

**IMPLEMENTATION OF STABLE
ISOTOPE LIPOPROTEIN
KINETIC STUDIES:
EFFECTS ON HDL METABOLISM
OF A MEDITERRANEAN-TYPE DIET
RICH IN MUFAs
FROM VIRGIN OLIVE OIL**

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**DOCTORAL THESIS
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**UNITAT DE RECERCA DE LÍPIDS I ARTERIOSCLEROSI
DEPARTAMENT DE MEDICINA I CIRURGIA
FACULTAT DE MEDICINA I CIÈNCIES DE LA SALUT
UNIVERSITAT ROVIRA I VIRGILI
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"Wait & hope"

Le Comte de Monte-Cristo

Alexandre Dumas

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RESUM/SUMMARY

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Resum

Els efectes antiaterogènics atribuïts a una dieta de tipus mediterrani, rica en àcids grassos monoinsaturats (AGM), aportats per oli d'oliva verge, són deguts, en part, a l'augment o al manteniment de les concentracions plasmàtiques de colesterol de les lipoproteïnes d'alta densitat (HDL). No obstant i fins al moment, no es coneixen del tot els mecanismes que expliquen aquestes concentracions. L'apolipoproteïna (apo) A-I (apoA-I) és l'apolipoproteïna majoritària de les HDL i els seus paràmetres cinètics, com ara la taxa de producció i la taxa de catabolisme, reflexen la cinètica de les partícules d'HDL. La nostra hipòtesi de treball és la següent: una dieta Mediterrània rica en AGM aportats per oli d'oliva verge, comparada amb una dieta STEP II, incrementa o manté els nivells de colesterol de les HDL degut a un augment de la producció i no a una disminució de la degradació d'apoA-I. Possiblement, la realització d'estudis de cinètiques utilitzant isòtops estables és la forma més fisiològica d'avaluar la producció i la degradació d'apolipoproteïnes en l'home.

Objectiu: Implementar la metodologia necessària per realitzar cinètiques d'apo B-100 i, especialment, d'apoA-I i d'apoA-II en voluntaris *in vivo* utilitzant isòtops estables com a marcatge de les proteïnes. Aplicar aquesta metodologia per comparar els efectes d'una dieta Mediterrània rica en oli d'oliva verge amb una dieta pobre en greixos STEP II sobre el metabolisme de les HDL i de les lipoproteïnes de baixa densitat (LDL).

Disseny: Estudi creuat randomitzat de 4 setmanes d'intervenció dietètica, amb un període de rentat entre elles. 10 voluntaris, homes sans, moderadament hipercolesterolèmics van seguir ambdues dietes. Aquest estudi va ser aprovat pel Comitè d'Ètica i d'Investigació Clínica de l'Hospital Universitari de Sant Joan de Reus.

Instrumentalització: Estudi cinètic mitjançant la injecció i perfusió d'isòtop estable $^2\text{H}_3$ -L-leucina en forma d'un *bolus* inicial i de perfusió durant 16h al final de cada dieta. Les diferents fraccions lipoproteïques es van separar per ultracentrifugació. La detecció d'isòtop incorporat va ser mitjançant GC-MS. Les dades obtingudes es van analitzar amb models multicompartimentals i el programa SAAM II. Els estudis cinètics d'apoA-I i d'apoA-II es van posar a punt i realitzats a la nostra Unitat de Recerca a Reus. Els estudis d'apo B-100 i la modelització de les dades cinètiques es van realitzar en col·laboració amb el grup de recerca de la *Vascular Biochemistry Section, Division of Cardiovascular & Medical Sciences, Royal Infirmary, University of Glasgow, Glasgow*.

Resultats: La dieta Mediterrània, comparada amb la dieta STEP II, incrementa significativament la concentració plasmàtica d'apoA-I. S'han realitzat 17 cinètiques però, degut a la complexitat de la metodologia, en aquesta tesi es presenten els resultats de les 7 cinètiques (4 després de la dieta Mediterrània i 3 després de la dieta STEP II) analitzades per GC-MS fins ara. A pesar de l'escàs nombre de cinètiques, s'observen certes tendències. La dieta Mediterrània, comparada amb la dieta STEP II, presenta una major taxa de producció d'apoA-I de les HDL, així com una major taxa de producció i de catabolisme de l'apo B-100 de les LDL.

Conclusions: S'ha implementat la metodologia

dels estudis de cinètiques amb isòtops estables de l'apoA-I i l'apoA-II de les HDL i de l'apo B-100 de les VLDL₁, VLDL₂, IDL i LDL. El determinant de les concentracions més elevades d'apoA-I en plasma és una major taxa de producció d'aquesta apo i no diferències en el seu catabolisme. Les aportacions de les cinètiques de les lipoproteïnes permetran avançar en els mecanismes lipídics involucrats en les malalties vasculars i aportar nous aspectes sobre les dianes nutricionals i farmacològiques.

Summary

The anti-atherogenic effects ascribed to a Mediterranean-type diet rich in monounsaturated fatty acids (MUFAs) from virgin olive oil are due, partly, to an increase in, or maintenance of, plasma concentrations of high density lipoprotein (HDL) cholesterol. However, the underlying mechanisms that may explain these concentrations are not well characterised, to-date. Apolipoprotein (apo) A-I (apoA-I) is the major HDL apo and its kinetic parameters, such as production rate and catabolic rate, reflect the kinetics of the HDL particle. Our working hypothesis is as follows: a Mediterranean-type diet rich in MUFAs from virgin olive oil, compared to a STEP II diet, increases or preserves HDL cholesterol concentrations due to an increase in apoA-I production and not to a decrease in apoA-I catabolism. Kinetic studies using stable isotopes are, perhaps, the best approach in physiologically evaluating apo production and catabolism in humans. This methodology has not as yet been implemented in Spain. Objectives: to implement the necessary methodology to perform kinetic studies of apo B-100 and, especially, of apoA-I and apoA-II in volunteers *in vivo* using stable isotopes to label proteins *in vivo*. Further, we used this methodology to analyse the overall effects of a Mediterranean-type diet rich in monounsaturated fatty acids from virgin olive oil compared with a low-fat, STEP II diet, on HDL and low density lipoprotein (LDL) metabolism. Design: we conducted a crossover, randomised study with dietary intervention periods of 4 weeks, interspersed with a washout period between diets. A total of 10 healthy, moderately hypercholesterolaemic, male volunteers consumed the two diets. The project was approved by the Clinical Research Ethical Committee of the *Hospital Universitari de Sant Joan*, Reus. Instrumentation: kinetic studies were performed at the end of each diet using a 16h primed constant infusion of stable isotope $^2\text{H}_3$ -L-leucine. Lipoprotein fractions were separated using ultracentrifugation technique. Stable isotope incorporated into proteins was measured using GC-MS. The data obtained were analysed applying multi-compartmental modelling technique with the SAAM II program. ApoA-I and A-II kinetic studies were conducted in our Research Unit in Reus. Apo B-100 kinetic studies and kinetic parameter modelling were performed in collaboration with Dr. Caslake and Professor Packard of the Vascular Biochemistry Section, Division of Cardiovascular & Medical Sciences, Royal Infirmary, University of Glasgow, Glasgow, Scotland. Results: the Mediterranean diet, compared to the STEP II diet, significantly increases apoA-I plasma concentrations. A total of 17 kinetic studies have been performed but, due to methodological complexity, only the results of 7 kinetic studies (4 following a Mediterranean diet and 3 following a STEP II diet) that have been analysed in a GC-MS to-date, are presented in this thesis. Despite the limitation of the low number of kinetic studies analysed, we are able to document that the Mediterranean diet induces a high apoA-I HDL production rate compared to the STEP II diet. Also, the Mediterranean diet induces a high apo B-100 LDL production rate and fractional catabolic rate compared to the STEP II diet. Conclusions: we have, for the first time in Spain, implemented the necessary

methodology to perform apo B-100, apoA-I and A-II kinetic studies *in vivo* using stable isotopes in human subjects. A high apoA-I production rate is the main determinant of high plasma concentrations of apoA-I, and not variations in its catabolism. Lipoprotein kinetic studies enable the monitoring of lipoprotein metabolic parameters and the investigation of nutritional and pharmacologic interventions in the primary and secondary prevention of cardiovascular disease targets.

What is known about the topic?

The anti-atherogenic effects ascribed to a Mediterranean-type diet rich in monounsaturated fatty acids (MUFAs) from virgin olive oil are due, partly, to an increase in, or maintenance of, plasma concentrations of high density lipoprotein (HDL) cholesterol. However, the underlying mechanisms that may explain these concentrations are not well characterised, to-date. Apolipoprotein (apo) A-I (apoA-I) is the major HDL apo and its kinetic parameters such as production rate and catabolic rate reflect the kinetic of HDL particle. Changes in HDL concentrations can be induced by an increase in apoA-I production rate, or by a decrease in apoA-I catabolic rate, or by both parameters simultaneously. Kinetic studies using stable isotopes are, perhaps, the best approach in physiologically evaluating apolipoprotein production and catabolism in humans.

Which are the present thesis contributions?

The present study is the first to compare the impact of a Mediterranean-type diet high in MUFAs from virgin olive oil, compared to a STEP II diet using stable isotopes kinetics.

The Mediterranean diet, compared with the STEP II diet, significantly increases plasma concentrations of apoA-I and shows a trend, albeit not significant, towards an increase in plasma concentrations of HDL cholesterol, and significantly decreases the apo B-100/apoA-I ratio. Both diets induce similar decreases in LDL cholesterol plasma concentrations, albeit statistically not significantly.

Because of the labour-intensive nature of the studies and the need for high-technology equipment, our lipoprotein kinetic study has the limitation of the low number of analysed kinetic studies, to-date. Despite these limitations, we are able to document that:

- * the Mediterranean diet induces a high apoA-I HDL production rate compared to the STEP II diet.
- * the Mediterranean diet induces a high apo B-100 LDL production rate and fractional catabolic rate compared to the STEP II diet.

Our data suggest that a high apoA-I production rate is the main determinant of high plasma concentrations of apoA-I, and not variations in its catabolism.

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INTRODUCTION

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1. The pathogenesis of atherosclerosis

1.1 Atherosclerosis: general insight of a complex aethiology

Current world-wide estimates suggest that most deaths (approximately 32 million) are attributable to non-communicable diseases and that just over half of these (17 million) are the result of cardiovascular disease (CVD). Further, CVD is also responsible for disability and decrease in life expectancy. CVD can be defined as any abnormal condition characterised by dysfunction of the heart and blood vessels. CVD includes coronary heart disease (CHD), cerebrovascular disease (stroke), transient ischaemic attack and peripheral arterial disease (PAD). In developed countries, heart disease and stroke are the 1st and 2nd leading causes of death for adult men and women. In Europe, CVD is still the main cause of death. In Spain, each year, 125,000 deaths are attributable to atherosclerosis, 5 million people are admitted to hospital because of the disease and more than 560,000 patients are discharged from hospital with the diagnosis of CVD. Current predictions indicate that, by the year 2020, CVD atherosclerosis in particular will have become the leading global cause of overall disease burden. Progressive ageing of the world's population and an unhealthy lifestyle (high-fat, high-calorie diets and lack of regular physical activity) will have contributed to this process ¹⁻⁴.

Atherosclerosis is the principal cause of CVD. Etymologically, atherosclerosis means "hardening of arteries". It is a multifactorial and progressive disease. It is the result of genetic and environmental factors and is characterised by the accumulation of lipids and fibrous elements within the arterial vessel wall. Atherosclerosis begins early in childhood, progresses asymptotically throughout adulthood, and becomes clinically manifested later in life ⁵⁻¹⁰.

