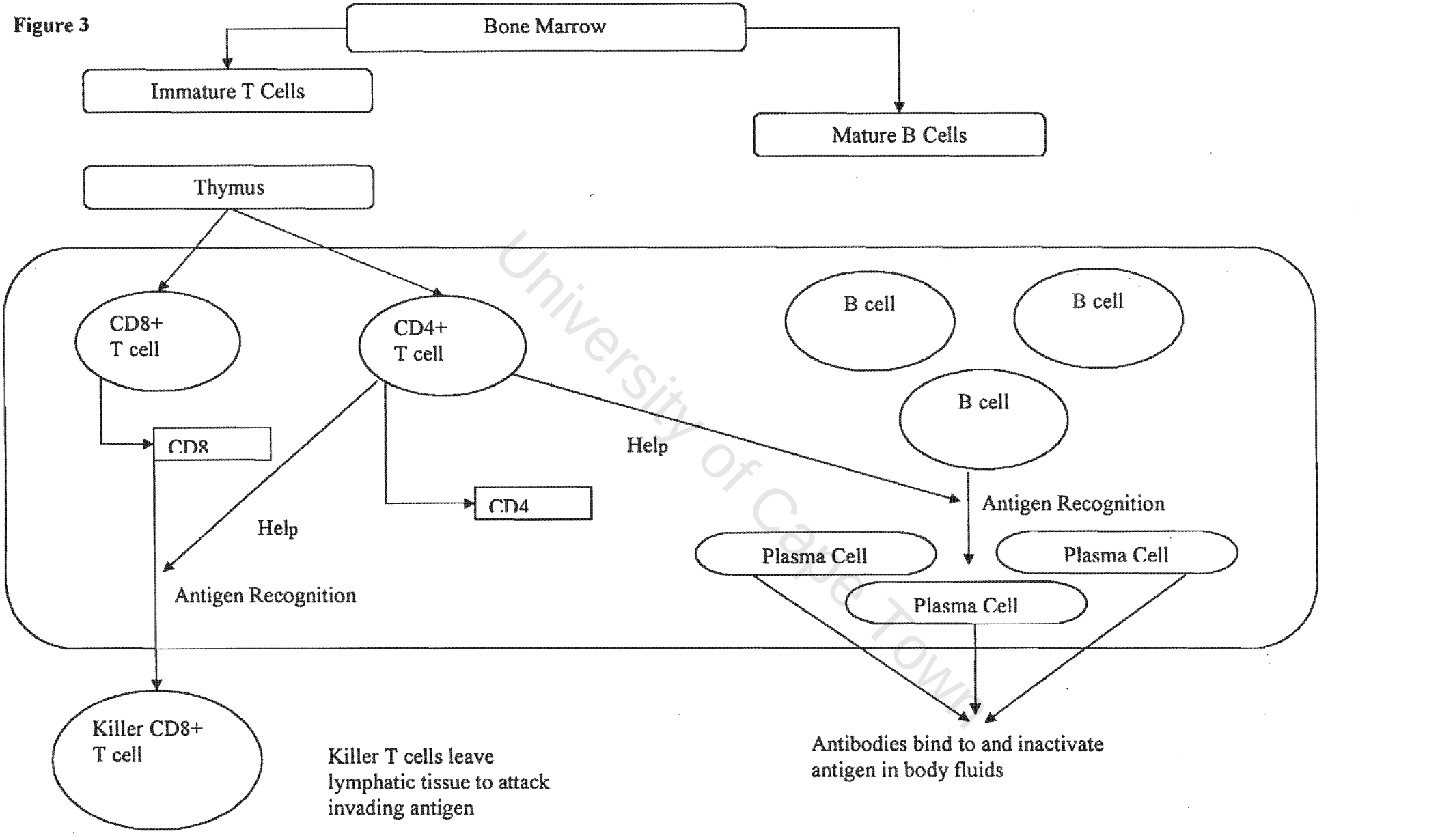
The cell mediated pathway involves T-lymphocytes (T cells). Cellular immunity has an important role in resistance to viruses, fungi, tumor cells and other foreign cells. When antigens stimulate T cells, they produce lymphokines and cytokines. These substances regulate the activities of other cells or cause damage to target cells, resulting in the destruction of antigens. There are three types of T cells: helper T cells,

cytotoxic T cells and suppressor T cells. T helper cells can be further differentiated into TH1 and TH2 cells. TH1 cells produce lymphokines to enhance the immune system response to viruses, bacteria, fungi and parasites; to activate the cytotoxic T cells or suppressor T cells that again kill abnormal cells infected with bacteria, viruses and cancer. TH1 cells also control the activity of the B cells. both TH1 and TH2 cells express CD4 molecules on their surface. TH2 cells are important in helping cells making antibody responses by synthesizing and secreting cytokines that promote the activation, growth and differentiation of B cells. T suppressor cells express CD8 molecules on their surface and counterbalance the effects of T cell help and suppress immune responses. Therefore, T cells control the activity of one another and other cells of the immune system through cytokine production. The measurement of the T cells expressing either CD4 or CD8 and their cytokine profiles has become clinically important as changes in the natural balance between helper and suppressor cells indicate changes in the healthy functioning of the immune system (Stainers et al., 1993).

The immune response can also be divided into early, non-specific innate immunity that is followed by an adaptive, antigen -specific immune response. Monocyte and macrophages form part of innate immunity and can influence the subsequent adaptive immune response, largely by the type of cytokines secreted, as shown in figure 4. Monocytes can secrete IL-10 and IL-12 cytokines, that promote the differentiation of TH2 and TH1 adaptive immune responses, respectively. Thus, evaluation of the balance of IL-10 and IL-12 cytokines and of the impact of any immuno-modulatory substances on this balance, can provide important information on the nature of adaptive immune responses that are likely to follow. Furthermore, significant heterogeneity exists in circulating monocyte population that can be divided into 4 subsets on the basis of co-expression of cell surface markers, namely CD14

[Lipopolysaccharide (LPS) receptor] and CD16 (Fc-γR111)-see figure 5. The CD14+16+ subset has been shown to produce large amounts of the pro-inflammatory cytokine TNF-α, and increased levels of this subset has been described in a number of conditions including HIV, TB, and hyperlipidaemia. Therefore, evaluation of the number and function of this pro-inflammatory subset can provide important clinical information, while substances that can modulate this are of potential therapeutic value. Finally, TH1 and TH2 subsets are defined by the nature of their signature cytokines, IFN-γ and IL-4 or IL-5 respectively. Conventionally, this has been done by stimulating cells and measuring concentrations of secreted cytokines in ELIZA assays. More recently, flow cytometry has provided a powerful tool to document intracellular cytokines and thus define TH subsets at the single cell level, which is more sensitive than conventional ELISA assays.

Figure 3



CELL MEDIATED IMMUNE RESPONSES
Directed against intracellular pathogens, such as viruses, some cancer cells & tissue transplants

ANTIBODY MEDIATED IMMUNE RESPONSES Directed against extra cellular pathogens, such as bacteria

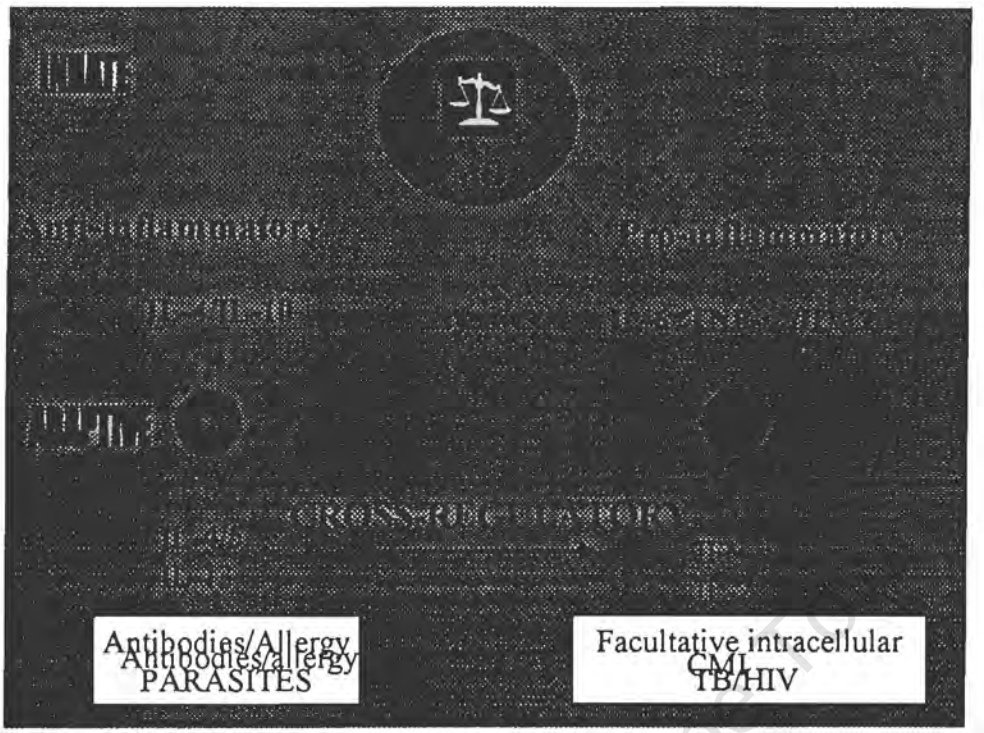


Figure 4: Th1/2 Adaptive immunity: Functional Relevance

Receptors	Subsets	Function
CD 14 CD 16	CD14++16-	Dominant peripheral blood
	CD14++16+	Intermediate
	CD14+16+	Pro-inflammotory TNFα
	CD14+16-	Divergent

Figure 5: Blood monocyte subset heterogeneity

4.1.20 PHYTOSTEROLS AND IMMUNE SYSTEM FUNCTIONING

Moducare® is a supplement that is widely sold over the counter in pharmacies in South Africa, following claims that it may enhance immune function. Moducare® contains phytosterols, namely B-sitosterol and its glycoside (sterolin). One capsule of Moducare® contains about 20mg of phytosterols, whereas the dose of sterols in the margarine is 1.6g per 20g consumed. Studies have been done on Moducare®, reporting immune modulatory effects that may be beneficial in patients with tuberculosis and HIV infections (Donald et al., 1997; Bouic, 1997).

As an immune modulator, Moducare® is said to influence cellular proliferation of T-lymphocytes and there was an enhanced response to mitogens (Bouic PJD et al., 1996). Cytokine secretion was also affected by this formulation as IFN-gamma, a cytokine belonging to TH1 was increased. Lymphocytes belonging to the TH1-type helper cells were increased, whereas those associated with the TH2- type cells were inhibited or remained unchanged. The increased TH1 response is vital for the clearance of certain pathogens while decreased TH2 response will improve the balance in allergies in autoimmune diseases.

A clinical study of the beta-sitosterol(BSS):beta-sitosterol glycoside(BSSG) complex on patients with culture proven TB showed an improvement in haematological parameters which included higher lymphocyte, eosinophil and monocyte counts and weight gain (Donald et al., 1997). An open-labelled study done on HIV patients showed a stabilisation of CD4 counts in HIV patients over a 36 month period, compared to patients not receiving the mixture who showed a decline in CD4 count (Bouic, 1997). Another study done on HIV patients involving 3 groups based on CD4 counts, showed that the Moducare® was most beneficial in the group with a CD4 count of greater than 500, whereas the group with the lowest CD4 count (less than

200) showed a decrease in CD4 count. Therefore the Moducare® was viewed as the most beneficial in recently infected HIV patients and immune intact patients.

Since phytosterols margarine supplements large doses, it is of interest to study the immunomodulatory effects in this thesis.

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5.1. CRITERIA FOR PATIENTS ADMITTED TO STUDY

5.1.1. INCLUSION CRITERIA

1. Males and females between the age of 21 and 70 years.
2. Familial hypercholesterolaemia diagnosed as a clinical phenotype or by genotyping. The clinical phenotype is that of an untreated LDL hypercholesterolaemia of >5.5mmol/L with a tendon xanthoma in the patient or a first degree relative. This will include the familial ligand defective apolipoprotein B100 as an FH phenotype for the purposes of this study. The patients can also be genotypically diagnosed with the locally prevalent founder effect mutations and in this setting a tendon xanthoma is not required.