Progression of atherosclerosis begins in response to an endothelial injury in large arteries, followed by the accumulation of lipids and fibrous elements at the injury site. This leads to a series of inflammatory responses that include recruitment of macrophages and lymphocytes and the accumulation of lipoproteins in the arterial wall together with proliferative and apoptotic cellular events that produce complex plaques in the arterial wall ⁵⁻¹³. Deposition of fatty material in the arterial wall results in narrowing of the arterial lumen and eventual impairment of blood flow (Figure 1).

As such, plasma lipoproteins are important factors in the atherosclerosis process.

Preceding the formation of atherosclerotic lesions, endothelial injury and dysfunction increases endothelial vascular permeability to lipoproteins and this leads to the earliest lesions (sub-endothelial accumulations of foam cells). Briefly, lipoprotein particles and their aggregate particles accumulate within the intima of the artery. The stimulated endothelial and smooth muscle cells (SMCs) synthesise cell adhesion molecules, chemotactic proteins and growth factors that result in the recruitment of lymphocytes and monocytes to the arterial wall ^{11, 14-24}. Monocytes adhere to the

surface of the endothelium and transmigrate across the endothelial monolayer into the intima. Some specific cytokines activate these inflammatory cells and transform monocytes into macrophages. Modified low-density lipoprotein (LDL), for example oxidised LDL, within the intima are taken-up by macrophages, leading to the formation of cholesterol-filled macrophages, or foam cells; the mechanism by which LDL promote atherogenesis being related to oxidative modifications of lipids and proteins of these particles within the artery wall, and which provide an inflammatory stimulus. When foam cells die, their lipid content contributes to the core of the lesion. Lymphocytes, as well as endothelial cells, secrete cytokines and growth factors that can promote the migration and proliferation of SMCs from the medial layer of the artery, which then accumulate in the plaque and secrete fibrous elements (depending on changes in their phenotype) (Figure 2). The lesion continues to grow as a result of the migration of new cells, cell proliferation, extracellular matrix production and accumulation of extracellular lipids ²⁵⁻³⁵. These early atheroma plaques are characterised by a fibrous cap consisting of SMCs and extracellular matrix that enclose a lipid-rich necrotic core. Such fatty streak lesions are the precursors of more advanced lesions containing a greater accumulation of lipid-rich necrotic debris and SMCs. Further, atheromatous plaques can become increasingly complex (including calcification). Although advanced lesions can block blood flow through the lumen, the most important clinical complication is an acute occlusion or blockage due to the formation of a thrombus and which, when occurring in the coronary arteries, results in a myocardial infarction or when in the cerebral arteries, results in a stroke. Usually, a thrombosis results from the rupture of a vulnerable plaque that has a thin fibrous cap (Figure 3) ^{24, 36-40}.

Figure 1. Atherosclerotic lesion progression ⁵.

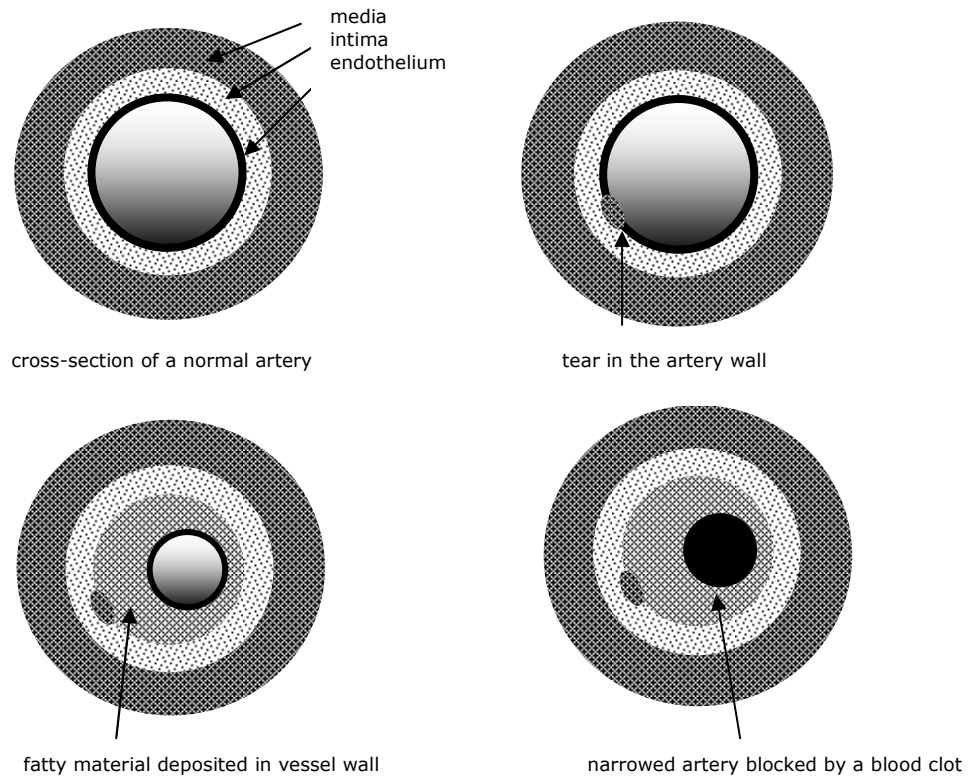


Figure 2. Endothelial dysfunction, increased LDL permeability and atherosclerotic lesion formation ⁸.

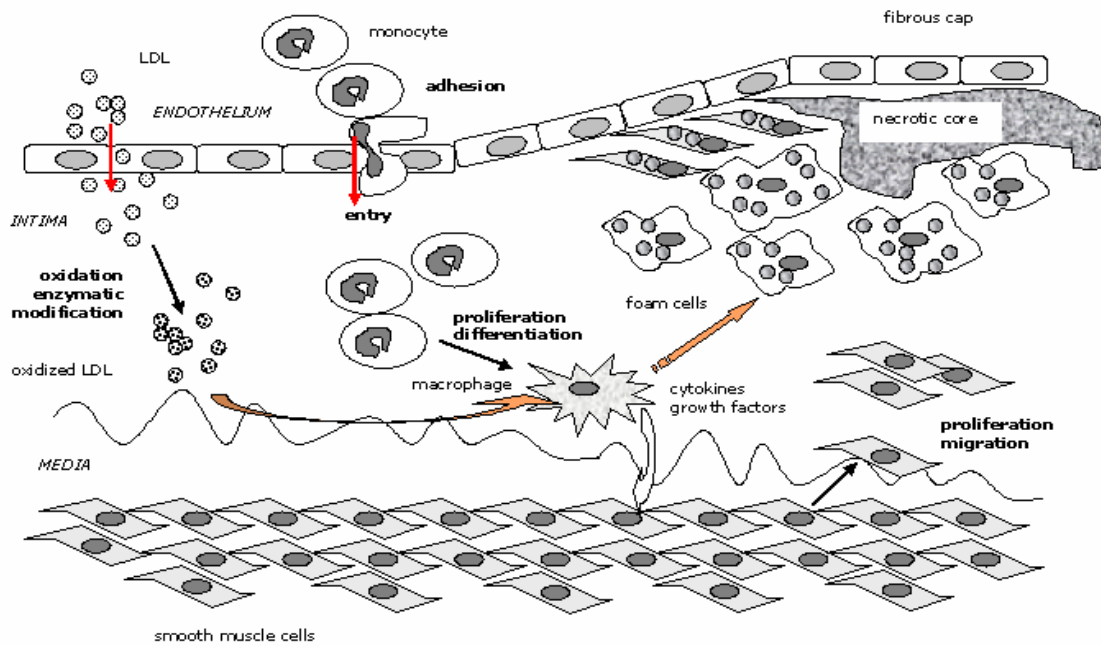
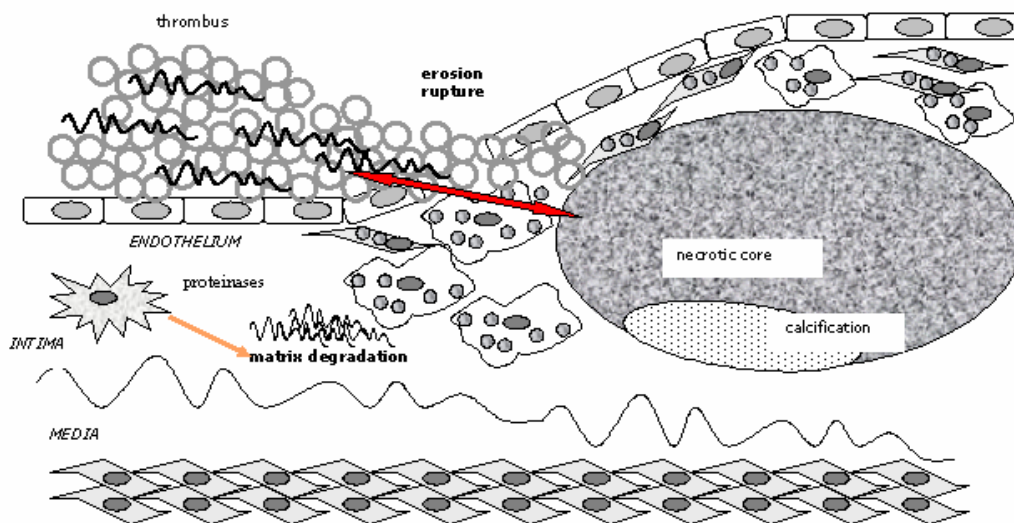


Figure 3. Advanced atherosclerotic lesion ⁸.



1.2 The concept of atherosclerosis risk factors

Over the past decade, there have been substantial improvements in the assessment of CVD risk. A CVD risk factor is a characteristic, or condition, or behaviour that increases the individual's susceptibility to heart disease. Epidemiological studies have identified numerous risk factors for atherosclerosis. A better knowledge of the atherogenic effects of well known cardiovascular risk factors has been accompanied by the awareness that the interaction of these factors, described as the global risk profile, provides better predictive power than any single traditional risk factor. A number of recently identified and, as such less well-known, factors have received intense attention over recent years ²⁴.

Traditional, or classical, CVD risk factors include non-modifiable risk factors (such as age, gender, race, family history and genetic features), as well as modifiable risk factors (such as plasma lipoprotein concentrations, smoking habit, hypertension, insulin resistance, obesity, life-style, chronic infections). Recently, several novel or non-traditional risk factors have been indentified as have new biochemical markers of risk. These include impaired fasting glucose, triglycerides (TG) and TG-rich lipoprotein remnants, lipoprotein (a), homocysteine, fibrinogen and high sensitivity C reactive protein; all are considered as contributing to an increased CVD risk ^{24, 41-44}, albeit the weight of evidence supporting these risk factors differing considerably. Factors that participate in the atherosclerosis process need to be distinguished from those that may serve merely as markers of risk, without direct involvement in the pathogenesis of the disease.

The established major risk factors are ^{24, 45-52}:

- * cigarette smoking.
- * hypertension (blood pressure $\geq 140/90$ mmHg).
- * low high-density lipoprotein (HDL) cholesterol.
- * high LDL cholesterol.
- * diabetes mellitus.
- * family history of premature CVD (in male first-degree relative < 55 years of age and in female first-degree relative < 65 years of age).
- * age (male ≥ 45 years of age and female ≥ 55 years of age).
- * life-style risk factors: obesity (body mass index ≥ 30 kg/m²), lack of physical activity and an atherogenic diet.

The emerging risk factors include ^{24, 45-52}:

- * lipoprotein (a) (Lp(a)): Lp(a) has similar characteristics as LDL but it has an apo (a) in its composition. Lp(a) is an homolog of plasminogen and might inhibit fibrinolysis by competition. Although individuals with high Lp(a) concentrations have increased CVD risk, Lp(a) concentrations do not predict CVD risk in populations.

- * homocysteine: high concentrations of homocysteine may promote thrombosis; the pathophysiology of this association is still uncertain.
- * prothrombotic markers: for example, high concentrations of fibrinogen and plasminogen-activator inhibitor 1 (PAI1) are related to blood clotting; albeit their plasma concentrations have not been proven to add information to other risk factors.
- * proinflammatory markers: for example, plasma concentrations of the marker of overall chronic inflammation, especially high-sensitivity C-reactive protein (hs CRP) which appears to add predictive information to other risk factors (high CRP concentrations may reflect overall atherosclerosis burden, and/or inflammation status that could contribute to the atherosclerosis process).
- * impaired fasting glucose.
- * sub-clinical atherogenesis.

CVD mortality is on the increase and there is a global trend towards greater atherosclerotic disease. Decreases in the number of risk factors have been shown to be associated with a decrease in CVD mortality in men and women. It is important that individuals adopt a healthy life-style. As such, the prevention of atherosclerosis becomes a long-term challenge to healthcare professionals and politicians. The classical definitions used in healthcare distinguish between primary, secondary and tertiary prevention. Primary prevention implies measures taken to avoid a disease before it occurs; secondary prevention is application of measures to pre-empt recurrence of a disease that already has been diagnosed, and tertiary prevention is the reduction in the amount of disability caused by a disease so as to achieve the highest level of function for the patient. Within the overall problem of atherosclerosis the key to prevention is primary care ⁵³⁻⁵⁸.

1.2.1 An attempt to define the significance of various risk factors

Several attempts have been made to define the relative significance of the various CVD risk factors in populations, and to develop new concepts of categorising patients at highest risk. The objective has been to reduce the incidence of the first, or recurrent, clinical event due to CHD such as ischaemic stroke and PAD and, further, to focus on disability and the prevention of premature death. Total CVD risk is the likelihood of a person suffering a fatal cardiovascular event within a defined period of time. Preclinical atherosclerosis reflects the integrated effects of multiple risk factors over time. Considering the impact on clinical practice, it is essential for clinicians to be able to make a rapid assessment of risk rapidly with enough accuracy to enable evidence-based management decisions to be taken ⁵⁹⁻⁶². Detection of preclinical atherosclerosis provides a strong argument in justifying the decision to treat a patient, especially in primary prevention. New methods of detection of preclinical atherosclerosis are, preferably, non-invasive. These methods include measuring the

thickening and stiffening of the walls of the larger arteries using high-resolution ultrasonography or echo-tracking techniques, coronary artery calcification using electron beam computed tomography and endothelium-dependent vasoactivity ^{63, 64}.

Another approach to determine CVD risk is the risk chart, published in 1994 and 1998 for implementation of its recommendations in Europe. This chart, developed from a concept pioneered by Anderson et al (1991) used age, gender, smoking status, total cholesterol (C) and systolic blood pressure to estimate the risk of a CHD event, fatal or non fatal, over the subsequent 10 years. A 10-year risk of $\geq 20\%$ was used as an arbitrary threshold for intensive risk-factor intervention. Several problems were associated with this risk chart. Firstly, it was derived from American data of the Framingham study and its applicability to European populations was uncertain. Secondly, the dataset used for the chart was fairly small. Thirdly, the definition of a non-fatal endpoint differed from that used in many other studies, making validation of the chart difficult ^{65, 66}.

A new model for total risk estimation based on the Systematic Coronary Risk Evaluation (SCORE) system was developed subsequently and presented several advantages over the previous chart. The SCORE risk assessment system is derived from a large dataset from prospective European studies and attempts to predict fatal CVD events over a 10-year period. Separate charts have since been developed for higher- and lower-risk areas of Europe. The SCORE system integrates the following risk factors: age, gender, smoking habit, systolic blood pressure, total C or total C/HDLC ratio. Since this chart predicts fatal events, the high-risk threshold is defined as $\geq 5\%$ instead of the previous $\geq 20\%$ in charts using composite coronary endpoints. In Spain, the proposal has been to adapt the SCORE system, although there are other total risk estimation models such as the *Registre Gironí del Cor* (REGICOR) system which was developed using data generated in Catalunya. Currently, several research groups are developing tools with which to estimate overall CHD risk in Spain ⁶⁷⁻⁷⁰.

1.2.1.1 Lipoprotein, lipid and apolipoprotein risk factors

Abnormalities in plasma lipoprotein concentrations and derangements in lipid metabolism are the best-established risk factors for atherosclerosis. Several expert panels have provided guidelines for the screening and management of lipid disorders.

Lipids are transported in blood within lipoprotein molecules. The relative concentrations of the different plasma lipoprotein particles appears to be of primary importance in atherosclerosis; a high level of atherogenic lipoproteins being a prerequisite for the disease ^{8, 12, 19, 24, 71}.

1.2.1.1.1 Lipoproteins

1.2.1.1.1.1 General structure and classification of lipoproteins

Lipids are a heterogeneous group of fat-like substances (all containing aliphatic hydrocarbons as a major constituent) which are characterised by being insoluble in water but soluble in organic solvents. Lipids, which are easily stored in the body, serve as source of energy and as essential structural components with biological functions in living cells. In animals, lipids are composed mainly of C, TG and phospholipids (PL). In plasma, lipids are transported within lipid-protein complex particles termed lipoproteins which consist of a spherical hydrophobic core of TG and cholesteryl esters (CE) surrounded by PL, unesterified C and apolipoproteins (apos) (Figure 4) ^{24, 72-75}.

Plasma lipoproteins are divided into four major classes based on their relative densities (determined by the amount of lipid and protein per particle): chylomicrons (Cm), very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL and HDL (Table 1). Each lipoprotein class, in turn, can be divided into subclasses that vary slightly in their density (determined by ultracentrifugation), diameter (determined by gel electrophoresis), electrophoretic mobility (determined by agarose gel electrophoresis) and protein composition.

Most TG is transported in Cm or VLDL and most C is carried as CE in LDL and HDL. Apos are required for the assembly and structure of lipoproteins. Apos serve to activate important enzymes and to mediate the binding of lipoproteins to cell-surface receptors (Table 2).

Figure 4. Scheme of a lipoprotein particle ⁷².

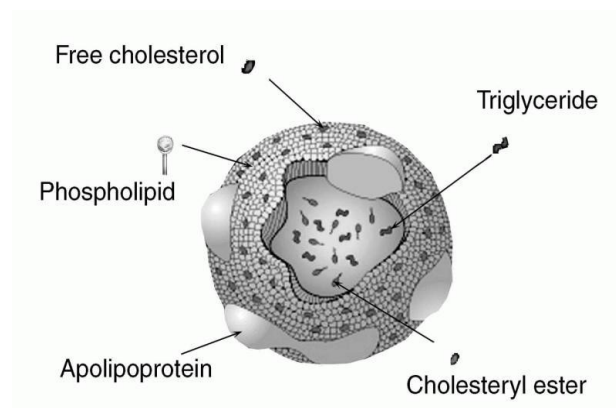


Table 1. Characteristics (A) and composition (B) of plasma lipoproteins particles ^{73, 74}.

(A)

Lipoprotein	Diameter (nm)	Density (kg/l)	Electrophoretic mobility
Cm	80-500	<0.93	None
VLDL	30-80	0.93-1.006	Pre-β
IDL	25-30	1.006-1.019	Slow pre-β
LDL	19-25	1.019-1.063	β
HDL2	8-11	1.063-1.125	α
HDL3	6-9	1.125-1.210	α

(B)

Lipoprotein	Percentage composition				
	CE	FC	TG	PL	Prot
Cm	1-3	1	86-94	3-8	1-2
VLDL	12-14	6-8	55-65	12-18	8-15
IDL	20-35	7-11	25-40	15-22	12-19
LDL	35-45	6-10	6-12	20-25	20-25
HDL2	15-20	4-6	3-8	30-40	35-40
HDL3	10-18	1-4	3-6	25-35	45-55

Cm=chylomicron; VLDL=very low-density lipoprotein; IDL=intermediate-density lipoprotein; LDL=low-density lipoprotein; HDL=high-density lipoprotein; CE=cholesteryl ester; FC=free cholesterol; TG=triglycerides; PL=phospholipids; Prot=protein.

Table 2. Apolipoprotein composition of the lipoproteins, and their putative function ²⁴.

Lipoprotein	Major	Other	Apos Function	Other constituents
Cm	Apo B-48	ApoA-I, A-IV, C-I, C-II, C-III, E	Apo B-48 → structural protein ApoC-II → LPL cofactor ApoC-III → inhibits lipoprotein binding to receptors Apo-E → LDL receptor ligand	retinyl esters
VLDL	Apo B-100	Apo-E, A-I, A-II, A-V, C-I, C-II, C-III	Apo B-100 → structural protein and LDL receptor ligand	vitamin E
IDL	Apo B-100	Apo-E, C-I, C-II, C-III	ApoC-II → LPL cofactor ApoC-III → inhibits lipoprotein binding to receptors Apo-E → LDL receptor ligand	vitamin E
LDL	Apo B-100		Apo B-100 → structural protein and LDL receptor ligand	vitamin E
HDL	ApoA-I	ApoA-II, A-IV, E, C-III	ApoA-I → structural protein and LCAT activator ApoA-II → structural protein	LCAT, CETP, paroxonase

LPL=lipoprotein lipase; CETP=cholesteryl ester transfer protein;
 HL=hepatic lipase; LCAT=lecithin cholesterol acyltransferase.
 Protein nomenclature according to UniProtKB/SwissProt
 (<http://www.expasy.org/uniprot/>).

1.2.1.1.1.2 Lipoprotein metabolism

The body can regulate plasma lipoprotein concentrations (and, hence, the lipid concentrations) by increasing, or decreasing, lipoprotein production and catabolic rates. In plasma, lipoprotein metabolism is regulated by specific apos, receptors, lipolytic enzymes and transfer proteins. The liver has a central role in this regulation. Lipoprotein transport can be described as having an exogenous pathway (for the transport of dietary lipids), an endogenous pathway (for liver-derived lipoproteins) and reverse cholesterol transport ^{24, 72-75}.

1.2.1.1.2.1 Exogenous pathway or dietary lipid transport

The exogenous pathway of lipoprotein metabolism enables an efficient transport of dietary lipids (Figure 5)²⁴. CM are the lipoproteins formed to mobilise dietary lipids for transport to the rest of the body.

In order for the body to make use of dietary lipids, they must first be absorbed from the small intestine. Lipids are insoluble in the aqueous environment of the intestine, and bile acids from the liver solubilise (or emulsify) dietary lipids. The emulsification of dietary fats renders them accessible to pancreatic lipases (lipase and phospholipase A₂). These enzymes generate free (unesterified) C, free fatty acids (FFAs) and glycerols that diffuse into enterocytes.

CM are assembled in enterocytes. Inside the enterocyte, dietary C is esterified with fatty acids (FAs) to form CE and dietary FAs are incorporated into TG. Then, CE and TG are packaged with apo B-48, FC and PL to form CM. Finally, CM leave the intestine via the lymphatic system and enter the systemic circulation. In the bloodstream, CM acquire apoC-II and apo-E from plasma HDL. Nascent CM are processed by peripheral tissues before reaching the liver. TG from CM are hydrolysed into FAs by lipoprotein lipase (LPL), a TG hydrolase found on the surface of endothelial cells of the capillaries. LPL is activated by its cofactor, apoC-II from CM, in the presence of PL. FFAs are then taken-up by tissues for their internal energy requirements. The resultant CM remnants (containing primarily CE, apo-E and apo B-48) are rapidly removed and taken-up by the liver through interaction with the CM remnant receptor (which requires apo-E for recognition). In this manner, CM deliver dietary TG to body tissues and dietary C to the liver⁷⁶⁻⁷⁹.