5.1.2. EXCLUSION CRITERIA

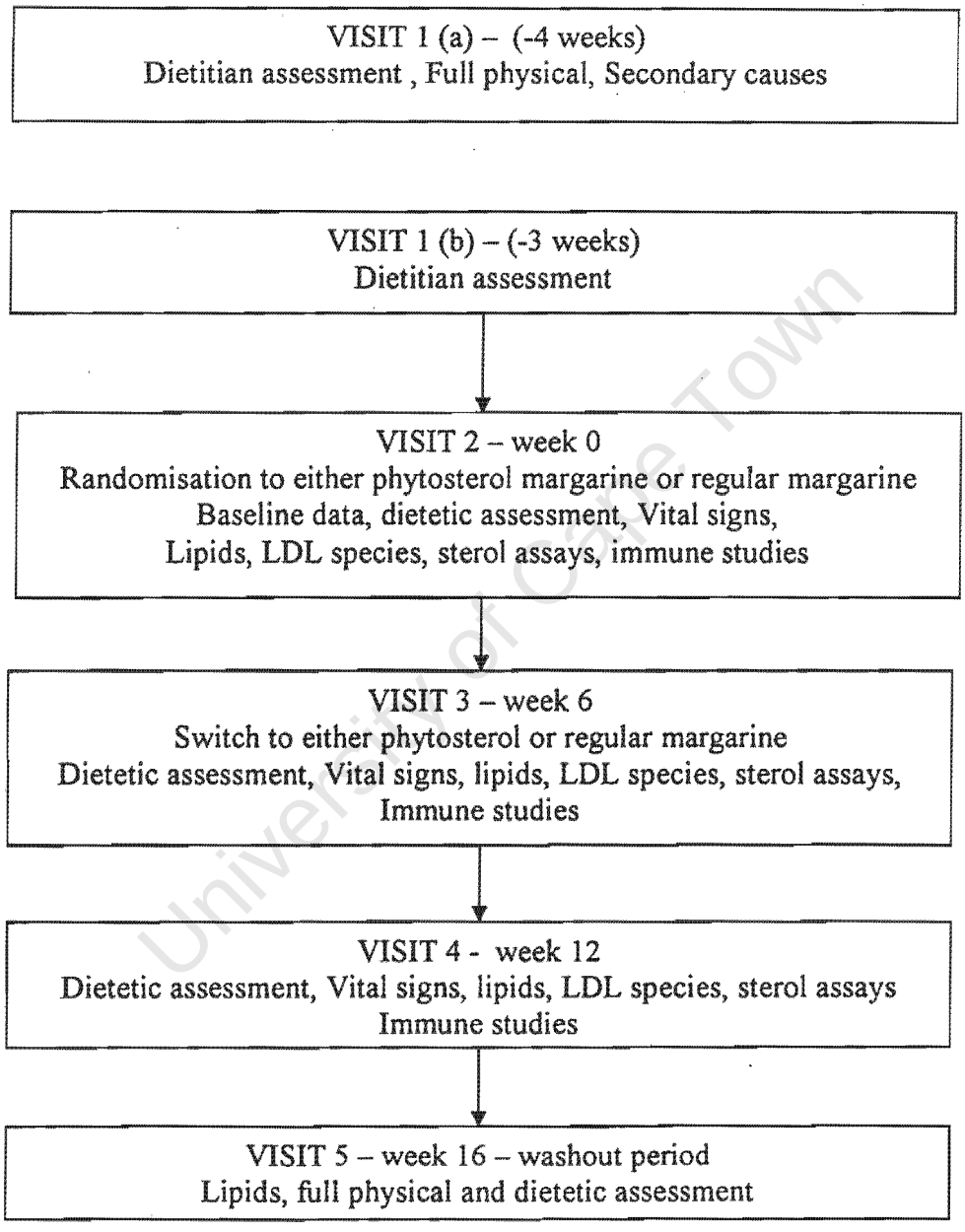
1. Alterations in drug treatment during the period of four weeks prior to randomisation. Patients were acceptable for randomisation on any dose of lipid-modifying treatment that had not been altered for at least four weeks and which was judged to be the most suitable dose in the constraints of the clinic. This medication was to be continued for the duration of this study uninterruptedly.
2. Patients who had an acute phase response in the three months prior to randomisation on to this study. This includes myocardial infarction, surgery other than superficial procedures and any severe illness with fever and requiring an antibiotic.
3. Patients judged as having a likelihood of being non-compliant with instructions for whatever reason, including those with a history of poor compliance and drug abuse.

4. Patients with allergy to margarines or not consuming adequate margarine for substitution.
5. Females with high risk of pregnancy or who planned pregnancy.
6. Patients found to be hypothyroid or diabetic at the screening visit.
7. Patients who took phytosterol supplementation including products such as Moducare. These substances may not be taken during the study.

6.1.1 STUDY DESIGN

The following study plan gives a summary of the study design. The visits are explained.

Figure 6



Dietetic assessment included – collection of diet histories, checking the histories for completeness, measurement of waist-hip circumference, bioelectrical impedance, weight, height and finally checking compliance with margarine.

Full physical initially included – assessment of physical signs of dyslipidemia (arcus cornealis, xanthelasma, cutaneous and tendinous xanthomata). Cardiac examination included the recording of pulse rate and rhythm as well as the presence of all known pulses and whether there were bruits. Blood pressure was recorded and cardiac auscultation performed. Respiratory and abdominal examination were done.

Vital signs were done at follow up visits and included recording of blood pressure, pulse rate and rhythm. Patients were questioned on any problems or complaints and asked to elaborate on them. Secondary causes included detection of any symptoms of renal or endocrine disorders.

Selections of heterozygous FH patients according to the defined clinical and/or genetic criteria were recruited from the Lipid Clinic of Groote Schuur Hospital. Patients were contacted telephonically and received a verbal briefing on the study.

The study was a double blind randomized crossover study in which subjects took regular or phytosterol margarine for 6 weeks and were later followed for a washout effect.

Recruited patients had reinforcement of dietary control before taking regular or phytosterols margarine for 6 weeks. A 4 week follow up after the two margarine phases were included to detect a possible carry-over effect.

6.1.2. COLLECTION OF DATA AND MEASUREMENTS PERFORMED AT EACH OF THE VISITS

During the initial screening visit, (V1), the medical history and physical findings of the patient were reviewed by the doctors as well as the inclusion and exclusion criteria. Medication was noted and a family pedigree was taken to verify the familial nature and premature heart disease associated with FH. Patients were also reviewed for secondary causes of hypercholesterolaemia. Patients signed an informed consent form. Patients were seen by a dietitian and were counseled to complete a 3-day diet record. The records were to be completed at each visit and the week before the visit. Nasco Food models and common household utensils were used to teach portion sizes and volumes of foodstuffs consumed. Patients were also issued with written instructions on how to complete the dietary records (see appendix A).

At visit 1b, diets were checked to see whether patients were at least adhering to a Step1 diet. Patients returned the completed 3 day diet records which were then analysed. If they were on modified diets they were advised to adhere to previously counselled low fat diets and not to change the diet significantly during the study period.

If their diets were not modified, they were counseled to adhere to a Step 1 diet and advised to adhere to the diet throughout the study. This was done prior to the study, to be certain that any changes in lipid values would have occurred before the start of the study.

At this visit, fasting lipid profiles and anthropometric data were also collected: mass, height, waist and hip circumference and bioelectrical impedance.

At visit 2 randomisation occurred for the double blind regular and Pro-Activ margarine. A fasted lipid profile was taken. The dietitian interviewed the patients to detect problems, answer questions, and counseled the substitution of 20g of margarine into the patient's diets. A diet recall sheet was supplied for the visit. Vital signs were assessed, and anthropometry again done including mass, waist amd hip circumference and bioelectrical impedance.

The margarine was supplied in 20g aliquots in identical plastic tubs. 7 days of extra margarine was given to cover a reasonable period of a delayed visit. The containers (used and unused) were returned for compliance counting. The margarine tubs were coded into either A or B for each patient. The coding of the tubs was done by Unilever. The randomization of the patients was also done by the company. The margarine was supplied in boxes with patient numbers. There were two packets, one with the A coded tubs and one with the B coded tubs. The A tubs could have either have been the regular or phytosterol margarine, the same with the B tubs. The A tubs were given for the first randomization and the B tubs for the next study period. The company had a list before the study which patients had the A tubs containing phytosterol and which had the B tubs containing the phytosterol. The list was only given to the reviewers once the study was finally completed and the results had to be analysed.

At visit 3, a fasted lipid sample was again taken with a brief clinical assessment and the crossover occurred to the second margarine form. The margarine tubs that the patient returned were counted. The dietitian received the previous diet recall and checked it for completeness. Anthropometric assessments were again done. A diet recall sheet was supplied for the visit to be returned at the next visit.

At visit 4, the patients again had a fasting lipid profile and returned all the margarine containers and diet records. A brief clinical evaluation and a dietetic assessment were performed. A diet recall sheet was supplied for this visit to be returned at the next visit. A washout period followed to assess whether there may be a carry-over effect of the phytosterol.

At visit 5 there was a final fasting blood sample, a final clinical evaluation as well as dietetic assessment.

METHODS

Selections of heterozygous FH patients according to the defined clinical genetic criteria were recruited from the Lipid Clinic of Groote Schuur Hospital. Patients were contacted telephonically and received a verbal briefing on the study.

The investigations to exclude secondary causes of dyslipidaemia were a fasting glucose and thyroid-stimulating hormone assay. A triglyceride assay was done and hypertriglyceridaemia of >3.5mM/L was taken as a marker of a secondary cause or additional gene modulating lipoprotein metabolism and served as an exclusion criterion.

6.1.3. LIPID TESTS

The lipid tests included triglyceride, total cholesterol, HDL cholesterol after precipitation of apoB-containing lipoproteins, and a calculated LDL by the Friedewald equation. These analyses were performed in a routine chemical pathology laboratory at Groote Schuur Hospital using conventional enzymatic assays in an auto-analysis.

A routine chemical pathology laboratory practicing conventional quality control was used to measure the lipids and perform the other routine tests. The samples were taken after the subject had fasted overnight (12hours) and had assumed a sitting position for 15 minutes before a tourniquet was applied for a short period to perform venesection with a commercially available vacuum tube system. The blood was drawn into ethylene diamine tetraacetic acid (EDTA). The samples were processed within 2 hours and analysed in an automated analyser using the same commercially available kits and standards for the entire study. The cholesterol assay utilises cholesterol esterase and oxidase from which the hydrogen peroxide generated produces a coloured end-product. The triglyceride assay uses a microbial lipase and

glycerol is generated before being phosphorylatd by glycerokinase and ATP. The glycerophosphate yields hydrogen peroxide when acted upon by an oxidase and thus generates a colour product. No glycerol blank was used. The HDL cholesterol was determined in the supernatent after precipitating the apolipoprotein B-containing lipoproteins with a commercial kit containing polyethylene glycol. The LDL cholesterol was calculated by the Friedewald equation. The LDL size was determined in the lipid laboratory by a method briefly described previously and in detail more recently. In brief, it categorises LDL into 5 categories, with A being the largest and I being intermediate and B the smallest, and A/I and IB to describe those in-between. Samples can be frozen and samples at different time points from the same subject were run in adjacent lanes for best comparison. The gel was designed to separate apolipoprotein B-containing lipoproteins and the LDL-like particles are spread between retardation factors of 0.85 and 1. The standardisation is done by carrying a reference large (A) and small species (B) from gel to gel (Marais et al., 1997; Blom et al., 2003).

6.1.4. IMMUNE STUDIES METHODS

The venesection at V1 (for secondary causes of dyslipidaemia requires 10ml of blood) and at visits 2 to 4, 15ml of blood was taken. At V5, only 10ml was taken. The total for the study will be 65ml.

Four colour Flow Cytometric analysis of Monocyte subset distribution, and lymphocyte activation marker expression, and intracellular cytokine production were evaluated ex-vivo and following stimulation by lipopolysaccharide (LPS) and phorbol 12 myristate 13 acetate (PMA) and ionomycin respectively. The method of whole-blood culture to induce cytokines in monocytes and for measuring cellular cytokines in lymphocytes were employed.

Whole blood was stimulated with 25ng/ml PMA and 1mcg/ml Ionomycin and 10mcg/ml Brefeldin A (BFA) was added to prevent export of cytokines from the cytoplasm.

An unstimulated control consisting of whole blood and BFA only was set up for each sample

The stimulated and unstimulated blood was incubated for 4hrs at 37 degrees celsius in a 5% carbon dioxide humidified incubator with the lids loosely closed to allow exchange of gases.

After incubation 50mcl of the whole blood was mixed with the appropriate volume of surface monoclonal antibodies according to supplier's instructions. The antibodies used were directly conjugated to fluorochromes as follows: CD3 to PerCP, and CD4-APC or CD8-APC.

After mixing gently the blood and surface antibodies were incubated at room temperature for 20 minutes in the dark.

The red cells were subsequently lysed using FACS Lysing solution for 10 minutes at room temperature.

After washing in wash buffer the cells were permeabilized using FACS permeabilizing solution for 10 minutes then washed once.

The cells were then stained for Interleukin (IL4) and interferon gamma (IFN-g) using monoclonal antibodies IL-4 PE and IFN-g for 30 minutes at room temperature in the dark.

The cells were washed in whole blood then resuspended in 0.5mls 1% paraformaldehyde and aquired on a FACS Calibur Flow Cytometer using Cellquest software.

The intracellular cytokines were analysed within CD3+/CD8+ OR CD3+/4+ lymphocytes.

For monocytes, the cytokines evaluated were TNF-α (pro-inflammatory), IL-10 (anti-inflammatory/ suppressive) and IL-12 (inducer of IFN-γ production/type 1 T-cell responses). The analysis for monocytes were similar except blood was stimulated with 1mcg/ML lipopolysaccharide. monocytes expressing CD14 were analysed.

For lymphocytes, the percentage of CD3+ / 4+ T-cells and CD3+ / 8+ T-cells that produce IFN-g (type 1) or IL-4 (type 2) were measured. This provided a reliable indication of any skewing in the Th1/2 ratio induced by phytosterol administration.

Cells were analysed in a FACScalibur (BD) flow cytometer. CD14 vs. side scatter were identified monocytes morphologically, according to light scatter properties. Monocytes were further classified according to intensity of expression into $CD14^{bright}$ or $CD14^{dim}$ subsets and those co-expressing CD16 marker.

All data were saved in listmode and analysed using cellquest software.

This protocol provides information on any changes in the distribution of the three major monocytes subsets, their pro-inflammatory capacity, and ability to induce type 1 T-cell responses.