1.2.1.1.2.2 Endogenous, or hepatic lipoprotein, transport pathway

The exogenous pathway of lipoprotein metabolism refers to the hepatic secretion and metabolism of VLDL, IDL and LDL (Figure 5)²⁴.

VLDL are assembled in hepatocytes. Within hepatocytes, TG are packaged with vitamin E, apo B-100, CE, FC and PL to form VLDL; the enzyme microsomal transfer protein (MTP) being required for the process. VLDL are secreted from the liver into the circulation. In the bloodstream, VLDL acquire apo-C's and apo-E and are processed by peripheral tissues before reaching the liver. VLDL, therefore, transport endogenously-derived TG to body tissues for storage or the generation of energy through oxidation. TG from VLDL are hydrolysed into FAs by LPL. This process is coupled to a loss of apo-C's that converts VLDL to VLDL remnants^{80, 81}.

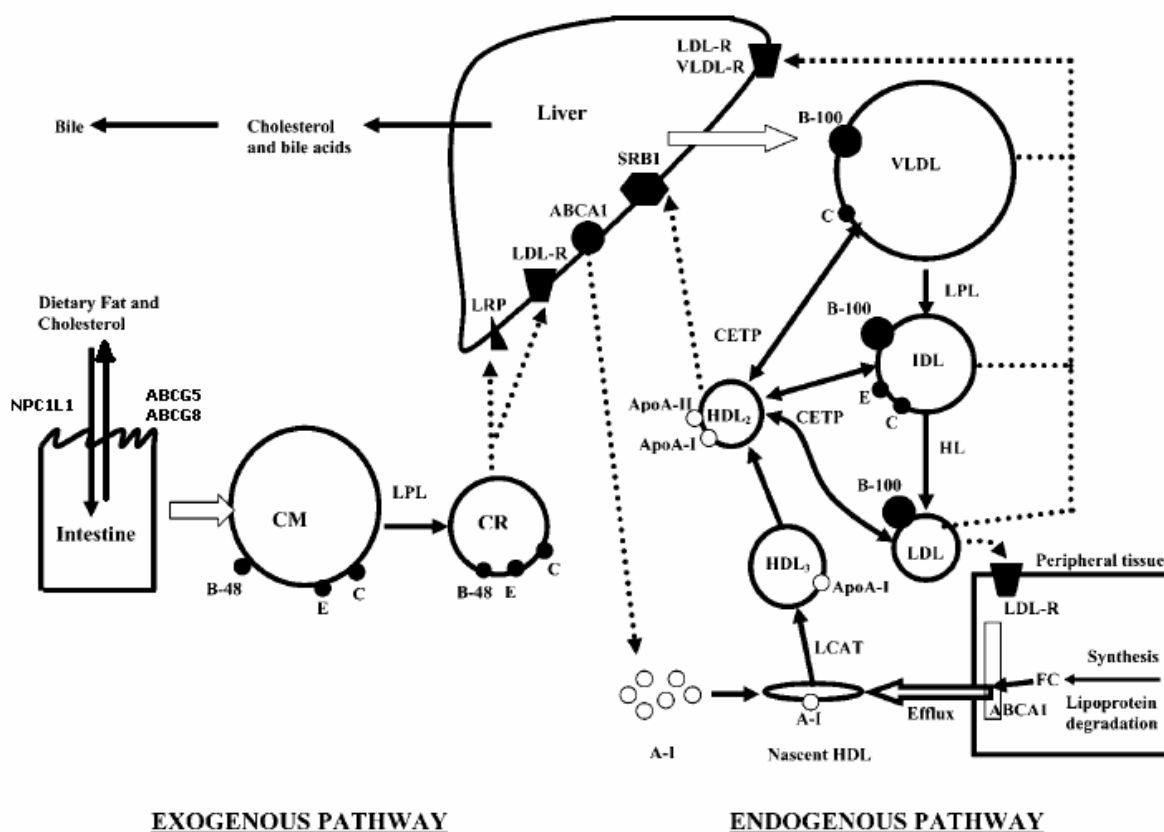
As VLDL remnants undergo further TG hydrolysis, they become IDL. Due to loss of apo-C's, the remaining apols in IDL are predominantly apo B-100 and apo-E. IDL are converted either to LDL (by further loss of TG) or are taken-up directly by the liver.

The liver takes-up VLDL remnants and IDL by VLDL- and/or LDL-receptor-mediated cell endocytosis. These interactions require the presence of apo-E ⁸²⁻⁸⁶.

The rest of the IDL are remodelled to form LDL by the action of hepatic lipase (HL). HL is a lipase synthesised in the liver and bound to its cells surfaces. Most LDL TG are hydrolysed and LDL apos are transferred to other lipoproteins, except apo B-100. LDLc accounts for 70% of plasma C. The uptake of LDL occurs predominantly in the liver (75%). As with IDL, LDL are taken-up by cells via LDL-receptor-mediated endocytosis (interaction requiring the presence of apo B-100). LDL taken-up by cells are deposited in lysosomes, where apos are degraded and C is made available for cells ⁸⁷⁻⁹².

Although the liver secretes mainly VLDL, it also appears to directly secrete a minor portion of IDL and LDL particles ⁹².

Figure 5. Plasma lipoprotein metabolism: endogenous and exogenous pathways ⁹³.



EXOGENOUS PATHWAY

ENDOGENOUS PATHWAY

CR=chylomicron remnants; LRP=LDL receptor-related protein; LDLR=LDL receptor; SRBI=scavenger receptor type BI; VLDLR=VLDL receptor; LPL=lipoprotein lipase; CETP=cholesteryl ester transfer protein; H=hepatic lipase; LCAT=lecithin cholesterol acyltransferase; ABCA1=membrane ATP binding cassette protein A1; membrane ATP binding cassette protein G5 and G8=ABCG5 and ABCG8; NPC1L1=Niemann-Pick C1-like 1 protein.

1.2.1.1.1.2.3 HDL metabolism and reverse cholesterol transport

All cells synthesise C, although specifically hepatocytes metabolise and excrete it from the body. C from peripheral cells is transported to the liver for its secretion into bile acids by a process mediated by HDL called reverse cholesterol transport (RCT) (Figure 5)²⁴.

Nascent HDL are synthesised by the liver and small intestine as protein-rich disc-shaped particles. The newly formed HDL are depleted of FC and CE but contain PL (mainly lecithin) and apoA-I. Nascent HDL acquire unesterified C and PL from peripheral tissues through action of the membrane ATP binding cassette protein A1 (ABCA1). C is then esterified through action of the HDL-associated enzyme, lecithin:cholesterol acyltransferase (LCAT)^{80, 94-96}. HDL also acquire additional apos and lipids transferred from the surface of Cm and VLDL during their lipolysis. As a result, HDL are converted into spherical lipoprotein particles through the accumulation of CE.

HDLC is transported to hepatocytes by a direct as well as an indirect pathway. HDL CE can be transferred to apo B-100-rich lipoproteins in exchange for TG through action of HDL-associated enzyme, cholesterol ester transfer protein (CETP)^{97, 98}. This allows CE to be removed by the liver through the LDL receptor mediated endocytosis pathway. HDLC can also be removed directly by the liver through scavenger receptor BI (SRBI). SRBI mediates selective uptake of CE from HDL without lysosomal degradation of lipoprotein particles (resecreted)⁹⁹⁻¹⁰⁵.

In this manner, HDL undergo extensive remodelling within the plasma as they transfer lipids and apos to lipoproteins and cells. PL can also be transferred to HDL by action of phospholipid transfer protein (PLTP). After CETP-mediated lipid exchange, TG-enriched HDL become a substrate for HL. HL hydrolyses TG and PL and generates smaller HDL particles^{88, 106}.

Once absorbed, C may be packaged into Cm or effluxed via ABCA1 to lipid-free apoA-I. ABCG5 and ABCG8 are also expressed by enterocytes and promote transport of C back into the intestinal lumen, thus directly influencing the efficiency of C absorption. Recent data suggest that Niemann-Pick C1-like 1 protein (NPC1L1) is a key molecule in intestinal C absorption. Biliary C can be reabsorbed from the intestinal lumen (between 50 and 80%)¹⁰⁷⁻¹¹².

1.2.1.1.2 Lipids

1.2.1.1.2.1 Total, LDL and HDL cholesterol

The most clinically relevant plasma lipid is C, a sterol. C is an important component of cell membranes. It is also the major precursor for the synthesis of vitamin D, of

several steroid hormones (cortisol, cortisone and aldosterone), of sex hormones (progesterone, oestrogen and testosterone), oxysterol and bile acids⁷².

Epidemiologic studies and clinical trials have established that elevated concentrations of LDLC are associated with increased CVD risk; the higher the C concentrations, the greater the CVD risk. As such, lowering total and LDLC concentrations significantly reduces CVD. As an independent risk factor, LDLC reductions in primary and secondary prevention of CVD had been associated with significant benefits with respect to mortality and morbidity rates^{26, 42, 44, 53, 54, 59-62, 68, 71, 113-118}.

Approximately 25 to 35% of plasma C is transported by HDL. These lipoproteins transport C from tissues back to the liver, where it is secreted into bile. Prospective studies have established that low HDLC concentrations are strongly, and inversely, associated with CVD risk. As such, high HDLC is seen as having a protective effect against CVD^{59-62, 113-118}.

In the United States (US), the Third Report of the Expert Panel on Detection, Evaluation and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III; or ATP III) presented the National Cholesterol Education Program's (NCEP) updated recommendations for C testing and management. This report continues to identify LDLC as the primary goal of therapy. CVD risk is calculated and individuals are assigned to one of three treatment categories according to LDLC goals (Table 3). Individuals are recommended to improve their life-style, and to consider medication, depending on their CVD risk^{24, 40-42, 44, 59-62}.

Recently, the Third Task Force has provided European guidelines and Expert Consensus documents with the objective of reducing the incidence of first, or recurrent, clinical CVD events. The current guidelines address the role of life-style changes, the management of major cardiovascular risk factors and the use of different prophylactic drug therapies for the prevention of clinical CVD. LDLC goals related to CVD risk categories are summarised in Table 4. Treatment goals for lipid management in asymptomatic subjects are summarised in Figure 6^{40, 59-62, 67-70, 119-122}.

No specific treatment goals are defined for HDLC, although its concentrations are used as markers of increased risk. HDLC <1.0 mmol/l (40 mg/dl) in men and <1.2 mmol/l (46 mg/dl) in women serve as cut-off values of increased CVD risk⁶⁷⁻⁷⁰.

Table 3. ATP III LDLC goals and cut-off points ²⁴.

Risk category	LDLC goal (mg/dl)	LDLC levels at which to initiate therapeutic lyfe- style changes (mg/dl)	LDLC levels at which to consider drug therapy (mg/dl)
0-2 risk factors	<160	≥160	≥190 (drug optional between 160-189)
2+ risk factors (10-years risk ≤ 20%)	<130	≥130	10-years risk 10% → ≥160 10-years risk 10-20% → ≥130
CVD or CVD risk equivalent*	<100	≥100	≥130 (drug optional between 100-129)

* as diabetes mellitus.