6.1.5. ANALYSIS OF MARKERS FOR STEROL METABOLISM

Plasma was frozen as a 1.5ml aliquot for analysis of markers of sterol metabolism by gas chromatography, lathosterol/cholesterol ratio could be employed to reflect cholesterol synthesis. Individual phytosterol concentrations can also be assayed by this technique. These investigations was performed by arrangement with the Hammersmith Hospital.

The margarine was also analysed by the Lipid Laboratory gas chromatography to confirm the presence of phytosterol in the margarine. This was done by the Lipid

Laboratory and the method employed was supplied by Prof G Thompson from Hammersmith Hospital in London.

6.1.6 METHOD FOR GAS CHROMOTOGRAPHY

The method involves extraction, derivitisation and isolation as well as detection.

The saponification process is a standard process. In a strong alcohol milieu, heating will cause ester bonds to break in cholesterol ester as well as glycerol esters.

To 100ul of plasma or serum 10ul of internal standard (5 cholestane, 0.5micorgrams/microlitre in absolute ethanol.Sima C8003, C27H48. FW372.7 amu.was added. This was hydrolysed for 1 hour at 55 degrees celsius with 1ml of saponification solution. 1ml of water and 3ml of hexane was added and mixed well. Organic and aqueous phases were allowed to separate. Extraction 2-3 more times with 3ml of hexane was done and pooled. The hexane fraction was dried under the nitrogen jet and transferred to a small capped glass vial where drying was continued.

For derivitisation 100 ul of trisyl TBT was added. The cap was replaced and mixed, this was left on a heat block for 60min at 60°C. This was cooled to room temperature and injected without delay. Isolation on column, the gas chromograph was a Pye Unicam Phillips 4500 with a flame ionization detection system and a Hewlett Packard Intergrator and printer. The column is 0.25mm fused silica capillary column, Restek Xti5. The lab used an injection temperature of 277°C, column at 300°C and FID 300°C and an isothermal run.1ul of the dissolved material was aspirated with a syringe and the syringe was inserted perpendicularly through the septum and to the full length of the needle. It was injected forcibly and quickly and the intergrator was switched on. The data was then recorded.

6.1.7. DIETARY ANALYSIS

The dietary analysis was performed using a computerised dietary analysis program; Food Fundi Proffessional program developed by the Medical Research Council and Penta Medical Systems. The information received from the dietary records were entered by the dietitian for each visit. The dietary information was checked at the time it was received for completeness, to see whether portion sizes were recorded and to question any other information that was not clear. The program analysed all the nutrients, but only those nutrients of interest to the purpose were selected for the study, as shown in Table 6.

6.1.8. ANTHROPOMETRICAL ANALYSIS

A calibrated Seca scale was used to weigh patients at each visit. Patients were weighed with no shoes and as little clothes as possible, weight was measured to the nearest 0.1kg.

Stature was measured to the nearest 0.1cm using a stadiometer.

Waist and hip measurements were taken to estimate the upper and lower body fat distribution. The waist measurement was recorded at the minimal circumference of the abdomen and the hip circumference was measured at the maximal gluteal protuberance of the buttocks. Subjects stood upright and relaxed with mass evenly distributed.

A new bioelectrical impedance machine (Bodystat 1500) was purchased to measure lean body weight, percentage of body fat and water and estimated average requirements as well as basal metabolic rate.

The physical principle of the bioelectrical impedance machine technique is that the body's lean (muscle, bone, water) compartment, comprising approximately 60-75% electrolytic water, conducts electricity far better then the body's fat compartment

which is lower in body water content (5-10%). The two compartments have very different impedance values due to high frequency electric signals. The single impedance measure reflects the degree of resistance to the flow of current in the body, water being a good conductor and fat being a bad conductor.

When using the machine, patients had to be in a supine position. Two electrodes were placed on the right hand: one at the distal portion of the second metacarpal and one at the distal portion of the ulna and the radius. Another two electrodes were placed on the right foot: distal portion of the the second metatarsal and between the two maleolli. Electrodes were then attached to exposed tabs on electrodes once appropriate data of gender, height, weight, age were entered into the unit. After the enter button was keyed results on body composition were available in three seconds (Bodystat 1500 instruction manual).

Compared to other methods for measuring body composition, it was the most practical and accurate method for this study. Skin fold measurements could have been done, however, considerable experience is needed for consistent results. If not within the population for predictive equation, can be off by 200%. Hydrostatic weighing and Dual X Energy X-Ray Absorptometry are more accurate methods for measuring body composition, however these methods are not practical and are expensive. Therefore the bioelectrical impedance measures were used, since when used under the correct conditions, as mentioned above is a non-invasive, indirect, easy means for general assessment for body composition (Burgess, 1997).

6.1.9. STATISTICAL ANALYSIS

CALCULATION OF SAMPLE SIZE

The power of the study to resolve whether a change would be detected between the two margarines was evaluated with the software (Statmate, Graphpad Software, San Diego USA). A comparison by t tests accepting an two tailed value 0.01 and a standard deviation of 0.5mmol/L will have 99% power to resolve difference of 0.6mmol/L of total cholesterol in 36 subjects. Because of a possible fall-out of subjects after recruitment it was deemed necessary to recruit 50 subjects.

PROCESSING OF DATA

Data capturing was done on Corel Paradox fields and units. The data was then imported into a spreadsheet (Quattro Pro). The information was captured as follows: age, plasma TG (visits 1-5), glucose and thyroid hormones (visit1), TC, HDL AND LDL (visits2-5), weight, height, waist, hip, activity, body fat percentage, lean body weight, estimated energy requirements, resting energy expenditure (all visit 1-5), and dietary information for baseline visit and visits 3, 4 and 5 (dietary energy (kJ), total fat (g), saturated fat (g), polyunsaturated fat (g), monounsaturated fat (g), cholesterol (mmol/l), carbohydrate (g), protein (g), fibre(g) and alcohol (g)). Further calculations done in Quattro Pro included (Body mass index (BMI-w/h^2) and waist-hip ratio (waist/hip) .

STATISTICAL ANALYSIS

The data was then imported into Graphpad Prism. Data were tested for normality. Basic descriptive statistics including means, medians, standard deviations, standard errors and 95% confidence intervals were calculated for all five visits.

The aim of the study was to test the difference in lipid values between the regular and phytosterol margarine since the two periods were exactly similar (i.e. substitution of 20g of margarine into their diets, one period being the regular margarine and the other period containing the phytosterols). This was the most crucial analysis. Therefore, student t tests were performed in Graphpad Prism between all the variables of the regular and phytosterol periods. P value of < 0.05 were regarded as significant.

The baseline data was also compared to all the visits to detect any changes. One-way ANOVA tests were done on Graphpad Prism and Bonferoni post-tests were used to test for any significant differences between the five periods.

Uni-variate correlations using linear regression in Graphpad Prism were done on all the variables to observe changes from baseline and changes between the regular and phytosterol margarines.

Calculations were done to test whether there was a period effect. The period effect takes into account whether the order in which the patients received the margarines made a difference to the plasma lipid values (whether a difference in plasma lipid values was detected if they received the phytosterol margarine at visit 2 or 3). Calculations to test for a carry- over effect with the sterol containing margarine was also done. The equations are given below

Period effect

$T = (d1-d2) / SE (d1+d2)$

where $SE (d1+d2) = t = d1 + d2 / sd2 (1/n1 + 1/n2)$

d1 = difference (period R – PS)

d2 = difference (period PS – R)

sd2 = pooled within groups estimate of variance

Carry over effect equation

$$Se(e1 - e2) = Se2\ (1/n1 + 1/n2)$$

E1 = sum (period R + period S)

E2 = sum (period S + period R)

Se2 = pooled within groups estimate of variance.

The statistical methods including the above equations were checked with Ms Monique Hanslo, statistician from the University of Cape Town Statistics Department.

STATISTICAL ANALYSIS OF PLASMA PLANT STEROLS

The ratios of the sitosterol, campesterol and lathosterol to cholesterol, expressed below as □mol x100/mmol cholesterol, had wide variations. Data were selected to include only those from subjects who had successful chromatographic analyses at all the visits so that more sensitive analyses for changes could be performed by paired or repeated measures. The unblinding of the interventions with the regular and phytosterol-enriched spreads allowed pooling into appropriate cohorts to compare the sterol ratios for changes in the following groups of interest: (1) before and after the phytosterol spread was taken by the whole cohort, (2) the change over the first 6 weeks after the phytosterol spread was discontinued by the whole cohort , and (3) the changes in the cohort that took the phytosterol-enriched spread first, and thus created opportunities to examine their plasma after 6 and 12 weeks of discontinuing the phytosterol-enriched spread.

The data was pooled for all the sterol analyses on plasma before and after the use of the phytosterol-enriched spread and were analysed by the paired t-test, for 23 subjects.

Reference

1. P. Armitage and G. Berry (1991). Statistical methods in Medical Research (2nd Edition).

2. Burgess ML. Body Composition Lecture Notes. 24/11/97

3. Marais AD, Firth JC, Bateman ME, Byrnes P, Martens P. Atorvastatin, an effective lipid modifying agent in familial hypercholesterolemia. Arteriosclerosis, Thrombosis and Vascular Biology 1997;17:1527-1531

4.. Blom DJ, Byrnes P, Jones S, Marais AD. Non-denaturing polyacrylamide gradient gel electrophoresis for the diagnosis of dysbetalipoproteinaemia. Journal of Lipid Research 2003; 44:212-217.

7. RESULTS

The results section will be presented. Firstly the changes that occurred on the regular, phytosterol and washout periods will be reported as changes occurred from baseline. Thereafter the comparison between the regular and phytosterol will be presented, which is the most crucial comparison since the two periods were exactly similar with respect to the amount of margarine consumed, diets were similar on the two periods and medication were the same.

7.1.1 SUBJECTS

Of the 49 patients recruited, 5 did not meet the inclusion criteria. Forty-four patients were therefore eligible to participate in the study, but 2 patients withdrew from the study before visit 3. A total of 42 patients completed the study. At visit 2, 22 patients were issued with the regular margarine and 20 patients were issued with the phytosterol-containing margarine. At visit 3, 20 patients were issued with the regular margarine and 22 were issued with the phytosterol-containing margarine. After consideration of compliance (diet (n=3), margarine (n=4) and medication (n=2)), and unexplained large variances in serum cholesterol values that were suggestive of non-compliance with medication (n=3), 12 patients were disqualified from the final analysis. Therefore, only 30 patients were included in the final analysis. These results will be presented.

The average age of the patients was 45 ± 11.52 years old. There were 18 females and 12 males. Patients presented with TG of 1.4 ± 0.83mmol/l, TC of 7.43 ± 2.20mmol/l, HDLC of 1.28 ± 0.48and LDLC of 5.56 ± 2.18mmol/l. The presentation glucose was 5.2 ± 0.55 mmol/l and TSH of 1.76 ± 0.80. The average BMI was 26.10 ± 6.28 and

waist hip ratio of 0.89 ± 0.12. The average body fat percentage was 30.40 ± 11.03% and lean body weight of 51 ± 12.37%.

All patients were on lipid lowering therapy except for four patients who were managed on diet only. No changes were made to the medical management of patients during the study. Medication was obtained from the Day Hospitals where occasionally patients may not be issued with their prescribed medication. Such patients were excluded from the final analysis. The average dose of Atorvastatin used in the study was 20mg.

Patients at the clinic receive dietary counseling at admission to the clinic, since medical management will not be instituted unless dietary and other lifestyle modifications are made. Patients dietary compliance was checked at each visit to the clinic.

7.1.2 COMPLIANCE

The compliance (assessed by counting used containers) of the patients was relatively good. There was a 97% compliance with the regular margarine and 94% compliance with the sterol margarine (not statistically significant). A randomly selected batch of tubs was evaluated to confirm the completeness of margarine use. The margarine in the full containers weighed 21± 0.16g. There was 0.24g ± 0.14g of margarine remaining in the sterol margarine period and 0.22 ± 0.12g of margarine remaining in the regular margarine period (two periods not statistically significant).

The margarine contains 1.6g sitosterol per 20g, as an ester (linoleic acid). This means that the average daily intake of the sterol in the margarine in the study was 1.66g in 20.76g of margarine.

7.1.3 TASTE PREFERENCES

Of the 30 subjects analysed, 15 could not taste a difference between the regular and sterol containing margarine, 11 preferred the taste of the sterol margarine and 4 preferred the taste of the regular margarine. Patients were asked at visit 4 whether they like the taste of the margarine they were given at visit 2 or visit 3. When the study was analysed, note was taken of which margarine was preferred and whether it was the phytosterol or regular containing margarine.

7.1.4. ANTHROPOMETRY

Table 2 shows the baseline data, compared with the different margarine periods and the washout period for 30 subjects. It shows that the anthropometric variables did not change significantly during the two margarine periods. BMI, waist- hip , lean body weight and body fat percentage did not change.

Table 3 shows the comparison between the two margarine periods for 30 subjects. This is the clearest way to distinguish a specific effect of phytosterol enrichment.

It shows that there were no significant changes in the anthropometry from baseline on the regular and phytosterol margarine.

TABLE 2 - Anthropometry for each visit compared with baseline (n=30).

	Period	Mean (at end of treatment)	Standard deviation	Confidence interval	Statistic p value
Body Mass Index	Baseline	26.10	6.45	23.76-28.45	
	Regular	25.97	6.28	23.62-28.32	0.27
	Sterol	25.99	6.24	23.66-28.32	0.25
	End	25.90	6.35	23.48-28.32	0.23
Waist Hip Ratio	Baseline	0.89	0.12	0.84-0.93	
	Regular	0.89	0.12	0.84-0.94	0.37
	Sterol	0.89	0.12	0.85-0.94	0.37
	End	0.89	0.12	0.84-0.94	0.37
%Body Fat	Baseline	30.40	11.03	25.53-33.56	
	Regular	29.90	10.56	25.79-34.00	0.22
	Sterol	29.98	11.00	25.92-34.04	0.27
	End	30.05	11.11	25.83-34.28	0.65
% Lean Body Weight	Baseline	49.84	12.01	45.27-54.41	
	Regular	50.27	11.78	45.87-54.67	0.57
	Sterol	50.25	11.94	45.79-54.71	0.66
	End	50.33	12.04	45.75-54.91	0.57

TABLE 3 – Comparison of anthropometry between the regular and phytosterol margarine.(n=30)

Variable	Comparison	Mean (at end of treatment)	Standard deviation	Confidence interval	Statistic p value
Body Mass Index	Regular	25.97	6.28	23.62-28.31	0.44
	Sterol	25.99	6.24	23.66-28.32	
Waist Hip Ratio	Regular	0.89	0.13	0.85-0.94	0.21
	Sterol	0.89	0.13	0.85-0.94	
% body fat	Regular	29.90	11.00	25.8-34	0.65
	Sterol	29.98	10.88	25.92-34.04	
% Lean Body Weight	Regular	50.27	11.94	45.79-54.71	0.90
	Sterol	50.25	12.04	45.75-54.91	

7.1.5. LIPID CHANGES

Table 4 shows that LDL was reduced on the regular margarine from baseline by 8%, which was statistically significant (p=0.02).

The total cholesterol was reduced from baseline on the regular margarine by 4%, although not statistically significant. TC did change significantly on the phytosterols margarine from baseline by 10%, (p<0.001).

When comparing the actual difference between the regular and phytosterol margarine period (Table 5), the plasma LDL cholesterol was lowered by 6.5% (p=0.01) and total cholesterol by 6.4%(p=0.00) during the sterol period. Triglycerides were also reduced by 18% (p=0.01). Plasma HDL-C did not change significantly during the two periods.

TABLE 4 -Lipid changes on each visit compared to baseline. (n=30)

Variable	Period	Mean	SD	95% confidence interval	p value	% diff where significant
Total cholesterol (mmol/L)	Baseline	7.43	2.20	6.60-8.25		
	Regular	7.15	2.02	6.39-7.89	0.25	
	Sterol	6.69	1.90	5.95-7.39	< 0.0001	10.00
	End	7.11	1.78	6.45-7.77	0.06	
LDL Cholesterol (mmol/l)	Baseline	5.56	2.18	4.73-6.39		
	Regular	5.10	1.91	4.38-5.80	0.02	8.00
	Sterol	4.76	1.91	4.05-5.47	< 0.0001	14.00
	End	5.10	1.81	4.42-5.78	0.01	8.00
Triglycerides (mmol/l)	Baseline	1.33	0.83	0.95-1.71		
	Regular	1.56	1.02	1.19-1.94	0.08	
	Sterol	1.24	0.98	0.99-1.49	0.63	
	End	1.35	0.67	1.09-1.60	0.78	
Hdl Cholesterol (mmol/l)	Baseline	1.28	0.48	1.10-1.46		
	Regular	1.35	0.40	1.19-1.49	0.18	
	Sterol	1.36	0.45	1.19-1.53	0.15	
	End	1.36	0.43	1.22-1.54	0.15	

TABLE 5– Comparison of lipid values between the regular and phytosterol margarine periods. (n=30)

Variable	Comparison	Mean (at end of treatment)	Standard deviation	Confidence interval	Statistic p value	% difference where significant
Total Cholesterol (mmol/l)	Regular	7.15	2.02	6.40-7.90	0.00	6.40%
	Sterol	6.69	1.90	5.98-7.40		
Ldl-Cholesterol (mmol/l)	Regular	5.09	1.91	4.34-5.80	0.01	6.50%
	Sterol	4.76	1.91	4.00-5.47		
Hdl-Cholesterol (mmol/l)	Regular	1.35	0.40	1.20-1.50	0.54	
	Sterol	1.36	0.40	1.20-1.53		
Triglyceride (mmol/l)	Regular	1.57	0.99	1.19-1.93	0.01	18%
	Sterol	1.24	0.67	0.98-1.48		

7.1.6. DIETARY ANALYSIS

From Table 6, it can be seen that the diet followed at entry to the study was significantly higher in total and saturated fat intake. The diet was significantly higher in carbohydrate on the regular and phytosterol margarine than baseline.

The energy, fat, protein and carbohydrate intake did not change significantly during the study. The fat intakes were 25% of energy with saturated fat 7%; polyunsaturated fat 7% and monounsaturated fat 8%, the cholesterol intakes were less than 110-126mg. The patients were therefore adhering to a Step 2 diet during the study period.

TABLE 6 - Dietary information on each visit compared with baseline. (n=30)

Variable	Comparison	Mean (at end of treatment)	Standard deviation	Confidence interval	Statistic p value	% diff where significant
Total Fat (g)	Baseline	47.00	11.35	42.75-51.24		
	Regular	37.67	7.20	35.20-40.70	<0.001	20.00
	Sterol	35.44	5.96	33.20-37.70	<0.001	24.00
	End	36.00	10.00	32.52-40.15	0.01	23.00
Saturated Fat (g)	Baseline	14.00	4.93	12.39-15.03		
	Regular	11.30	3.95	9.96-12.73	0.01	19.00
	Sterol	11.00	2.93	9.49-11.75	<0.001	21.00
	End	11.73	4.23	10.12-13.34	0.06	
Polyunsaturated Fat (g)	Baseline	11.00	2.87	9.78-11.93		
	Regular	10.00	2.14	9.00-10.50	0.78	
	Sterol	9.00	2.34	8.30-10.00	0.22	
	End	9.00	2.92	7.89-10.10	0.07	
Monounsaturated Fat (g)	Baseline	16.00	5.66	13.93-16.89		
	Regular	12.00	3.69	10.20-13.00	<0.001	25.00
	Sterol	11.00	2.96	9.83-12.00	< 0.001	31.00
	End	11.00	4.14	9.56-12.74		
Energy (Kj)	Baseline	5,942.00	1,915.00	5227-6657		
	Regular	5,576.00	1,324.00	5082-6071	0.21	
	Sterol	5,449.00	1,330.00	4952-5945	0.14	
	End	5,523.00	1,492.00	4955-6091	0.23	
Protein (g)	Baseline	60.71	3.35	56.34-65.08		
	Regular	57.30	4.23	52.00-62.00	0.89	
	Sterol	57.90	5.45	51.00-64.00	0.91	
	End	57.56	5.35	50.97-64.19	0.80	
Carbohydrate (g)	Baseline	181.00	32.01	175.62-184.49		
	Regular	199.00	29.06	187.00-209.00	<0.001	10.00
	Sterol	195.00	23.30	186.00-204.00	0.01	8.00
	End	184.00	30.44	173.38-195.50	0.07	
Cholesterol (mg)	Baseline	158.00	84.40	126-90-189.90		
	Regular	126.00	46.27	108.30-142.80	0.05	
	Sterol	110.00	39.68	95.18-124.80	0.02	30.00
	End	125.00	53.59	100.90-141.60	0.05	

TABLE 7 - Dietary information comparison between the regular and phytosterol margarine. (n=30)

Variable	Comparison	Mean (at end of treatment)	Standard deviation	Confidence interval	Statistic p value
Total Fat (g)	Regular	38.00	7.20	35.20-40.70	0.17
	Sterol	35.00	6.00	33.20-37.70	
Saturated Fat (g)	Regular	11.30	3.70	9.96-12.73	0.43
	Sterol	10.64	2.93	9.49-11.75	
Polyunsaturated Fat (g)	Regular	10.00	1.42	9.00-10.50	0.18
	Sterol	9.20	1.59	8.30-10.00	
Monounsaturated Fat (g)	Regular	11.60	2.45	10.20-13.00	0.53
	Sterol	11.00	2.01	9.83-12.00	
Cholesterol (mg)	Regular	125.50	46.27	108.30-142.80	0.08
	Sterol	110.00	39.68	95.18-124.00	
Energy (kj)	Regular	5,576.00	1,324.00	5082-6071	0.17
	Sterol	5,449.00	1,330.00	4952-5945	
Fibre (g)	Regular	17.67	6.80	15.10-20.21	0.70
	Sterol	17.33	9.67	14.80-19.95	
Alcohol (g)	Regular	1.93	4.50	0.25-3.61	0.64
	Sterol	1.63	3.80	0.20-3.06	
Carbohydrate (g)	Regular	199.00	29.00	187.00-209.00	0.06
	Sterol	195.00	24.00	186.00-204.00	
Protein (g)	Regular	57.00	13.80	52.00-62.00	0.51
	Sterol	57.00	17.40	51.00-64.00	

7.1.7 CARRY OVER AND PERIOD EFFECT

TABLE 8

Calculated value for the equations for the period effect and carry over effect. The p value is given in each case.

	Equation Value	p value
Triglycerides		
Period	0.45	>0.05
Carry Over	0.58	>0.05
Ldl – cholesterol		
Period	0.54	>0.05
Carry Over	0.7	>0.05
Total Cholesterol		
Period	0.12	>0.05
Carry Over	0.28	>0.05

7.1.8 PLASMA PLANT STEROL ANALYSIS

The phytosterol/cholesterol ratio increased significantly from 254 ± 123 (SEM) to 302 ± 28 ($p < 0.007$) and the campesterol/cholesterol ratio increased significantly from 341 ± 21 to 653 ± 54 ($p<0.0001$) whilst there was no significant change for the ratio of lathosterol to cholesterol: 73 ± 8 and 76 ± 8 ($p = 0.42$), see Table 9.

On the same cohort, comparisons were made between the values obtained at the end of the phytosterol spread consumption and the washout over 6 weeks . The phytosterol/cholesterol and campesterol/cholesterol ratios decreased statistically significantly to 241 ± 24 ($p < 0.02$) and 419 ± 58 ($p < 0.0002$) respectively, whereas the lathosterol/cholesterol ratio did not change significantly to 83 ± 14 ($p = 0.35$), see Table 9.

As it appeared that the campesterol/cholesterol ratio had not returned to normal, a comparison of the ratios of phytosterol, campesterol and lathosterol to cholesterol was undertaken in the cohort of 11 subjects who had data available for the ratios of these sterols at the end of the phytosterol treatment, and the end of the regular spread treatment that acted as a 6 weeks wash-out period, and the end of the designated 6 weeks washout. The phytosterol/cholesterol ratio decreased significantly from 303 ± 33 to 251 ± 27 and 241 ± 24 ($p<0.02$) and the campesterol/cholesterol ratio also decreased significantly, from 655 ± to 428 ± 50 and 419 ± 58 ($p<0.001$), see Table 10. The Bonferroni post-tests revealed that there were no significant differences between the 6 weeks and 12 weeks washout points for the campesterol/cholesterol ratios whereas there was a significant difference for the phytosterol/cholesterol ratio at these intervals.

Table 9.

Plant sterol:cholesterol ratios in the selected whole cohort of 23 subjects taking regular and phytosterol-enriched margarine, comparing the different phases of the study.

Ratios	Before intervention	After intervention	After 6 weeks
Phytosterol/cholesterol	254 ± 123	302 ± 28	241 ± 24
Campesterol/cholesterol	341 ± 29	653 ± 54	419 ± 58
Lathosterol/cholesterol	73 ± 8	76 ± 8	83 ± 14

Results are expressed means and SEM. The means are statistically significantly different for the phytosterol/cholesterol but not for the lathosterol/cholesterol ratios (ANOVA, $p<0.007$).

Table 10

Plant sterol:cholesterol ratios in the selected cohort of 11 subjects for the prolonged washout observations following phytosterol-enriched margarine.

Ratios	Before intervention	After intervention	After 6 weeks
Phytosterol/cholesterol	303 ± 33	251 ± 27	241 ± 24
Campesterol/cholesterol	655 ± 74	428 ± 50	419 ± 58
Lathosterol/cholesterol	79 ± 15	78 ± 12	83 ± 14

Results are expressed as means and SEM. The means are statistically significantly different for the phytosterol/cholesterol but not for the lathosterol/cholesterol ratios (ANOVA, $p<0.02$ for phytosterol and $p<0.001$ for campesterol).

APPENDIX F and G

Gas chromatography done by the Lipid Lab on the regular and phytosterol margarine indicates the presence of B sitosterol as seen by the peaks at 24s and 21min respectively, these peaks are not seen with the regular margarine.

IMMUNOLOGY

The influence of dietary phytosterols on monocyte numbers and function was evaluated for each subject after consuming the regular or phytosterol-enriched margarine, by measuring monocyte subset distribution and cytokine production.

All the tables that follow represent pooled processed data of the individual responses in all the study subjects. Table 11 indicates no significant difference in total WBC or in the percentage of monocytes within the total leukocyte population.. Furthermore, the percentage of the four circulating monocyte subsets (defined by variable co-expression of CD16 surface marker and variable intensity of CD14 surface marker expression) did not differ significantly between the two interventions. Following stimulation of monocytes (as defined by expression of CD14 lineage marker) with LPS there was no statistically significant difference between the two patient groups in the expression of the cytokines TNF-α, IL-10 or IL-12 (table 12). However, higher levels of spontaneous monocyte secretion of IL-12 (ex-vivo, unstimulated blood) was observed in the subjects on phytosterol enriched margarine (table 14).