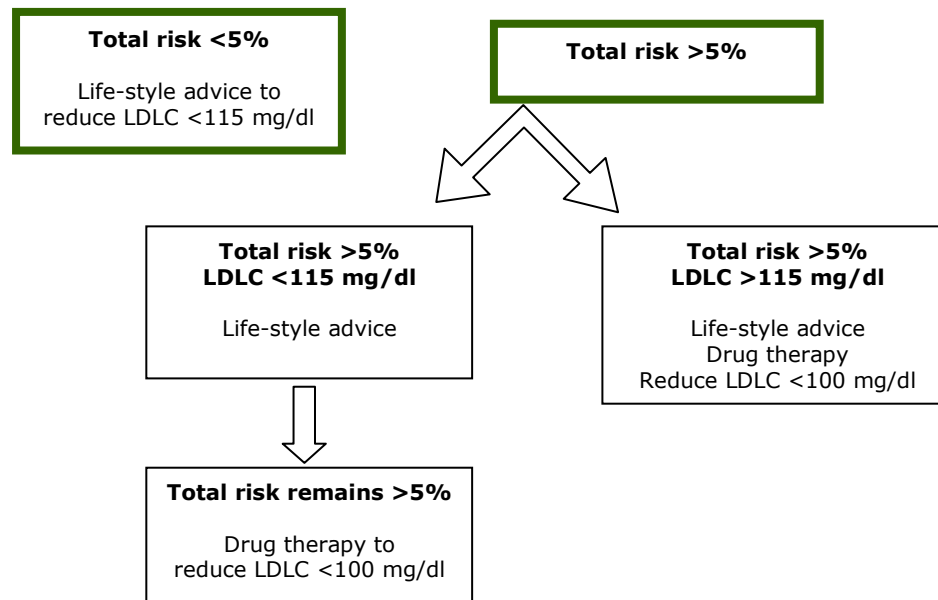
Table 4. Third Joint Task Force LDLC goals ^{68, 69}.

Risk category	LDLC goal (mg/dl)
10-years risk <5%	<115
10-years risk >5%	<100 (moderate dose drug therapy)
CVD or CVD risk equivalent *	<100 (drug therapy)

* as diabetes mellitus.

adapted and adopted in Spain ⁶⁹.

Figure 6. Third Joint Task Force treatment goals for asymptomatic individuals with a 10-year risk of death from CVD <5%>^{68, 69}.



adapted and adopted in Spain⁶⁹.

1.2.1.1.2.2 Triglycerides

TG are glycerides in which the glycerol is esterified with three FAs. TG play an important role in metabolism as a source of energy; they contain twice as much energy as carbohydrates (CH)⁷².

High plasma TG levels have been associated with CVD but they have not been established as an independent risk factor. Many individuals with CVD also have high plasma TG concentrations and also high total C, high LDLC and low HDLC concentrations (frequently with other major risk factors such as obesity, diabetes, and/or high blood pressure). The reciprocal association between TG and HDLC has been well established; with TG increase, HDLC is decreased. However, recent evidence suggest that high plasma TG levels are a significant CVD risk factor^{60-62, 121, 123-126}.

The ATP III guidelines of the NCEP establish the cut-off point for normal TG <150 mg/dl; placing more emphasis on moderate elevations^{24, 61}.

According to the European guidelines of the Third Joint Task Force, no specific treatment objectives are defined for TG. But fasting TG concentrations >150 mg/dl are used as a marker of increased CVD risk⁶⁷⁻⁷⁰.

1.2.1.1.2.3 Fatty acids

FAs are carboxylic acids with a long hydrocarbon linear chain. FAs can be classified according to their main carbon chain-lengths into: long-chain (>12 carbons), middle-chain (8-12 carbons) and short-chain (<8 carbons). FAs can also be classified based on the number of double bonds between carbon atoms into: no double bonds or saturated FAs (SFAs) and double bonds or unsaturated FAs. Unsaturated FAs can be further divided into: one double bond or monounsaturated FAs (MUFAs) and two or more double bonds or polyunsaturated FAs (PUFAs)⁷².

In the decade of the 1950's, studies suggested that the type of fat might be more important than the total amount of dietary fat. FAs are major factors that determine plasma lipid concentrations through regulation of C homeostasis and lipoprotein concentrations. Further, dietary FAs affect the levels of other risk factors, such as blood pressure, haemostasis and body weight¹²⁷⁻¹³¹. Food intake involves a mixture of SFAs, MUFAs and PUFAs which complicates any study of the effects of specific classes of FAs on CVD.

1.2.1.1.2.3.1 Saturated fatty acids

SFAs in the human diet are mainly derived from animal products (meat, dairy products), oils (coconut, palm oils) and cooking fats (lard, hard margarines). SFAs are directly related to total C, LDLC and HDLC concentrations, to the development of atherosclerosis, and to CVD. Several studies have shown that when SFAs are replaced by unsaturated fats, total plasma C is decreased. Further evidence for the benefits of lowering dietary SFAs has been provided by a corresponding lowering in CVD mortality¹³²⁻¹³⁶.

1.2.1.1.2.3.2 Polyunsaturated and monounsaturated fatty acids

Unsaturated FAs can be classified into PUFAs and MUFAs.

PUFAs are long chain FAs containing two or more carbon-carbon double bonds. PUFAs are grouped on the basis of the position of the terminal double bond with respect to the terminal carbon atom of the FA chain⁷². The two major groups are¹³⁷⁻¹⁴²:

- * ω -3 or n-3 FAs series (α -linolenic acid, eicosapentaenoic acid and docosahexaenoic acid): mainly present in seafood, fish oils, green leafy

vegetables, walnuts, grains, various seeds and seed oils. α -Linolenic acid can be n-3 group precursor (α -linolenic acid is converted to eicosapentaenoic acid and docosahexaenoic acid in the body).

* ω -6 or n-6 FAs series (linoleic acid and arachidonic acid): mainly present in vegetables, fruits, walnuts, grains, various seeds and seed oils.

α -Linolenic acid and linoleic acid are two essential FAs (not synthesised in the body and acquired from the diet). Many studies have shown the health benefits of PUFAs. There is a strong inverse association between PUFAs and CVD risk. Replacing SFAs with PUFAs decreases LDLC as well as HDLC concentrations. The recommendations are that n-3 and n-6 should be consumed in appropriate quantities as well as in the correct proportions (an optimal ratio of n-6 to n-3 is considered as 4:1).

MUFAs have one carbon-carbon double bond in the FAs chain. These fats are present in all animal and vegetable products (e.g. oils). Oleic acid, a non-essential FA, is the most common dietary MUFA. Major dietary sources include olive oil, canola oil, avocados, peanuts, hazelnuts, almonds, pistachios and various seeds ⁷². Several studies have found that replacing SFAs with MUFAs decreases LDLC concentrations. Replacing SFAs with MUFAs or CH not induce much change, or can increase HDLC plasma concentrations ¹⁴³⁻¹⁴⁷.

1.2.1.1.3 Apolipoproteins

Apos are structural components of lipoprotein particles (e.g. apo B-48, A-I, A-II), ligands of cell membrane receptors (e.g. apo B-100, E), activators or inhibitors of key enzymes (e.g. apoA-I, C-II, C-II) or transport systems (e.g. apoA-IV, F). The interaction of proteins on the surface of lipoprotein particles with each other, with enzymes and with specific apos on the cell's surface determines whether TG and C will be incorporated, or removed, from lipoproteins (Table 5) ¹⁴⁸⁻¹⁷¹.

Another approach to CVD risk assessment is to measure concentrations of apos in plasma (especially apo B-100, the sole apo in LDL; and apoA-I, the major apo in HDL) or to combine these values into ratios (such as the apo B-100/apoA-I ratio). Growing evidence indicates that apo B-100/apoA-I ratio is a better marker for CVD risk assessment than lipids or lipoproteins-to-lipid ratios. However, since measurements of some apos are not universally available, measurements of apos concentrations are not generally included as an integral part of the commonly-used guidelines for risk assessment ⁶⁷⁻⁷⁰.

1.2.1.1.3.1 Apo B-100

Apo B exists in human plasma as two isoforms of one gene; apo B-48 and apo B-100^{172, 173}. Apo B-48 is synthesised in the small intestine. It comprises the N-terminal region of apo B-100 and results from post-transcriptional changes. Apo B-48 is present in Cm and Cm remnants and plays a role in intestinal absorption of dietary fats. Apo B-100 is synthesised in the liver and it is required for the assembly of VLDL. Apo B-100 is also the major physiological ligand for the LDL receptor (LDLR). Unlike other apos, it does not interchange between lipoprotein particles and it is found in IDL and LDL particles; >90% of the LDL particle protein content being apo B-100.

Apo B-100 plays a central role in lipoprotein metabolism^{72, 154}. Concentrations of apo B-100 can be used as a direct measure of the concentration of atherogenic lipoproteins. As such, measurement of apo B-100 concentrations has been proposed as a better CVD risk marker than LDLC. Apo B-100 concentrations reflect not only LDL, but also that of other atherogenic lipoproteins (e.g. VLDL and IDL). Some observational studies have proposed apo B-100 as a more powerful independent CVD risk predictor than LDLC^{67-70, 157, 171, 174, 175}.

1.2.1.1.3.2 ApoA-I

ApoA-I is synthesised in the liver and the small intestine. ApoA-I is the major apo of HDL. It functions as an LCAT cofactor and promotes C efflux from cells¹⁷⁶⁻¹⁸⁴.

ApoA-I seems to be protective against atherosclerosis because of its role in HDL metabolism. Low concentrations of apoA-I are, like HDLC, associated with high CVD risk. As such, apoA-I has been proposed as a better marker of protection against CVD than HDLC^{67-70, 120, 124, 154, 185-188}.

1.2.1.1.3.3 ApoA-II

ApoA-II is synthesised in the liver. ApoA-II is the second most abundant protein of HDL particles. ApoA-II may affect HDL structure and metabolism (e.g. apoA-II might affect HL association and increase its activity, inhibit LCAT and CETP and hepatic CE uptake from HDL through SRBI, while it could displace apoA-I from HDL and accelerate its catabolism)¹⁸⁹⁻¹⁹¹.

As a major constituent of HDL particles, apoA-II might play an important role as a modulator in RCT and lipid metabolism. As yet, there is not much known regarding apoA-II biochemistry and, although the anti-atherogenic role of apoA-I has been clearly demonstrated, little is known on apoA-II function in HDL metabolism^{72, 124, 154, 192-203}.

Table 5. Characteristics and distribution of the major apos ^{73, 74, 148-171}.

Apo	Lipoprotein fraction						Tissue source	MW (Da)
	Plasma (mg/dl)	Cm	VLDL	LDL	HDL2	HDL3		
ApoA-I	100-150	0-5			70	65	Liver Intestine	29,016
ApoA-II	30-50	0-1			10	20	Liver	17,414
ApoA-IV	15	10				♦	Intestine	44,465
ApoA-V			♦				Liver	41,213
ApoB-48	5*	20-25					Intestine	241,000
Apo B-100	80-100		35-40	95-100			Liver	512,723
ApoC-I			♦		♦		Liver Intestine	9,332
ApoC-II	3-8	15	7-8	♦	♦	♦	Liver	8,900
ApoC-III	8-15	35-40	35-40	♦	10	5	Liver	8,800
Apo-D			♦	♦	5	5	Intestine Liver Pancreas	33,000
Apo-E	3-6	5	5-10	♦	1-10	1-2	Liver	34,145
Apo-F	2			♦	♦	♦	Intestine Liver	113,000
Apo-H	15	♦			♦	♦	Liver	50,000
Apo-J							Variety of tissues	52,495
Apo-L (I-VI)	0.9				♦	♦	Liver Pancreas	44,000
Apo-M			♦	♦	♦		Liver Kidney	21,253

*apo B-48 is present only during the post-pandrial period.

♦ apo present but no quantitative data available.

apo=apolipoprotein; Cm=chylomicron; VLDL=very low-density lipoprotein;

LDL=low-density lipoprotein; HDL=high-density lipoprotein; MW=molecular weight;

Da=daltons.

Protein name according to UniProtKB/SwissProt (<http://www.expasy.org/uniprot/>).