Lymphocyte activation was evaluated by measuring the whole blood proliferative response obtained following stimulation of T-cells in-vitro by PMA and ionomycin, and by measurement of T-cell expression of the IL-2 receptor (CD25) activation marker. The percentage of cells expressing either type 1 (IFN-γ) or type 2 (IL-4) cytokines was determined for both CD4+ and CD8+ lymphocyte subsets. These results are shown in table 13. They again demonstrate no statistically significant difference between the interventions. The percentage of CD4+ and CD8+ unstimulated lymphocytes (ex-vivo blood) in which type 1 (IFN-γ) or type 2 (IL-4) cytokines could be detected was also not significantly different (table 15). The balance of TH1/TH2 intracellular cytokines in whole blood can be expressed as a

ratio of these cytokines in unstimulated or PMA- ionomycin stimulated CD4+ and CD8+ lymphocyte subsets. This represents a highly sensitive way of detecting any modulation of type 1 and type 2 cytokines. As shown in table 16, no statistically significant difference in the ratio was observed in subjects on regular or phytosterol enriched margarine.

TABLE 11

CIRCULATING MONOCYTE SUBSETS

	Mean	Standard deviation	95% confidence interval	Statistic p value
Regular CD14+16+	4.78	2.42	3.24-6.32	0.20
Phytosterol CD14+16+	6.58	4.45	3.76-9.42	
Regular CD14++16-	77.05	6.20	73.10-80.99	0.34
Phytosterol CD14++16-	74.20	8.76	68.64-79.77	
Regular CD14++16+	13.57	5.23	10.25-16.89	0.68
Phytosterol CD14++16+	14.59	5.52	11.08-18.10	
Regular CD14+16-	4.59	2.02	3.30-5.88	0.97
Phytosterol CD14+16-	4.62	1.92	3.39-5.89	
Mono% regular	8.89	8.89	7.05-10.74	0.28
Mono% phytosterol	7.81	7.81	5.92-9.72	
WBC regular	6.04	1.59	5.03-4.71	1.0
WBC phytosterol	5.80	1.62	4.70-6.90	

All values represent the percentage of cells from the flow cytometric analysis, except for the WBC and monocte counts which were determined by a Coulter counter and are expressed as 10^9/L

TABLE 12

INTRACELLULAR CYTOKINE EXPRESSION BY MONOCYTES GIVEN AS PERCENTAGE OF CELLS EXPRESSING THE GIVEN CYTOKINE; CD14-GATED AGAINST SIDE SCATTER.

	Mean	Standard deviation	95% confidence interval	Statistic p value
Regular TNF	86.40	17.58	75.23-97.57	0.91
Phytosterol TNF	81.38	26.55	64.51-98.25	
Regular IL10	1.38	1.53	0.39-2.34	0.49
Phytosterol IL10	1.80	4.35	-0.96-4.56	
Regular IL2	21.63	17.27	10.65-32.60	0.27
Phytosterol IL2	9.59	10.20	3.11-16.07	

Values represent the percentage of cells expressing the given cytokine.

TABLE 13

LYMPHOCYTE PROLIFERATION ACTIVATION MARKERS (CD25) AND INTRACELLULAR CYTOKINE EXPRESSION FOLLOWING STIMULATION WITH PMA & IONOMYCIN. SI IS FOR STIMULATION INDEX (CPM-STIMULATED/CPM BACKGROUND).

	Mean	Standard deviation	95% confidence interval	Statistic p value
PMA regular	271.00	165.60	165.80-376.20	0.62
PMA-phyto	226.90	91.64	188.70-285.10	
CD25 regular	62.17	13.58	53.54-70.80	0.27
CD25 phyto%	66.17	16.49	55.69-76.64	
CD4 regIFN	12.75	8.53	7.33-18.17	0.11
CD4 phytoIFN	9.93	7.15	5.39-14.48	
CD4 reg IL4	3.10	2.08	1.77-4.42	0.09
CD4 phyto IL4	1.73	1.01	1.73-1.01	
CD8 reg IFN	42.16	14.38	33.02-51.30	1.0
CD8 phyt IFN	33.11	19.80	20.53-45.69	
CD8 reg IL4	1.55	1.53	0.58-2.53	0.21
CD8 phyto IL4	1.73	1.78	0.60-2.86	

Mean values of PMA + I are reported as the stimulation index (SI, cpm-stimulated/cpm background). All other mean values reflect percentage of cells expressing

TABLE 14

INTRACELLULAR CYTOKINE EXPRESSION IN UNSTIMULATED MONOCYTES EX VIVO.

	Mean	Standard deviation	95% confidence interval	Statistic	p value
Regular IL12	2.11	1.53	1.14-3.08		0.02
Phytosterol IL12	2.99	1.34	2.14-3.85		
Regular IL10	2.12	2.04	0.83-3.42		0.42
Phytosterol IL10	1.48	1.76	0.36-2.60		
Regular TNF	0.65	0.54	0.31-0.99		0.23
Phytosterol TNF	0.44	0.35	0.21-0.66		

Mean values represent percentage of cells expressing relevant cytokine

TABLE 15 - INTRACELLULAR CYTOKINE EXPRESSION IN EX VIVO UNSTIMULATED CD4+ AND CD8+ T-CELL SUBSETS

	Mean	Standard deviation	95% confidence interval	Statistic p value
Regular CD4 IL4	0.53	0.38	0.28-0.76	0.25
Phytosterol CD4 IL4	0.63	0.34	0.41-0.85	
Regular CD4 IFN	0.27	0.20	0.13-0.39	0.76
Phytosterol CD4 IFN	0.58	1.06	-0.09-1.24	
Regular CD8 IL4	0.60	0.29	0.41-0.78	0.68
Phytosterol CD8 IL4	0.57	0.45	0.28-0.85	
Regular CD8 IFN	0.48	0.28	0.31-0.66	0.32
Phytosterol CD8 IFN	0.78	0.70	0.33-1.23	

Mean values represent percentage of cells expressing relevant cytokine

TABLE 16 - Ratios OF IFN-GAMMA /IL4 in CD4 & CD8 LYMPHOCYTE SUBSETS

	Mean	Standard deviation	95% confidence interval	Statistic p value
Regular CD4 Unstimulated	1.31	1.14	0.59-2.04	0.25
Phytosterol CD4 Unstimulated	1.13	1.19	0.38-1.88	
Regular CD4 Stimulated	3.83	4.67	0.86-6.80	0.25
Phytosterol CD4 Stimulated	2.47	2.51	0.87-4.06	
Regular CD8 Unstimulated	1.19	1.15	0.46-1.92	0.28
Phytosterol CD8 Unstimulated	2.57	3.07	0.49-4.62	
Regular CD8 Stimulated	70.34	63.95	29.70-111.00	0.43
Phytosterol CD8 Stimulated	34.70	38.29	7.31-62.08	

8. DISCUSSION

8.1.1 LIPIDS

The objective of this study was achieved since the effect of the phytosterol containing margarine on lipid levels and immune function were clearly demonstrated.

This study shows a reduction in total cholesterol and LDL cholesterol whereas HDL remained unchanged in FH patients who consumed phytosterol margarine as compared to the regular margarine. The reduction in total cholesterol of 6.4% and LDL cholesterol of 6.5% is in keeping with other studies on patients with normal or mildly elevated cholesterol levels (Moghadasian and Frohlich, 1999).

LDLC was reduced on the regular margarine from baseline by 8%, which was statistically significant (p=0.02), but the difference when comparing directly to the regular and phytosterol margarine periods was 6.5% (p=0.01).

A possible explanation for the reduction of the LDL cholesterol with the regular margarine could be better compliance with the diet and to a lesser extent, the introduction of PUFA's. Also possibly drugs (while monitored) were taken more regularly, but that would apply to both groups. The patient's diets at entry to the study was significantly higher in total fat, saturated fat and monounsaturated fat than the diet consumed with the regular margarine and the sterol margarine.

When comparing for the most crucial difference between the regular and phytosterol margarine, the LDL cholesterol was significantly lower on the phytosterol margarine than the regular margarine, i.e. 6.5% lower (total reduction from baseline 14%). This reduction in LDL on the phytosterol margarine is probably due to the effect of the phyosterols since the diet and medication between the two margarine periods did not differ.

The total cholesterol was reduced from baseline on the regular margarine by 4%, although not statistically significant. Total cholesterol did change significantly on the phytosterol margarine from baseline by 10%.

When comparing the most crucial difference between the regular and phytosterol margarine, the total plasma cholesterol was lower on the phytosterol margarine than the regular margarine by 6.4 %.

When comparing the above results with previous studies, the reduction in plasma cholesterol and LDL and HDL are within expected values. In Moghadasian et al., (1999) review of 16 randomised controlled trials where various mixtures of phytostanol and phytosterol mixtures (between 1-3g/day) were used, the range for the reduction in total cholesterol ranges between 5-23% and LDL cholesterol reduction of 7-29%. However, most of these studies were done on normo and mildly hypercholesterolaemic(n=5) and moderately hypercholesterolaemic (n=7) subjects who were not on any drug treatment, while 3 were done on FH children. The responses in children were much greater (range for total cholesterol 11-29% and LDL cholesterol 15-23%). The reason for this could be due to the fact that the children receive much more phytosterol/kg than adults i.e. child of 35 kg consuming 1.6g of phytosterols/day would receive 0.05g phytosterol/kg compared to a 60kg adult who would receive 0.03g phytosterol/kg. However, the dose dependant study done by Hendricks et al, (1999) found that there was not any significant reduction in lowering of cholesterol with the three different doses of phytosterol (i.e. 0.83g/day, 1.61g/day and 3.24g/day) in adults. Therefore, the reason for the larger response in children could possibly not be due to the dose, perhaps there is a difference in the absorption in children, but this has not been investigated in any of the studies.

The largest response seen in children was a study done by Becker et al. (1992). The authors studied seven FH children. The children first followed dietary intervention for

three months, followed by phytosterol pastilles (3 x 2g/day) which resulted in lowering of total and LDL cholesterol of 17%, followed by bezafibrate for three months(11 to 22 mg/kg/day) resulting in cholesterol lowering of 18% in total and 28% for LDL cholesterol, followed by bezafibrate (200mg/day) and phytosterol(3 x 1g/day) for 24 months resulting in cholesterol lowering of 40% in total and 50% in LDL cholesterol. The lowering of the cholesterol on the phytosterol period is in keeping with other findings done in children. However, the combination of the bezafibrate with phytosterol does result in very high reductions in total cholesterol. This could be due to the fact that the study did not allow for any washout periods and the cholesterol lowering effect of the high dose of bezafibrate could have resulted in a carry over effect into the next study period resulting in the huge reductions.

When comparing this study to others in which statins are used, the following results were reported. In diabetic subjects with hypercholesterolaemia receiving Pravastatin, there was an additional 4% lowering with the consumption of 3g sitostanol/day (Gylling and Miettinen, 1996) and an additional 11% lowering in postmenopausal mildly hypercholesterolaemic women with CHD on Simvastatin (Gyling et al., 1997). In another study performed on FH subjects on statins, there was a 14% reduction in total and 20% reduction in LDL cholesterol in subjects consuming 2.24g of plant stanols (Vurio, 2000). However, the study design was different to this study in that it was not a randomized controlled cross-over study, also there is no report on the dietary composition that the subjects consumed on baseline nor on the test margarine period. The reduction therefore does appear to be larger than in our study, but there is no evidence to suggest that this reduction could have also been due to changes in diet or medication compliance. It could also be due to the higher dose of plant stanols, however, as discussed before, Hendricks et al. (1999) did not find any differences in cholesterol lowering with three different doses of plant stanols.

There was a triglyceride increase of 18% on the regular margarine, although not statistically significant. This was not seen on the phytosterol margarine or at the end of the study. This increase could be related to the increased carbohydrate intake (10%) on the regular margarine. Although the carbohydrate was similar between the regular and phytosterol margarines, there was no increase in the triglyceride on the phytosterol margarine.

When comparing the regular and phytosterol margarine periods, there was a significant decrease in triglycerides on the sterol margarine by 18%. This decrease was not due to the effect of phytosterols, since there was not significant difference on the phytosterol margarine from baseline, but rather due to an increase in triglycerides on the regular margarine.

However, when one looks at the actual values, there is only a 0.32 mmol/l plasma triglyceride difference between the regular and phytosterol periods, but because the plasma triglyceride values are so small, the percentage is large in comparison. Another reason why the triglycerides could have increased on the regular margarine could be due to differences in testing in the chemical laboratory.

LDL REDUCTION

The mechanism for the reduction in LDL cholesterol on the phytosterol margarine has been explained in detail in the review section and will be briefly summarised. The phytosterol competes with cholesterol for incorporation into mixed micelles. This results in less cholesterol entering enterocytes. Less (free and esterified) cholesterol is incorporated into chylomicrons. Less intestinally derived cholesterol is then transported through the circulation to the liver. Consequently an upregulation of the LDL receptor and HMG-CoAReductase.. The increased hepatic uptake of LDL by LDLR lowers LDL plasma cholesterol. The transporters involved in the process have

been explained. Since they are active in exporting phytosterol from the enterocyte little is absorbed. The small amount that enters the circulation in chylomicrons is taken up by the liver and, like in the enterocyte is exported to the bile by ABCG5 and ABCG8.

The factors that might govern the LDL response were examined by looking for correlations between the absolute and relative changes in LDL cholesterol (Appendix D). In this study, the LDL response (i.e. the reduction between the regular and phytosterol) margarine negatively correlate with age indicating that there is a smaller response in older people (Appendix D – Table B). This was not affected by gender. There was also a negative association with waist hip ratio, indicating that there is a better response in leaner people.

Plasma LDL concentration positively correlated with triglycerides probably reflecting the lipoprotein production rate (Appendix D – Table A). The liver is the primary site of production of VLDL, which is the precursor for LDL, and is the organ responsible for the bulk of LDL-receptor mediated clearance of LDL from the plasma. Within the hepatocyte, excess cholesterol is converted to cholesterol esters (CE) through the action of ACAT2. Formation of excess CE may potentiate VLDL secretion and reduced availability of cholesterol esters may reduce secretion (Dodson and Barnett, 1999).

TRIGLYCERIDE CHANGE

There was a positive correlation with lean body weight (Appendix D- Table E). The correlation explains a 30% variance and is similar between the two periods (regular and phytosterol margarine).

A positive correlation with BMR and EER could be explained by obesity. Obese individuals have a higher BMR and higher EER because of their greater weight.

Obese people also have higher plasma triglyceride values. In obese individuals, lipolysis of adipose tissue is often accelerated, due to insulin resistance. There is also a direct release of free fatty acids in portal circulation. Therefore, the greater influx of triglyceride precursors to the liver could lead to VLDL overproduction. Turnover studies also show increased synthesis of both VLDL cholesterol and ApoB. More VLDL particles, enlarged because TG enriched, are found in obese subjects (Kelly DE, 2002).

PLASMA TRIGLYCERIDE

There is a negative association with HDL (Appendix D-Table D). This is expected. It is generally assumed that by defective breakdown of very low-density lipoprotein triglycerides (VLDL-TG), a lower amount of free cholesterol will be moved from the lipoprotein surface to the HDL density range, thus in turn lowering HDL. The VLDL is reduced by stimulating their catabolism and in turn increasing HDL levels.

There is also a positive correlation with waist-hip ratio also again due to obesity.

Again a positive correlation with lean body weight.

8.1.2 CARRY OVER AND PERIOD EFFECT

There were no significant differences as a carry over effect for the period resulting from the randomisation between the regular and phytosterol margarines ($p>0.1$) The period effect takes into account the order in which the patients received the two treatments. The carry over effect takes into account any carry over in the response to the phytosterols margarine into the next period.

8.1.3 PLASMA PLANT STEROLS

The phytosterol-enriched spread has been analysed and was found to contain phytosterol, campesterol and stigmasterol in the following molar ratios: phytosterol 47: campesterol 26: stigmasterol 19 (Dr F O' Neill, personal communication). Thus, the phytosterol-enriched spread supplied additional phytosterol and campesterol to the background diet.

The increased ratio of both phytosterols to cholesterol in the plasma suggests that significant amounts of these sterols must be absorbed as they cannot be synthesised in animals. The sterols competitively inhibit cholesterol absorption at the mixed micelle level and by means of as yet unidentified mechanism at the enterocyte. The resultant diminished contribution of the absorbed cholesterol to the pool of cholesterol in the hepatocyte could induce the increased clearance of LDL from the circulation as well as the increased synthesis of cholesterol by upregulation of LDL receptors and HMGCoA respectively. The lathosterol/cholesterol ratio is expected to increase with increased de novo synthesis but this was not found in this study. It is likely that there is an early increase in HMGCoA reductase activity together with the LDL receptor regulation but, if the latter compensates for the reduced cholesterol pool, HMGCoAR would be rapidly turned off by changes in its phosphorylation state or by down-regulation. Thus, it is possible that either an early or a small change in increased de novo cholesterol synthesis could be missed by testing only after 6 weeks. It may be possible to detect such early changes better with fasting mevalonic acid concentrations.

The results also suggest that the LDL cholesterol-lowering effect of the phytosterol spread is related to the interference with cholesterol absorption alone, as the LDL

cholesterol reverted to pre-internvention levels by the end of the washout despite the mildly increased phytosterol concentrations that may still pertain at 6 weeks, although this concentration may also be too low to exert a regulatory effect.

These analyses also indicate that there are differences in the absorption and clearance rates of campesterol and phytosterol. As indicated, both of these plant sterols are presented in larger amounts to the enterocyte when the subject is taking the phytosterol spread. The mechanisms of absorption and excretion are not fully understood, but it may be speculated that the ABC transporter G5-G8 is more efficient in excreting the phytosterol and in doing so selects campesterol for absorption. A similar phenomenon may also select the phytosterol for biliary secretion in the liver and thus promote the greater increase in campesterol into the plasma compartment whilst there is phytosterol present. The apparently more rapid clearance of campesterol as compared with phytosterol after cessation of the phytosterol-enriched spread remains to be confirmed and explained. Phytosterol may be mobilised more slowly from the body compartments during the washout as its stereochemistry differs more from that of cholesterol than campesterol, imposing a significant disadvantage to ABC transporters other than ABCG5-G8.

The interesting observation from this study is that, whilst there appears to be no carry-over effect of the phytosterol-enriched margarine in the washout phase(s), it is clear that the phytosterol concentrations had not returned to pre-intervention levels by the end of 6 weeks. This suggests that the influence of phytosterols is almost certainly only due to the lessened absorption of cholesterol. Additionally, this study hints at different rates of absorption and/or excretion for the structurally very closely related

phytosterol and campesterol: the latter increases more in relative concentration and also reaches steady-state more rapidly.

8.1.4 DIET

The reported energy intakes do seem low. This could reflect underreporting, but the reporting is consistent during the regular and sterol containing margarine periods. When the BMR to energy ratio is compared, the value obtained is 0.9. Any value less than 1.2 indicates that there is underreporting, therefore the patients on the study have underreported their intake.

Studies have shown that subjects who are obese, those with little formal education, those who are in lower socioeconomic groups and those who smoke but do not drink alcohol all underreport dietary intake (Ketch et al, 1999; Johnson et al, 1998; Kristal et al, 1998).

Behavioral factors are also thought to play a role. Kretsch et al suggested that dietary restraint , depression and elevated anxiety levels might affect reports of dietary intake. Studies have demonstrated that underreported intake may not affect all foods equally (Poppitt et al, 1998).

In this study, not all the characteristics mentioned above related to underreporting were investigated. All patients could read and write in order to complete the diet records, but information about level of education, socio-economic status, smoking, depression, behavioural or anxiety levels were not collected. It could be possible that some of these factors contributed to the underreporting.

The estimation of portion size rather than direct weighing is associated with imprecision at the individual level. In general, this is in the order of 50% (coefficient of variation) for foods and 20% for nutrients (Bingham et al, 1990).

The diet at entry to the study was significantly higher in total and saturated fat intake on the regular and phytosterol margarines and significantly higher in carbohydrate on the regular and phytosterol margarine than baseline. This could have been the reason that the LDL was reduced on the regular margarine by 8%.

When comparing the crucial difference between the regular and phytosterol margarine, dietary parameters also did not change significantly between the regular and phytosterol margarine periods during the study.

8.1.5 COST IMPLICATIONS

Patients with FH present with very high cholesterol and LDL plasma levels and have a very high risk of ischaemic heart disease. Dietary intervention is usually the first line of treatment, but would only reduce the cholesterol by approximately 1.5mmol/l in our experience at the Lipid Clinic. This will not reduce cholesterol levels to the target set by South African Clinical guidelines (South African Medical Association and Lipid and Atherosclerosis Society of Southern Africa Working Group, 2000) .. Therefore, drugs have to be instituted to reduce the cholesterol to within recommended guidelines. However, the cost of the drugs is beyond the budget allocated by the Provincial Administration for the goals to be met. The Lipid clinic thus due to budget constraints, cannot treat patients to reach their target lipid values. It is attractive therefore to analyse the cost efficacy of the phytosterol margarine in this setting as a means by which patients might improve lipid control.

The patients analyzed for the study had presentation LDL cholesterol levels of 7 mmol/l. If they were to receive medication to reduce their LDLC levels to recommended guidelines, a dose of 80mg of Atorvastatin has to be prescribed. Even at this dose, target LDL levels may still not be reached. The patients are on average only receiving 20mg Atorvastatin.

The effect Pro-Activ margarine in reducing LDL levels is equivalent to doubling the dose of the statins. The margarine costs R40 per month. The most cost-effective statin on the South African market at the time of the study was Atorvastatin. In the retail market the cost was R296 for 10mg per month, 20mg cost R421 per month and 40mg cost R540 per month. A patient on 20mg of Atorvastatin and still not on target would have to double the dose of the drug, which would cost the patient an additional R121. However, if the patient bought the margarine it would only cost R40. This amounts to a saving of R81. At much higher doses the savings are much greater as can be seen in the diagram (Figure 5). The margarine would also be a safer and more natural option for reducing the cholesterol than using more lipid lowering medication, which is associated with side effects.

In comparison with other margarines, the price of the margarine does seem expensive(appendix C). The high cost of the product is ascribed to the fact that 2500 parts of raw material is required to manufacture one part of sterol (Law M, 2000).

The extra light margarine and pro-activ margarine have a high moisture content, little fat, almost no trans fatty acids, and have a high polyunsaturated content (Appendix C). These features make the margarine suitable for use in patients with elevated cholesterol levels. The addition of the phytosterols in the Pro-activ has been proven to further lower cholesterol levels. These spreads could be used on bread or melted onto foodstuffs such as vegetables after their preparation.

The efficacy of phytosterols is not affected by heat. However, because of the high moisture content it is not recommended that these margarines be used for frying, a method of cooking which is not recommended as part of a healthy diet anyway.

The following illustration summarises the cost effectiveness of the phytosterol containing margarine when used before any drug treatment is initiated as well as

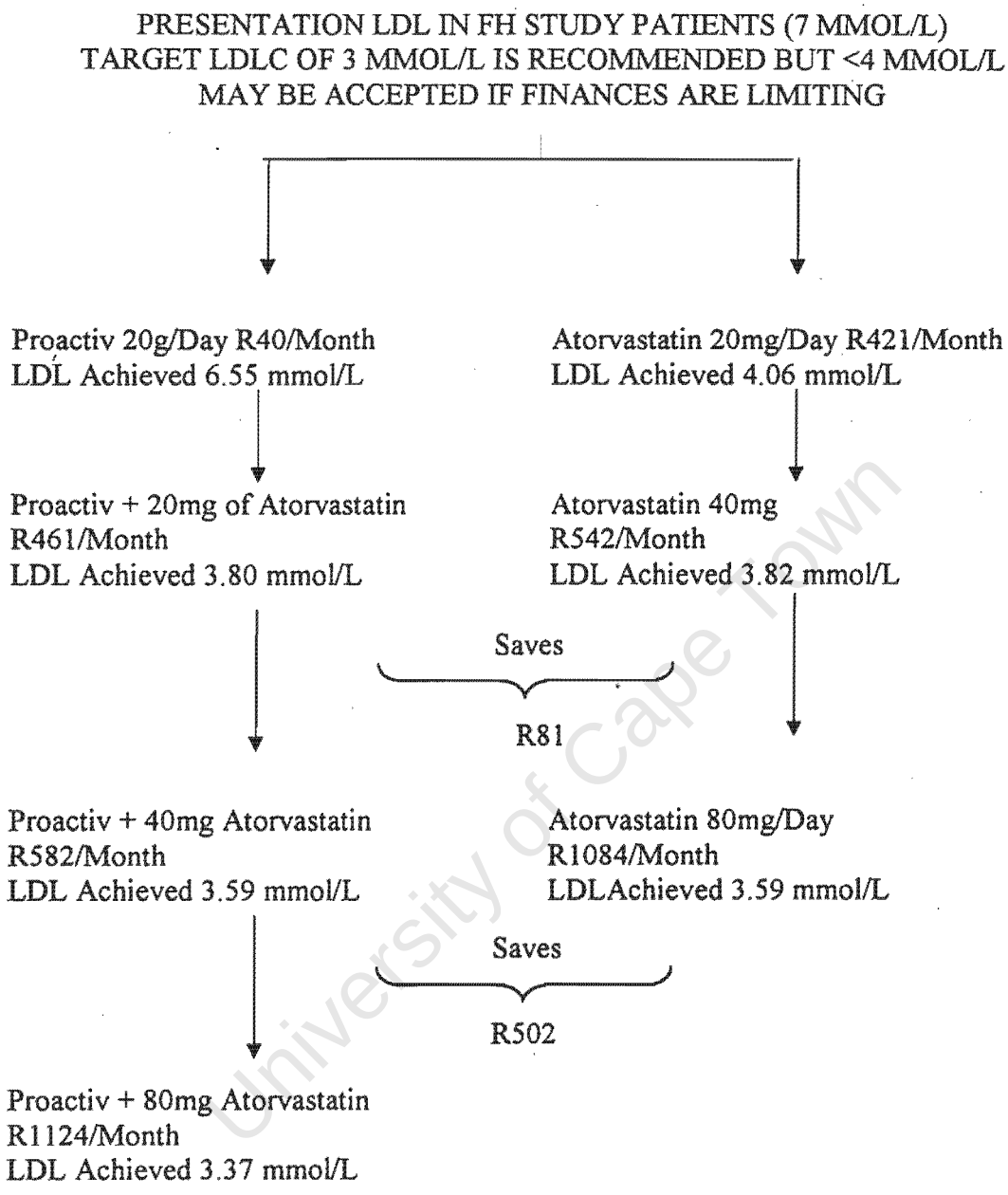
using the phytosterols margarine in addition to medication to reduce LDLC plasma levels instead of doubling the dose of the medication.