1.2.1.2 Life-style modifications

Dietary interventions are highly effective in the prevention of recurrent events in patients with established CVD. Hence, dietary changes are an integral part of the prevention and health-care management strategies. Diets act in many different aspects related to CVD such as in the regulation of plasma lipid concentrations, lipoprotein composition, lipid oxidation, arterial pressure, endothelial function, thrombogenesis and inflammation. The diet can be improved by modifying the amount and type of ingested fat (and is reflected in lipid profile) without adverse side effects ²⁰⁴⁻²¹¹.

In 1957, the American Heart Association (AHA) proposed that a reduction of dietary fat intake would reduce the incidence of CVD ⁴⁹. Since then, the AHA has issued several policy statements on diet and CVD as and when more reliable new information becomes available ^{2, 60-62, 212, 213}. Current AHA recommendations regarding diets in healthy life-style practices for the general population are based on evidence indicating that modifications of specific risk factors decrease the incidence of CVD ². These guidelines recommend:

- * eating a nutritionally-adequate diet consisting of a variety of foodstuffs.
- * reducing consumption of fat (especially SFAs and C).
- * achieving and maintaining an appropriate body weight (regular physical activity).
- * increasing consumption of complex CH and dietary fiber.
- * reducing salt intake.
- * consuming alcohol in moderation.

European organisations such as the Third Joint Task Force have issued guidelines and Expert Consensus documents ⁶⁷⁻⁷⁰. The focus on major risk factor changes in patients behaviour include: smoking, high levels of plasma C (especially LDLC), low levels of HDLC, high blood pressure, diabetes mellitus, obesity (especially visceral adiposity) and physical inactivity. The general European recommendations are:

- * eating a nutritionally-adequate diet consisting of a variety of foodstuffs.
- * reducing consumption of fat (total fat intake <30% of total energy intake), especially SFAs (not to exceed a third of total fat intake) and C (<300 mg/day).
- * replacing SFAs and trans-FAs with MUFAs and PUFAs of vegetable and seafood origin.
- * encouraging the consumption of fruits and vegetables, whole grain cereals and bread, low-fat dairy products, fish and lean meat.
- * consuming oily fish and n-3 FAs.
- * achieving and maintaining an appropriate body weight (BMI 20-25 kg/m²) and regular physical activity.
- * reducing intake of salt.

* consuming alcohol in moderation.

All these guidelines and recommendations indicate that individuals can improve their overall health, and to maintain it with a healthy life-style ^{2, 24}.

1.2.1.2.1 Dietary recommendations: their effect on lipoprotein metabolism

Dietary recommendations for populations have been made with the aim of reducing CVD risk ^{24, 214, 215}. Dietary modifications to achieve a healthy diet focus on limiting the intake of SFAs and C. High SFA diets increase total C and LDLC concentrations whereas high-MUFA and/or PUFA diets decrease total C and LDLC concentrations. The effects of these FAs on HDLC are not, as yet, clearly defined; SFAs substitution for CH decreases HDLC levels whereas SFAs substitution for MUFAs does not change HDLC concentrations, or even increase them.

The AHA has developed two diets, STEP I and II diets, intended to lower LDLC concentrations. Several studies have shown that these low-fat diets, especially STEP II, decrease LDLC (by having an impact on apo B-100 metabolism) but also HDLC (by having an impact on apoA-I metabolism). However, since low HDLC concentrations are a major risk factor for CVD, other types of diets, such as the Mediterranean diet which does not appear to decrease HDLC concentrations, are becoming a focus of interest in seeking a reduction of CVD risk ⁶⁰⁻⁶². The Mediterranean diet has a high MUFA content and would be a viable alternative to the diets recommended by the AHA.

1.2.1.2.1.1 STEP I and STEP II diets

The National Heart, Lung and Blood Institute (NHLBI) launched the NCEP in 1985. The goal of the NCEP was to reduce CVD in the US by reducing the proportion of individuals with high plasma C concentrations ^{2, 24, 60-62, 216}. STEP I and STEP II diets were developed by the NCEP, and both were approved by the AHA. Both diets were designed to reduce CVD risk by reducing high plasma C concentrations. STEP I diet was considered the starting point for individuals at a lower risk of atherosclerotic disease. STEP II diet goals were considered for individuals already on STEP I diet, for patients with high risk C concentration (≥ 240 mg/dl) and/or for patients who had already had a heart attack.

In May 2001, the NCEP released new guidelines for C management. The AHA no longer uses the terms "STEP I" and "STEP II" in reference to "heart-healthy" diets, but prefers "Therapeutic Life-style Changes" (TLC) which include dietary modifications. TLC diet has a total fat content ranging from 25 to 35% of total

calories. This is achieved by increasing the amount of MUFAs up to 20% of total calories.

Currently, ATP III recommends a more intensive life-style intervention. TLC places more emphasis on the shedding of excess body weight and increasing daily physical activity, than do earlier guidelines (Table 6) ^{213, 217-219}.

Table 6. Therapeutic Life-style Changes recommendations (A) and diet characteristics (B) in ATP III ².

(A)

ATP III recommendations

Reduced intake of SFAs and C
Therapeutic dietary options for enhancing LDL lowering (plants stanols, sterols and increased soluble fibre)
Weight reduction
Increased regular physical activity

(B)

Nutrient	Recommended intake as percentage of total calories
Total Fat	25–35%
SFAs	<7%
PUFAs	up to 10%
MUFAs	up to 20%
C	<200 mg per day
Prot	approximately 15%
CH	50–60% of total calories (food rich in complex carbohydrates)
Total calories	balance energy intake and expenditure to maintain desirable body weight and prevent weight gain (include at least moderate physical activity)

C=cholesterol; Prot=proteins; CH=carbohydrates.

1.2.1.2.1.2 Mediterranean diet

Recently, there has been increasing scientific evidence for positive health benefits from diets that have a high intake of fruit, vegetables, legumes, whole grains, fish, nuts and low-fat dairy products. Such diets do not need to be restrictive with respect to total fat, provided there is no excessive intake of total calories (with emphasis on

vegetable oils, low SFAs). These dietary characteristics are present in the traditional Mediterranean diet ²²⁰⁻²²³.

The term "Mediterranean diet" was coined by Willett et al (1995) and reflected food patterns typical of Crete, much of the rest of Greece and southern Italy in the early 1960's. Variations of Mediterranean diet exist elsewhere in the region and is closely tied to traditional areas of olive cultivation and are termed Mediterranean-type diets. The diet first came to attention in the 1950's and 1960's with The Seven Countries Study and was based on three lines of evidence ²²⁴⁻²²⁶:

- * adult life-expectancy for populations in these areas was among the highest in the world and rates of CVD, certain cancers, and some other diet related chronic diseases were among the lowest.
- * data on food availability and dietary intake in the Mediterranean region described dietary patterns with many common characteristics.
- * the dietary patterns were associated with low rates of chronic diseases and high adult-life expectancy in numerous epidemiological studies conducted world-wide.

Characteristics of Mediterranean diet at the time of the Seven Countries Study were ²²⁴⁻²²⁶:

- * abundance of plant food (fruit, vegetables, cereals, potatoes, beans, nuts and seeds).
- * minimally-processed, seasonally-fresh and locally-grown foods.
- * olive oil as the principal source of fat.
- * dairy products (principally cheese and yoghurt).
- * consumption of fish, poultry and eggs.
- * consumption of low amounts of red meat.
- * consumption of wine in moderate amounts, normally with meals.

Traditional Mediterranean diet is a high-fat diet with a low amount of SFAs (<15% of energy). The amount of total fat ranges from <25 to >35% of total calories, depending on the geographical area, with a high consumption of MUFAs (mainly from virgin olive oil) ²²⁵. This dietary pattern is attractive for its palatability, as well as for its health benefits. The Mediterranean diet represents a life-style including regular physical activity which, until now, has been associated with low obesity rates ²²⁰⁻²²⁷.

Mediterranean-type diets reduce atherosclerosis by reducing SFAs and by substituting them with MUFAs, together with a high consumption of vegetables, fruit and whole grains. This improves the plasma lipid profile (lowering LDLC while increasing or maintaining HDLC) and lipid oxidation, decreases atherothrombosis risk and improves endothelial function and insulin resistance ²²⁸⁻²⁴⁹. Evidence suggest that apart from the C-lowering properties of MUFAs (mainly oleic acid from virgin olive oil), other cardio-protective benefits are derived from nutrients from the Mediterranean diet. For example, minor components of virgin olive oil such as polyphenols, vitamin E and

vegetal sterols have beneficial effects on CVD due to their antioxidant and vasodilatation properties ²⁴⁹⁻²⁵².

2. Kinetic studies: application in the investigation of lipoprotein metabolism

Metabolism covers all biochemical processes that occur within any living organism. Metabolism consists of anabolism (production) and catabolism (degradation). The complexity of lipoprotein metabolism can be studied using different techniques to characterise the underlying mechanisms (Table 7) ⁷². The understanding of metabolic processes requires knowledge of the metabolic pathways *in vitro* (quantified in the laboratory) but also *in vivo* (conducted in living systems). The data so derived can be analysed within a framework that provides a detailed understanding of the metabolic pathway under investigation.

Table 7. Some techniques used to study metabolism ²⁵³⁻²⁵⁵.

<p>PHYSICAL TECHNIQUES</p> <ul style="list-style-type: none">▪ mass spectrometry (MS)▪ X-ray crystallography▪ nuclear magnetic resonance (NMR)▪ ultracentrifugation
<p>PHOTOMETRIC TECHNIQUES</p> <ul style="list-style-type: none">▪ spectrophotometry▪ turbidimetry
<p>CHROMATOGRAPHIC AND ELECTROPHORETIC TECHNIQUES</p> <ul style="list-style-type: none">▪ ion exchange chromatography▪ gas chromatography (GC)▪ gel electrophoresis
<p>BIOCHEMICAL, CELLULAR AND MOLECULAR TECHNIQUES</p> <ul style="list-style-type: none">▪ enzymology<ul style="list-style-type: none">immunochemical<ul style="list-style-type: none">enzyme linked immunosorbent assay (ELISA)enzymatic assays<ul style="list-style-type: none">enzyme kinetics (Km)▪ cell/tissue culture▪ DNA/RNA structural and functional analysis<ul style="list-style-type: none">genetic screening techniques<ul style="list-style-type: none">arraystransgenic animal modelsknockout animal models▪ protein structural and functional analysis<ul style="list-style-type: none">Western Blot
<p>BIOINFORMATIC TECHNIQUES</p>
<p>BIOSTATISTIC TECHNIQUES</p>

Traditionally, the measurement of plasma lipids and lipoproteins have been used to characterise lipoprotein metabolism. However, static measures of either lipid or lipoprotein concentrations do not provide information on the underlying mechanisms (plasma concentrations being a result of a balance between input and output of the system i.e. as rates of production and catabolism of lipoprotein particles). Due to the specific features of human lipoprotein metabolism, *in vivo* studies are performed, preferably, in human subjects ²⁵⁶⁻²⁶⁰. Lipoprotein kinetic studies in humans provide a powerful approach to the understanding of mechanisms that modulate lipoprotein metabolism and provide further insight into metabolic disorders and the effects of treatment ^{24, 64, 85, 261-277}. For example, assessment of the differences between a "normal" and a "diseased" state could provide a rational basis for the search for a remedy for the disease. They can also be applied to nutritional intervention studies to examine the reason why nutritional interventions might favourably regulate disorders of lipoprotein metabolism and, hence, decrease CVD risk. Currently, stable isotope tracers and multi-compartmental models are increasingly being used for lipoprotein kinetic studies.