The statin reduction will be coded as follows (Nawrocki et al., 1995):

1. LDLC lowering of 42% for 20mg of Atorvastatin
2. LDLC lowering of 48% for 40 mg
3. LDLC lowering of 54% for 80mg

The margarine has a reduction of 6.4%.

FIGURE 7 - COST IMPLICATIONS



It has been estimated that every 1% reduction in LDLC translates into a 2% reduction in CVS events. From this point of view, the subjects not having reached target, would also benefit from the margarine consumption.

The cost implications for moderate LDL hypercholesterolemia also need consideration. In this setting, according the landmark statin studies such as the 4S, CARE and LIPID, secondary prevention is achieved by lowering LDLC by between 25-35% (Scandanavian Simvastatin Study Group, 1994; LIPID Study Group, 1998). In primary prevention studies with statins e.g. WOSCOPS and AFCAPS, 26% reductions of LDLC were effective (Shepherd et al., 1995; Downs et al., 1998).

The dose-response curve of the statins is not linear: the bulk of the change in LDLC occurs near the lowest dose marketed. It has been accepted that a 6% rule applies to doubling the dose of a statin, eg. Atorvastatin 10mg lowers LDLC by 36% and higher doses lowers LDLC as previously indicated (Knopp, 1999). Consequently, 5mg of atorvastatin will lower LDLC by 30%. In a person who requires 36% lowering of LDLC to achieve a target LDLC of 3mmol/L or to conform with 4S, it would be possible to take ½ of a 20mg tablet so that R300/month is reduced to R150/month, while adding the margarine at R40/month could achieve the same target at R190/month and in doing so could save R110/per month.

8.1.6 SAFETY

Plasma vitamin E levels were not assayed, but were unlikely to be a problem because the phytosterols margarine was fortified with 66mg of vitamin E that the regular margarine did not contain. In addition, changes in LDL also reflect changes in plasma vitamin E as discussed in the literature review.

In subjects with phytosterolaemia (homozygous) there is a markedly increase(>30 fold) in absorption of phytosterol, stigmasterol and campesterol in their plasma. In

normal healthy individuals, only 5% of phytosterol is absorbed, almost all of the ingested sitosterol is secreted into the bile with trace amounts of sterols remaining in the plasma. In phytosterolaemia, however, subjects absorb between 15-60% of phytosterols and secrete little into the bile. In addition to plant sterols, there is also hyper absorption of cholesterol (Berge et al., 2000). The use of the margarine would clearly be contraindicated for patients that are homozygous for the condition.

However, subjects that are heterozygous carriers for this disorder are clinically and biochemically normal. According to a study performed on heterozygous carriers of phytosterolaemia,, the levels of plant sterols increased to levels as found in normal individuals. In addition, plasma cholesterol levels were also reduced to levels to the same extent as in normal individuals (Stalenhoef, 2001).

8.1.7 IMMUNOLOGY

Boiuc (1996) has suggested that BSS and BSSG have immune-modulating properties. He found BSS and BSSG beneficial in enhancing immune function by demonstrating enhanced NK activity, proliferation following stimulation by submitogenic doses of mitogen and cytokine production both ex vivo and in vivo. This was also extended to HIV-positive patients. The mechanism by which immune function was modulated was proposed to be by targeting specific T- helper lymphocytes, the TH1 and TH2 cells, normalizing their function and resulting in improved T-lymphocyte and natural killer cell activity.

Using sensitive flow cytometry-based assays evaluating monocyte and lymphocyte subsets and intracellular cytokine expression, this study does not find any support for a beneficial effect on immune function by phytosterols in the dose consumed in 20g of the margarine.

There is 1.6g of phytosterol as ester in the margarine, while in Boiuc's study the formulation was 60mg BSS and BSSG 0.6mg. Bouic contends that the ratio of 100/1 was proven to be critical to have an immune modulatory effect from in vivo and in vitro studies. Since he demonstrated a dose-response effect over a wide range of this phytosterol blend, the very high dose of sitosterol in the margarine would have been expected to exert some effect unless the glycoside was a critical requirement.

CD14+16+ were considered as pro-inflammatory state on monocytes and there was no significant difference between the regular and phytosterol margarines on the percentage of cell monocytes expressing these markers.

T cells are divided into two distinct non-overlapping populations: a subset which carries the CD4 marker and mainly helps to induce immune responses, and a subset which carries the CD8 marker and is mainly cytotoxic.CD4 TH cells have two different profiles of cytokine production (TH1 and TH2) and these patterns mediate distinct responses. TH2 activity is reflected by IL-10 and IL-4. TH2 cytokines are associated with regulation of strong antibody responses and allergic reactions. TH1 activity is reflected by IL-12, TNF and IFN-GAMMA as well as T cell activation marker (CD25 (IL2 receptor) and increased ratio of IFN/IL4. TH1 cytokines activate cytotoxic, inflammatory and delayed hypersensitivity reactions. Again neither of these parameters was significant, except for baseline IL12 (which in isolation is unlikely to be meaningful).

In conclusion, this immune study has not confirmed any augmentation of TH1 function as proposed by Bouic. this study did not , however, apply the identical assays but performed assays generally regarded as highly sensitive for evaluating TH1 function. This study also used sitosterol ester which may not be equivalent to BSS and BSSG. It is unknown whether these are differently metabolised and absorbed

when compared with sitosterol ester. Future studies should address the exact requirements for immune modulation before fortified margarines are put to the test.

9. CONCLUSION

This study shows that the Flora Pro-activ margarine containing 1.6g of phytosterol significantly reduces total and LDL cholesterol levels in FH patients on partial lipid lowering treatment. The response to dietary modification (14% reduction in LDL and 10% in total cholesterol), underlies the importance of repeated counseling, the effect of the phytosterols margarine without dietary changes is (6.5% in LDL and TC of 6.4%). Triglyceride was increased from baseline on the regular margarine period, but not on the phytosterols margarine. HDL remained unchanged.

It is also evident from this study that for persons close to the target LDLC concentration, the phytosterols margarine may permit the achievement of target at a reduced cost and no extra risk of a higher dose of statins.

The study also shows no augmentation of TH1 function, therefore immune function was not affected by the phytosterol margarine.

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10. RECOMMENDATIONS

From this study it is evident that margarine containing phytosterols reduces cholesterol in familial hypercholesterolaemia patients already on medication and modified diets. When comparing the cost of the margarine to the cost of statins it is clear that the addition of the margarine to the management with a statin will aid the achievement of target and may represent a cost saving.

Based on the research findings the following recommendations can be made:

1. A dietitian should counsel all patients with FH at the time of diagnosis; subsequent follow-ups are essential to ensure patients are adhering to lipid lowering diets.
2. Phytosterol margarine should be included in the nutrition care plan for FH patients. Patients should therefore be advised on diet and margarine before statins are commenced. In this way the dose of the statins used would be much less, thereby reducing drugs costs and limiting side effects.
3. The margarine containing phytosterols is very expensive and is not affordable by most patients of lower socio-economic status that attend the Lipid Clinic at GSH. However, it is affordable by an ever-increasing cohort of patients whose medical insurance limits pharmaceuticals.
4. Since the margarine is not affordable to most patients, various options should be investigated. One option would be to allow patients to buy the margarine at a reduced cost if the government subsidises the cost thereof or if the company producing the margarine can investigate methods of reducing overall manufacturing costs and therefore reduce the consumer price. Government would not be able to provide the margarine free of charge due to drastic budget cuts in healthcare. Perhaps negotiations with government and the

company manufacturing the margarine would allow the margarine to be affordable to most patients.

5. Further studies would be required into the use of immune modulating medication since this study did not show any benefit of phytosterols in immune function. This medication is quite expensive and has only been studied by one author.
6. The long term safety of the phytosterol margarine is still not established as there may be cumulative effects in tissues over decades.

11. LIMITATIONS

COMPLIANCE

Of the 42 patients that were included in the study, 12 patients were disqualified from the final analysis due to compliance either with diet, medication or margarine consumption, although the 30 patients analysed were viewed as representative of the sample. The results of the study might have been different if these patients were included.

DIETARY REPORTING

The patients in this study has under-reported their intake since the energy to BMR ratio is less than 1.2, although the under-reporting is consistent throughout the study and would therefore not have affected serum lipid values. Patients weights were also consistent during the study. The study did not take into account any error inherent in the food composition tables nor the ability of patients to recall the food consumed as previously discussed in 7.1.3 . Studies have found that the composition of food changes more rapidly than the nutrient data in the food tables and is therefore a source of variation that needs to be considered (Marshall and Judd, 1982). Also the

size of the database could affect the analysis since there are multiple entries available for similar foods (Dwyer and Suitor, 1984)

IMMUNE STUDIES

This study did not show any changes in the immune function of patients on the study. However when compared to Boiuc's studies, this study did not employ the same assays that were employed in his studies, but assays that are generally regarded as highly sensitive for TH1 function were employed. The dose of the phytosterol in the margarines is much higher and would therefore likely to exert an effect on immune function. This study used phytosterol ester whereas Boiuc's study used BSS and BSSG.

It is not easy to understand how the ratio of 2 poorly absorbed substances can influence systemic immune function whilst demonstrable changes in plasma phytosterol did not.

Future studies should employ the exact methods employed by him with the exact dose of BSS and BSSG to make direct comparisons.

References

1. Bouic PJD, Etsebeth S, Liebenberg RW, et al. Beta-sitosterol and Betasitosterol glycoside stimulate human peripheral blood lymphocyte proliferation: implications for their use as an immunomodulatory vitamin combination. Int J Immunopharmacol 1996; 18:693-700.
2. Dwyer J and Suitor CW. Caveat emptor: Assessing needs, evaluating computer options. J Am Diet Assoc 84: 1984:302.
3. Marshall MW, Judd JT. Calculated vs Analysed composition of four modified diets. J Am Diet Assoc 1982;80:537-549.

APPENDIX A

CONSENT FOR THE STUDY COMPARING REGULAR AND SITOSTEROL-ENRICHED MARGARINE

I, ..., hereby indicate that I understand the points made below regarding the testing of margarine in addition to regular lipid-lowering treatment. In addition, my signature below indicates my voluntary desire to partake in this study.

*The study will test a regular and new form of margarine. The new form of margarine has a plant product in it that has previously been shown to lower cholesterol when approximately 20ml (4 teaspoons) of margarine is consumed per day.

*There have been no side effects with the margarine.

*While on the study the diet should be consistent with that educated at the clinic and reinforced at the recruitment to the trial. Additionally, the lipid-modifying treatment that has been prescribed should be continued during the study.

*The visits should remain on schedule, but if there is a problem then a delay of up to seven days is acceptable.

*The margarine containers should be returned at each visit. This applies to both the used and unused containers.

* Blood will be taken for studies of immune function and blood will be taken for storage to measure plant sterols should it be deemed necessary.

*Any complaints that may be noticed during the study should be brought to the doctor's attention. You can contact Drs Wolmarans/Firth at 406 6370 or 4066642, or Ms Zarina Hamdulay 404 9111 Page 1443 or 404 4471.

*Any participant may withdraw from the study and this action will not jeopardise any future treatment.

All the above points have been explained to me by .....

Signed by ..... **Date** .....

Witnessed by .....

(NAME IN BLOCK LETTERS) ..... **Date** .....

Appendix 2.

APPENDIX B
INSTRUCTIONS FOR DIETARY RECORD DURING MARGARINE STUDY

1. It is important that accurate dietary information be kept during the study.
2. Please keep a record of everything that you eat or drink for **two weekdays** and **one weekend day** before your next scheduled visit.
3. Record the brand names of everything eaten. Include any modifications of the food item e.g.: low sodium, fat free, artificially sweetened.
4. Record alcoholic beverages.
5. The following items will help you to keep more accurate measurements and descriptions of the food and beverages eaten.

*** Record in cups (small, medium, large)**

All beverages
Potatoes, rice, cereals, soups
Chopped fruits, vegetables (cooked or canned)
Casseroles

***Record by number and size (small, medium, large)**

Breads, rolls, crackers
Raw fruits and vegetables
Cookies, snack items

*Record weight in grams and specify if raw or cooked weight

Meat, poultry, fish, cheese
If weight is unknown, use dimensions – length x width x depth

***Record in teaspoons or tablespoons**

Jelly, jam, sugar, syrup
Sauces, gravies
Salad dressing, butter, margarine, oil
Nuts, seeds

Give a detailed description of the food you ate and the beverages drank. Use the following as a checklist.

Were fats or oils used in cooking? If yes, what type of oil?

Was salad dressing or mayonnaise used in salads or sandwiches?

Was fat added in cooking vegetables?

What kind of milk was drunk or used in cooking?

What brand of margarine or butter used on bread, potatoes etc.

APPENDIX B (continued)

Were gravies, sauces or syrups added to the food you ate?
What cuts of meat was eaten, was the fat trimmed off?
Was the skin removed from the poultry?

What type of cheese was used?

What ingredients were used in mixed dishes?

Were whole eggs, egg whites used in ingredients?

Do you use salt in you cooking?

RECORDING YOUR INFORMATION

It is important that you do not change the food that you usually eat and drink in the days of keeping the record. We need to know what you usually eat and drink and not what you think you should be eating or drinking.

Record each meal after it was eaten.

In the time and place column, use H for meals eaten at home and A for meals eaten away from home. Also record if meal was for breakfast, lunch, supper, snack, brunch.