2.1 Aim of kinetic studies in lipoprotein metabolism

The aim of lipoprotein kinetic studies is to obtain information on the dynamics of the physiological processes. To fully understand lipoprotein metabolism, mathematical models are developed to obtain kinetic data which provide insight into the pathways that quantitatively describe parameters such as synthesis, degradation, and transport rates ^{24, 257, 262, 264, 276-285}. Lipoprotein metabolism depends, to a large extent, on apos. Thus, information on the rates of production and catabolism of the apos can be used to interpret the fate of the parent lipoprotein molecule.

Kinetic studies include:

- * hypothesis development.
- * experimental design.
- * statistical considerations.
- * tracer administration and sampling schedule.
- * development of compartmental models for interpretation of tracer data.

2.2 Stable isotope tracers and their use in kinetic studies

Some specific terminology is used in kinetic studies of the behaviour of endogenous molecules in a system. The "tracee" refers to the biological substance of interest in biological systems. The "tracer" refers to the labelled form of a biological substances (e.g. amino acids, glucose, lipoprotein particles) that are used to determine certain properties of this substance in biological systems. Tracers differ from tracees by their structures being labelled with isotopes. The "tracer" is introduced into the "system" to

infer information about the kinetics of the tracee. "System" refers to a biological construct (e.g. whole body, organ, cellular or subcellular levels) in which the substance under investigation is present. The system should be maintained in a steady state i.e. one in which the variables are maintained constant during the study so that the rates of input and output for a given unlabelled tracee substance are equal and time invariant. To achieve a steady state, the tracer has to be introduced into the system in small enough quantities to not perturb the steady state but, at the same time, to ensure sufficient tracer incorporated into the substance to enable its quantification ^{278, 279}.

2.2.1 Isotopes: radioisotopes versus stable isotopes

The atomic number (Z) of a chemical element is the number of protons in the nucleus of each of its atoms. This number is the defining characteristic of a given element, invariant for all atoms of that element. Atoms of an element might have different atomic mass number (A) due to differences in their number of neutrons. Atoms of the same Z but different A are called isotopes ²⁸⁵. Elements can exist as:

- * stable isotopes which show no tendency to undergo radioactive decay.
- * unstable or radioactive isotopes which have an unstable nucleus that achieve stability by the emission of radioactive particles.

The first studies using stable isotopes to investigate human metabolism *in vivo* were pioneered by Schoenheimer and Rittenberg in the 1930's, when they used deuterium to trace the fate of various molecules such as amino acids, FAs and glucose in animals and humans ^{286, 287}. The use of radiotracers became the method-of-choice due, mainly, to stable isotope production and detection having considerable technical problems. However, these problems have been resolved in great part and the use of stable isotopes has reached wider acceptance to-date ^{280, 288}.

Radiotracers had been used for lipoprotein kinetic studies in the decades of the 1960's-1980's. There were, however, disadvantages that limited their use. These included:

- * the need to isolate and radiolabel of the apos extra-corporeal, and which could induce modifications.
- * isolation and radiolabelling was technically difficult.
- * labelled proteins had to be re-introduced into the subject which may not have been physiologically identical and, as such, modifying their metabolism.
- * for ethical considerations such as radiation safety, the use of radiotracers was limited.

The use of stable isotopes in lipoprotein kinetic studies was a development during the decade of the 1990's. It was achieved, partly, by advances in the technology of incorporating stable isotopes into the synthesis of biological compounds and improvements in computer-supported mass spectrometry for their detection ^{262, 287, 289-292}. Stable isotopes enabled the endogenous labelling of molecules and made *in vivo* kinetic studies possible. Comparisons between radioactive and stable isotope tracers showed that both tracers lead, in general, to the same metabolic conclusions ^{257, 297-298} while endogenous labelling with stable isotopes had several theoretical advantages ^{262, 290}:

- * the biological nature of the apos was not altered by having been outside the body during their isolation and labelling.
- * since the incorporation of labelled tracers occurred in all newly synthesised proteins, the stable isotope approach enabled the direct determination of production rates of proteins (including the apos). As such, endogenous labelling provided a more direct measure of biosynthesis through tracer incorporation rates.
- * no radiotracers of certain elements are available for practical use.
- * less biological side effects than with corresponding radiotracer.
- * substrate content and isotopic enrichment can be measured simultaneously
- * very high assay specificity and sensitivity.
- * possible simultaneous, and repeated, use of several tracers in the same study subject.
- * simultaneous study of multiple proteins in the same individual.

Currently, several international research groups are investigating lipoprotein kinetics *in vivo*. These include the groups of Packard et al (Scotland), Barrett et al (Australia), Schaefer et al (US), Taskinen et al (Finland), Lamarche et al (Canada) and Patterson et al (Canada).

2.2.2 Characteristics of stable isotopes used in lipoprotein metabolic studies

An ideal tracer should be ^{278, 279, 299}:

- * a detectable substance.
- * a substance not perturbing the system when introduced into it.
- * a substance indistinguishable with respect to the properties of the one being studied (physically, chemically and biologically identical with the tracee).

Other criteria that need to be taken into account are ³⁰⁰⁻³⁰⁴:

- * safety of the tracer:

use of sterile and pyrogen-free intravenous injectable solutions.

research project aproved by ethical committees and patient consent.

- * reliable source of the tracer.
- * chemical purity being guaranteed and independently confirmed.
- * label stability.

These characteristics are fulfilled by stable isotopes such as ^2H , ^{13}C , ^{15}N which are usually used in endogenous labelling of amino acids for *in vivo* kinetic studies (Table 8). Deuterium or heavy water (^2H) is the stable isotope of hydrogen. The nucleus has one proton and one neutron whereas a normal hydrogen nucleus has just one proton. It occurs naturally as deuterium gas $^2\text{H}_2$ (Table 9) ³⁰⁵⁻³⁰⁸. Deuterium is frequently used as a tracer molecule to study metabolic pathways. Chemically it behaves identically to the more abundant "normal" hydrogen but it can be distinguished by its different mass using mass spectrometry. Usually, deuterium is used as a multiple labelled tracer in kinetic studies, as $^2\text{H}_3$ which contains three atoms of ^2H and which enables greater sensitivity in detection of low levels of infused tracer, and prevents interference from natural abundance (background) of stable isotopes when using mass spectrometry (Table 8).

There is no evidence that stable isotopes can be biologically harmful in the doses used for tracer studies. It is assumed that stable isotope tracers behave exactly as natural compounds, and they are widely used in human studies. For adults, 200-400 mg ^2H /kg body weight is the threshold for clinically-relevant side effects (e.g. vesical carcinoma). These ranges are well above the usual tracer dosage ²⁹²; the dose of ^2H used in *in vivo* studies being between 1 and 80 mg/kg body weight (Table 8). To-date, no alterations in the biological behaviour of apos have been shown when labelled with stable isotopes at the usual dose of 0.6-3 mg/kg body weight.

Table 8. Abundance of stable isotopes in nature, in human body, daily intakes and that usually administered in kinetic studies ^{300, 301}.

Element	Average natural abundance (%)	Abundance in human body (mg/kg body weight)	Intake with air, food, water (mg/kg)	Tracer amount (mg/kg body weight)
^2H	0.015	15	6.9	1-80
^{13}C	1.111	2,000	99.9	1-25
^{15}N	0.360	110	0.15	10-40
^{18}O	0.200	1,300	127.4	60-180

Table 9. Characteristics of hydrogen's isotopes²⁹⁹.

Isotope	Natural abundance (%)	Half-life	Radioactivity
¹ H (hydrogen)	99.985	stable	
² H (deuterium)	0.015	stable	
³ H (tritium)	radioactive isotope	12.33 years	β decay
⁴ H	radioactive isotope	unknown	neutron emission

2.2.3 Amino acids used as tracers

Tracers used in lipoprotein kinetic studies are, mainly, amino acids labelled with isotopes such as ²H₃-L-leucine, ¹⁵H-L-glycine and ¹³C-L-phenylalanine. Administration of a tracer enables proteins to be labelled endogenously such that the stable isotope labelled amino acid precursor is biosynthetically incorporated *in vivo*²⁶³.

Choice of amino acid for labelling with stable isotope is based on knowledge of amino acid metabolism, abundance in the protein-of-interest, and availability/cost of the tracer. However, selection of amino acid for tracer studies does not appear to affect the calculation of the lipoprotein kinetic parameters^{309, 310}.

2.2.3.1 Use of the amino acid leucine

Leucine has been considered the most appropriate apo tracer. Amino acids occur in two possible forms, "D" and "L" amino acids, with the L-amino acid form predominating in proteins. L-leucine is an essential amino acid with no *de novo* synthesis by cells and is acquired from the diet. It is hydrophobic and constitutes a structural element of proteins and enzymes. Deuterated labelled ²H₃-L-leucine (Figure 7) is used in kinetic studies because^{72, 262, 310-313}:

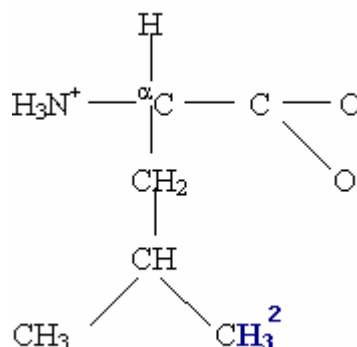
- * it is metabolised predominantly in muscle which reduces the possibility of amino acid recycling in the liver.
- * it is an essential amino acid and, as such, the enrichment of leucine pools in the liver is not affected by any endogenously synthesised leucine.
- * leucine is converted to its keto-acid and not to other amino acids. It has no intermediate metabolism and the products derived from its degradation are not reincorporated into other amino acids.

- * certain apos (such as apo B-100, apoA-I and A-II) contain more leucine than any other amino acids.
- * leucine labelled with stable isotopes (e.g. $^2\text{H}_3$ -L-leucine tracer) is commercially available.

There need to be certain assumptions regarding $^2\text{H}_3$ -L-leucine tracer ³¹⁰:

- * it is processed *in vivo* in an identical manner to endogenous unlabelled leucine.
- * it is not selectively incorporated into proteins or stored within specific amino acid pools in the liver.
- * the rate of tracer equilibrium within intracellular pools is relatively rapid so as to allow for an accurate estimation of apos production.

Figure 7. Structure of amino acid $^2\text{H}_3$ - L- leucine ³¹¹.



2.3 Analysis and modelling of stable isotope kinetic studies applied to lipoprotein metabolism

Lipoprotein heterogeneity requires complex data analysis. Several approaches are used to analyse lipoprotein kinetics, among which is multi-compartmental modelling.

Kinetic data contain more information than can be obtained by simpler methods of analysis such as non-compartmental models:

- * using algebraic or linear regression functions to describe enrichment curves. Algebraic functions are not appropriate and no longer used to describe lipoprotein tracer data because ^{310, 312}:
 - tracer data do not fit linear functions.

- delay associated with synthesis and secretion of apos into plasma fail to be accounted-for.
- enrichment of immediate precursor pool should be known (a variable not always available). Thus the assumption is that the plateau of VLDL apo B-100 enrichment (the first to be secreted into plasma) is the equivalent to the precursor pool.

For example, for the calculation of the fractional catabolic rate (FCR), the equation is presented as:

$$FCR = [(d\% \text{ APE}/h)/(d\% \text{ APE in plateau})] \times 24h$$

where $d\% \text{ APE}/h$ is obtained from the enrichment curve slope regression function.