Leave spaces between each meal.

Start a new day on a new page.

Do not write in the column titled 'reviewers comments'

APPENDIX B (continued)

DIETARY RECORD FOR MARGARINE STUDY

PATIENT No:_____ PATIENT INITIALS: _____

VISIT DATE: _____ VISIT NUMBER: _____

DATE HANDED BACK TO DIETITIAN:_____

Intake was 1- typical
2- more than usual
3- less than usual

Interviewers opinion 1- reliable
2- unreliable

Meal eaten B - breakfast
L - lunch
S - supper
SN – snack
BR – brunch

Day of diary _____
Date of diary_____

Place H (Home) A (Away)	Time meal was eaten Which meal (B,L,S,SN,BR)	Food and beverages eaten	Amount	Fat used in food preparation	Cooking method	Reviewers Comment

APPENDIX C
COMPOSITION OF DIFFERENT FAT SPREADS

Margarine	Flora ProActiv	Flora Extra Light	Flora regular	Brick margerine	Butter
Energy (kj)	1351	1354	3022	3056	3040
Total fat(g)	35	35	81	82.2	82
Sat fat(g)	8	9	22	18.92	48
Poly fat(g)	17.5	18	41	17.34	1.61
Mono fat(g)	9	8	17	42	21
Trans fat (g)	0.5	0	1	23.81	5.26
Cho (g)			0.4	0.9	0.3
Protein (g)	0.1		0.2	0.3	0.5
Plant sterol	8				
Vit A mg RE		691	691	750	754
Vit E mg tocopherol	66			15	1.58
Moist %	60	60	15	15	15
R cost per 100g	6	2	1.2	1	2.4

Sat fat – Saturated fat
Poly fat – Polyunsaturated fat
Mono fat – Monounsaturated fat
CHO – Carbohydrate
Vit A – Vitamin A
Vit E – Vitamin E

Margarine spreads given as on the label, brick margarine and butter according to the Food Composition Tables (Kruger et al, 39, 40, 1991)

Price calculated per 100 g in R in August 2001 at Pick and Pay in Cape Town.

APPENDIX D
CORRELATIONS

(A) LDL

VARIABLE	SLOPE	r2	p value
TG-baseline	positive	0.23	0.0089
TG-regular	positive	0.19	0.0188
TG-phytosterol	positive	0.18	0.0200

(B) LDL difference

VARIABLE	SLOPE	r2	p value
Age	negative	0.15	0.0344
WH-baseline	negative	0.15	0.0294
WH-phytosterol	negative	0.15	0.0294
WH-regular	negative	0.14	0.0285

(C)HDL

VARIABLE	SLOPE	r2	p value
TG-baseline	negative	0.22	0.0090
TG- regular	negative	0.22	0.0090
TG- phytosterol	negative	0.16	0.0185

(D)TRIGYLCERIDES

VARIABLE	SLOPE	r2	p value
HDL- baseline	negative	0.22	0.0086
HDL-phytosterol	negative	0.16	0.0310
HDLdifference	negative	0.15	0.0354
WH-baseline	positive	0.19	0.0136
WH- regular	positive	0.23	0.0067
WHS-phyotsterol	positive	0.25	0.0050
LEAN2-baseline	positive	0.15	0.0384
LEAN- regular	positive	0.16	0.0294
BMR- regular	positive	0.15	0.0322
BMR-phytosterol	positive	0.16	0.0248

APPENDIX D
CORRELATIONS (continued)

(E) TRIGLYCERIDE DIFFERENCE

VARIABLE	SLOPE	r2	p value
LEAN- baseline	positive	0.34	0.0009
LEAN- regular	positive	0.29	0.0019
LEANphytosterol	positive	0.32	0.0010
LEANDifference	negative	0.29	0.0022
BMR- regular	positive	0.34	0.0016
BMR-phytosterol	positive	0.33	0.0080
EER-baseline	positive	0.29	0.0023
EER- regular	positive	0.26	0.0042
EER-phytosterol	positive	0.29	0.0020

(F)Estimated Average Requirements

VARIABLE	SLOPE	r2	p value
AGE	negative	0.29	0.0090
TG- regular	positive	0.20	0.0100
TGDifference	positive	0.29	0.0020
WH- regular	positive	0.15	0.0100
WH-phytosterol	positive	0.19	0.0400
F%- baseline	negative	0.34	0.0080
F%- regular	negative	0.29	0.0020
F%-washout	negative	0.31	0.0010
LEAN- baseline	positive	0.94	<0.0001
LEAN- regular	positive	0.93	<0.0001
LEAN-phytosterol	positive	0.93	<0.0001
BMR- regular	positive	0.96	<0.0001
BMR-phytosterol	positive	0.96	<0.0001
FAT- regular	positive	0.23	<0.0001
FAT-phytosterol	positive	0.21	0.0020
FAT-washout	positive	0.16	0.0020
SFA regular	positive	0.16	0.0300

APPENDIX D
CORRELATIONS (continued)

SFA-phytosterol	positive	0.15	0.0400
EN- regular	positive	0.35	0.0007
EN-washout	positive	0.41	0.0002

(G) BODY FAT %

VARIABLE	SLOPE	r2	p value
AGE	positve	0.19	0.02
BMI- baseline	positve	0.47	<0.0001
BMI- regular	positve	0.46	<0.0001
BMI-phytosterol	positve	0.46	<0.0001
LEAN- baseline	negative	0.24	0.007
LEAN- regular	negative	0.24	0.007
LEAN-phyotsterol	negative	0.23	0.008
BMR- regular	negative	0.26	0.005
BMR-phyotsterol	negative	0.26	0.005
EER- regular	negative	0.35	0.0008
EER-phytosterol	negative	0.3	0.002
FAT- regular	negative	0.24	0.006
FAT-phytosterol	negative	0.29	0.003
FAT-washout	negative	0.41	0.002
SFA- regular	negative	0.23	0.002
SFA-phytosterol	negative	0.19	0.003
SFA-washout	negative	0.36	0.002
PUFA- regular	negative	0.14	0.04
PUFA-washout	negative	0.15	0.04
MUFA- regular	negative	0.24	0.006
MUFA-phytosterol	negative	0.24	0.006
MUFA-washout	negative	0.2	0.004
EN- regular	negative	0.3	0.002
Enphytosterol	negative	0.2	0.01
EN-washout	negative	0.29	0.002

APPENDIX D
CORRELATIONS (continued)

(H)LEAN BODY WEIGHT

VARIABLE	SLOPE	r2	p value
TG- baseline	positive	0.15	0.04
TG-regular	positive	0.28	0.005
TGDifference	positive	0.34	0.002
HDL- regular	negative	0.19	0.003
HDL-phytosterol	negative	0.16	0.04
WH-baseline	positive	0.16	0.04
WH- regular	positive	0.23	0.0069
WH-phytosterol	positive	0.24	0.0026
F%-baseline	negative	0.24	0.0068
F%- regular	negative	0.19	0.0165
F%-phytosterol	negative	0.21	0.0115
BMR- regular	positive	0.98	<0.0001
BMR-phytosterol	positive	0.97	<0.0001
EER- regular	positive	0.94	<0.0001
EER-phytosterol	positive	0.94	<0.0001
EER-washout	positive	0.95	<0.0001
FAT- regular	positive	0.21	0.0113
FAT-phytosterol	positive	0.23	0.0073
FAT-washout	positive	0.16	0.0315
MUFA- regular	positive	0.26	0.0052
MUFA-phytosterol	positive	0.26	0.0044
EN- regular	positive	0.25	0.0054

The above tables represent the correlations done for the variables, they are done for all the visits including baseline, phytosterol margarine period, regular margarine period and washout period.

Abbreviations used in the tables

EER-estimated average requirements

BMR- basal metabolic requirements

WH-waist hip ratio

FAT-Total fat intake

SFA-saturated fat

MUFA- monounsaturated fat

PUFA – polyunsaturated fat

EN - Energy

F% - Body fat percentage

TG- triglyceride

Tgdifference- triglyceride difference

LEAN- lean body weight percentage

APPENDIX E

VISIT DATE RECORDED: _____

VISIT NO: _____

PATIENT INITIALS: _____

PATIENT NO: _____

SITE: _____

RETURNED TO DIETITIAN: _____

DIETETIC RECORD

	Visit 1 (b)	Visit 2	Visit 3	Visit 4	Visit 5
Weight					
Height					

Anthro					
Biceps					
Mid-arm					
Triceps					
Supscap					
Supraileac					

B.E					
Body Fat					
Lean Mass					
Body Water					
BMR					
BMI					
EER					

Visit 1 (a)

Dietitian's Comment

Visit 1 (b)

Dietitian's Comment

APPENDIX E (continued)

VISIT DATE RECORDED: _____

VISIT NO: _____

PATIENT INITIALS: _____

PATIENT NO: _____

SITE: _____

RETURNED TO DIETITIAN: _____

DIETETIC RECORD

Visit 2
Dietitian's Comment

Visit 3
Dietitian's Comment

Visit 4
Dietitian's Comment

Visit 5
Dietitian's Comment

APPENDIX F

Gas chromatography for regular margarine.

AND GROOTE SCHUUR HOSPITAL

LIPID LABORATORY CHROMATOGRAPHY

Cape Heart Centre, Anzio Rd, Observatory, 7925 South Africa.
Tel: 27-21-4066166 Facsimile: 27-21-4066396

Chromatographer. BDR DTB ADM Run No. 101 1

Analysis requested by Z Hanidulay M Sc Student Unifoods Study. Lipid Lab.

Reason for Analysis. Confirm phytosterol in margarine under study.

SAMPLE Date. Aug 2000

Patient Name Margarine Folder Nr — DNA Nr —

Blood — Other samples Margarine Storage 4°C

Preparation Folch extraction, saponification (100 mg) Date 21.08.01

Derivitisation BSTFA Date 30.08.01

CHROMATOGRAPHY Date 14.09.01

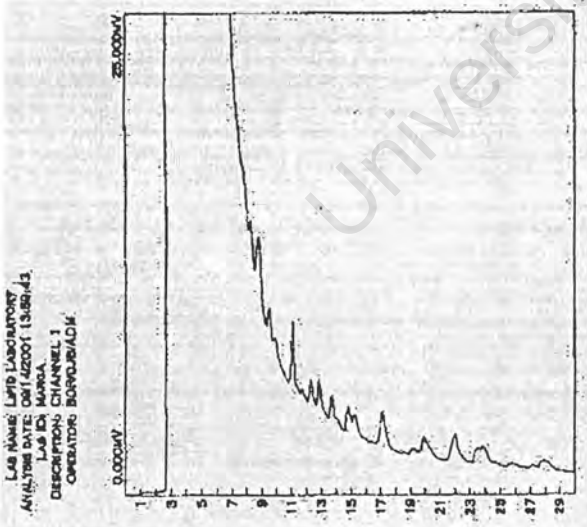
Machine Pye unicam Detection FID 300

Column XT.5 Injection °C 275 Column °C

Ramping 275 x 10', 12°/min → 310 x 20 min.

Injection: volume 2 µL solvent hexane

ANALYSIS



No significant peak seen at retention time (25') for β-sitosterol for which the detection limit is approx 2.5 µg/mL.

Date 24.10.01 Signature Duraraj

APPENDIX G

Gas chromatography for phytosterol margarine

AND GROOTE SCHUUR HOSPITAL

LIPID LABORATORY CHROMATOGRAPHY

Cape Heart Centre, Anzio Rd, Observatory, 7925 South Africa.
Tel: 27-21-4066166 Facsimile: 27-21-4066396

Chromatographer. BDR, OOB, ADM Run No. 102

Analysis requested by Z. Hamdulay MSc Student, Unifoods Study
Lipid Lab.

Reason for Analysis Confirm phytosterol in margarine under study

SAMPLE Date Aug 2000

Patient Name ..... Folder Nr. ..... DNA Nr. —

Blood — Other samples ..... Storage 4C

Preparation Folch (150 mg) extraction, saponification Date 29.05.01

Derivitisation BSTFA Date 30.05.01

CHROMATOGRAPHY Date 14.09.01

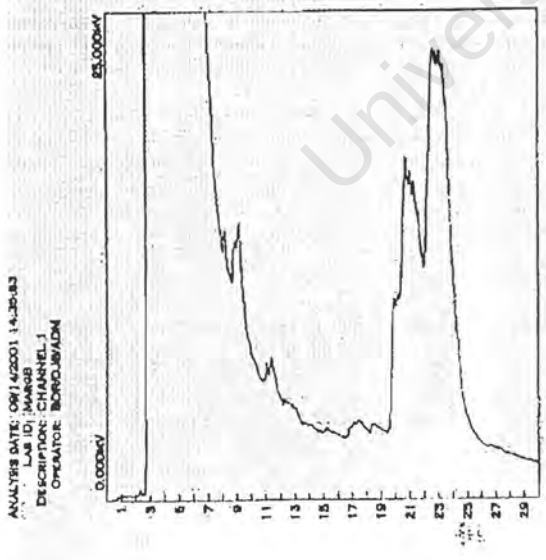
Machine Pye Unicam Detection FID

Column XTC 5 Injection °C 275 Column °C 310

Ramping 275 X 10', 12°/min to 310° X 20'

Injection: volume 2 µl. solvent hexane

ANALYSIS



Two major peaks seen suggestive of Sitosterol and Brassicasterol because retention times are 24.5 and 21 min respectively.

Date 24.10.01 Signature