* using mono-exponential functions to describe enrichment curves. Mono-exponential functions rely on the system under study to be in a steady state and that pools for tracer data to be kinetically homogeneous. This assumption is not valid for most lipoproteins which show heterogeneous kinetics. When the total appearance rate occurs into a non-sampled or accessible compartment, the non-compartmental approach is not acceptable e.g. when determining amino acid appearance rates ³¹⁴⁻³¹⁷. Physiologically, only a few substances appear directly in plasma, and most substances enter intracellular pools throughout the body. Moreover, isotopic enrichment cannot always fit suitably with mono-exponential functions. For example, the calculation of isotopic enrichment curves using the mono-exponential function:

$$E_t = b \times (1 - e^{-kt}) + E_0 \times e^{-kt}$$

where E_0 isotopic enrichment levels immediately before isotope injection.

Both approaches merely estimate kinetic parameters and provide limited quantitative information. Hence, multi-compartmental modelling and multi-exponential equation systems are needed to fit the observed isotope enrichment curves.

To-date, multi-compartmental modelling is considered the best approach, the most complex and the most appropriate in obtaining data for analysis from kinetic studies ^{309, 318, 319}. This approach is a powerful mathematical simulation via equations describing the flux of material between compartments and thereby enabling an estimation of quantitative or predictive information regarding system dynamics ²⁵⁷. However, a model that is excessively complex will not be supported by the experimental data and will have little predictive value ^{310, 320-322}.

2.3.1 Principles of multi-compartmental models

Tracer data generated in lipoprotein metabolism studies *in vivo* can be analysed using a variety of methodologies. However, multi-compartmental analysis is being used more frequently because of the increasingly complex experimental protocols being used. A "compartment" is defined as an amount of material that is well mixed, kinetically homogeneous and distinct from other material in the system. A "compartmental model" is a collection of compartments connected in a specific structure that allows movement of material between them³⁰⁹. A "kinetic model" is developed taking into account what is known regarding the kinetics of naturally-occurring substance in humans. A model enables measurements to be made of several kinetic events such as the incorporation of the studied substance into proteins, the release from proteins, and oxidation. Hence, modelling can be defined as the creation of a simplified representation of the biochemical world^{287, 314, 322-329}.

2.3.2 Tracer administration and sampling protocols

Selection of the type of administration of stable isotope and the sampling protocol has a considerable impact on data generated in kinetic studies. It affects the precision of measurement of the model's parameters, optimisation of the experiment's economic cost, the timing of intervention and the number of samples collected²⁵⁷.

The administration of the stable isotope tracer requires that several solutions be prepared. Tracers are chemically synthesised and can contain different contaminants, for example their unlabelled counterparts derived from natural sources. Hence, tracer solutions need to be tested for their content, identity and purity. These rely on certification provided by manufacturers in that, for example, labelled compounds for biological tracing are available with the heavy isotope representing 99% of the tracer element²⁶⁴. Solutions have to be sterile and pyrogen-free before use in humans; pharmacists or physicians need to take responsibility for this. When preparing solutions for human use, an aseptic procedure is an absolute requirement (e.g the use of sterile glassware and pyrogen-free solutions). The solution should be infused through a 0.22 µm filter to exclude possible bacterial contamination. Infused solutions need to be tested to ensure that the pyrogen load is below the human tolerance level^{291, 304}.

Patients (or volunteers) selected to undergo the lipoprotein kinetic study are admitted to a hospital room in the morning following an overnight fast. Typically, subjects are studied in a semi-recumbent position. Catheters are inserted into a vein in the forearm for isotope administration, on occasions via a filter as well. The catheterised vein in the contra-lateral is used for venous blood sampling at various time points, according to the study protocol. Infusion time can be variable as well as

the extraction of additional fasting blood samples over the days following the isotope administration so as to provide other kinetic information on lipoproteins. Thus, different time periods (e.g 48h, 96h or even 14d time-points) related to blood sampling may be needed, depending on protein turnover rates²⁶⁵.

Tracers can be administered intravenously using three approaches, either (Figure 8) 308, 313:

* a bolus injection or primed dose^{261, 262, 267, 304, 311-313, 323, 324, 329, 330}.

This approach provides information on the dynamics of inaccessible pools. In short,

- simplifies the measurement of particle inter-conversion.
- simplifies the detection of newly-synthesised particles (intracellular precursor enrichment is greater at the beginning of the study).
- enables frequent measurements of amino acid precursor enrichment in plasma.
- plasma enrichment is greater. The assumption is, however, that intracellular precursor pool enrichment is similar to that in plasma; there is an unlabelled amino acid dilution of intracellular pools due to protein turnover.

* a constant infusion³²³. This approach provides information on the degree to which equilibrium has been achieved (in a metabolic steady state) and, as such provides information on whole-body and end products. The constant infusion is often preceded by a bolus injection.

* a primed constant infusion^{313, 314, 329, 331}. The combination of bolus injection and constant infusion approach is termed "primed constant infusion". Searle et al first described, in 1954 when investigating glucose kinetics, the use of a bolus injection (primed dose) of tracer followed by a constant infusion of the tracer. Primed constant infusion provides more extensive information on the inter-related metabolic processes. It enables the measurements a number of integrated processes such as protein synthesis parameters to be performed in steady state conditions. The general acceptance the primed constant infusion technique is due to the simplified quantification of tracer appearance rate into plasma as well as the need for only a few blood samples once isotopic equilibrium has been achieved and, as such, minimising analytical time and patient discomfort.

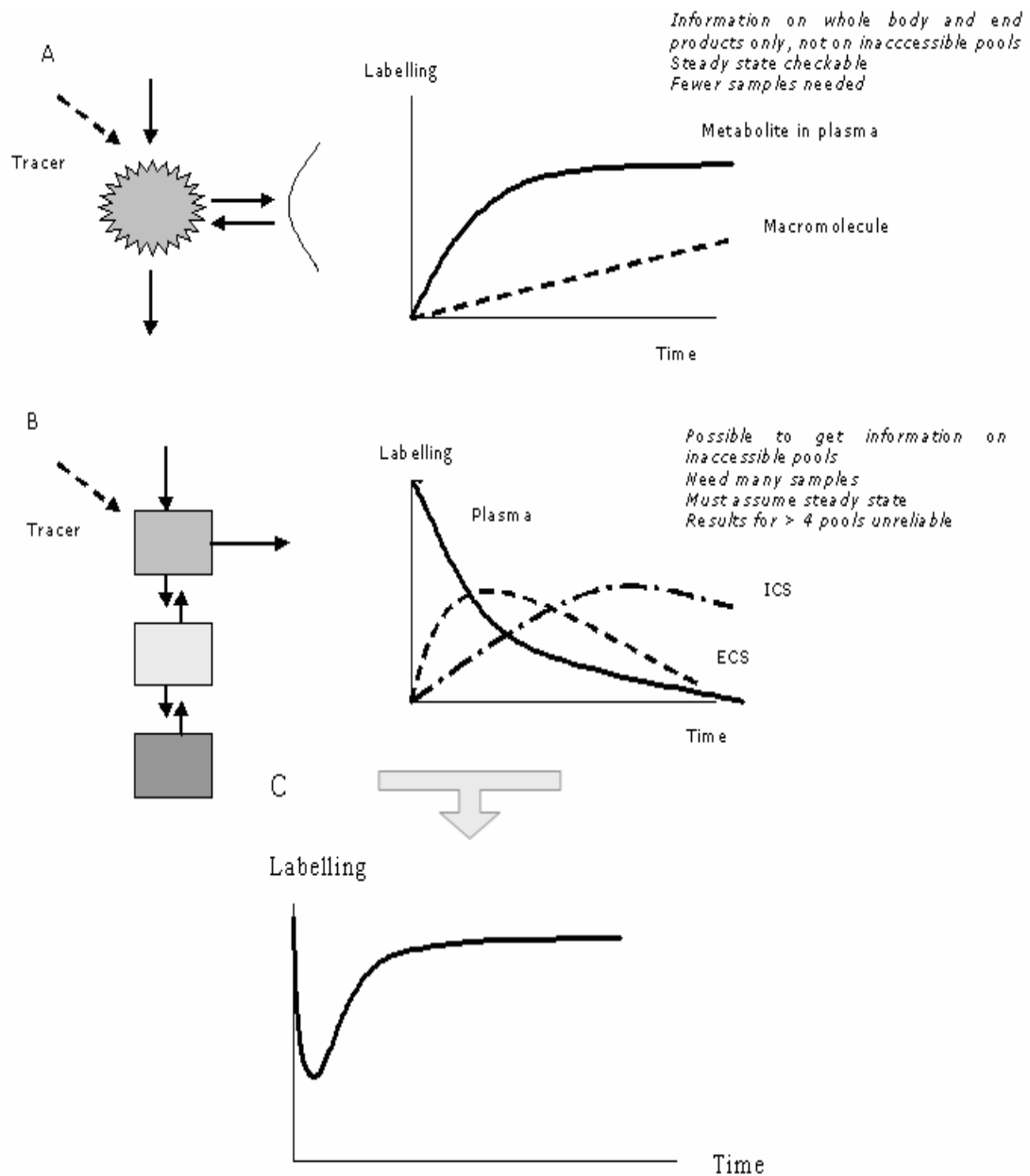
Primed constant infusion is widely used in lipoprotein kinetic studies. Its goal is to label, instantaneously, the total substance pool until equilibrium and, hence, reducing time required to reach isotopic equilibrium. If the pool size of a substance is large compared to the rate at which it is turning over, it may take several hours to reach an isotopic equilibrium using a constant infusion. It is often required that an isotopic equilibrium is reached in as short a time as possible. The primed constant infusion

creates a situation in which the sum of the decline in enrichment resulting from the bolus injection and the rise in enrichment due to the continuous infusion equals the final plateau enrichment (Figure 8). Thus, a bolus injection enables a high level of isotopic enrichment to be achieved at the start of the kinetic study, while the constant infusion keeps these levels constant over time, once the bolus effects have disappeared. All these considerations explain why a bolus injection is more suitable for studying lipoproteins of slow turnover (e.g. apoA-I HDL, apo B-100 LDL) or under conditions of kinetic heterogeneity (e.g. apo B-100 LDL), while a constant infusion is more suitable for proteins having rapid turnover (e.g. apo B-100 VLDL); the primed constant infusion combines both these requirements.

The use of a primed constant tracer infusion makes several assumptions ^{262, 267, 290, 304, 313, 322, 323, 330, 331, 332}.

- * a steady state is achieved (plasma levels of tracee should not change during the course of the study).
- * the dose of tracer will not have any effect on the metabolism of the tracee; albeit the isotopes need to be infused at rates sufficient to ensure enough plasma enrichment for accurate detection by mass spectrometry.
- * labelled and unlabelled amino acids are processed identically *in vivo*.
- * no significant recycling of isotope should take place. It is assumed that tracers are incorporated into body protein and will not return by protein turnover into the plasma amino acid pool during the course of the infusion (any returned tracer would increase enrichment and, therefore, impacting upon the kinetic measurements). Over a 24h period there is up to 30% recycling and so it is important to design the kinetic experiment to be as short as is feasible.
- * enrichment measured in plasma should be representative of that in the protein synthesis site (e.g. intracellular pool in the liver).
- * provide a constant enrichment of plasma leucine and precursor pool.

Figure 8. Constant infusion (A), bolus injection (B) and primed constant infusion (C) approaches for the administration of the tracer ³¹³.



ICS=intracellular space; ECS=extracellular space.

2.3.3 Acquisition of kinetic data

To obtain reliable kinetic data requires careful measurements and analytical methodologies appropriate for *in vivo* measurements. Stable isotope studies provide data from which models can be developed and tested against experimental data and

which can provide new insights into metabolic pathways. When using the modelling approach several aspects need to be taken into consideration. These include: experimental design, the number of compartments, certain tests to ensure that the model describes the experimental data and that the model parameters can be estimated with precision. Modelling programs provide an easy-to-use interface to develop and test multi-compartmental models against experimental models²⁵⁷.

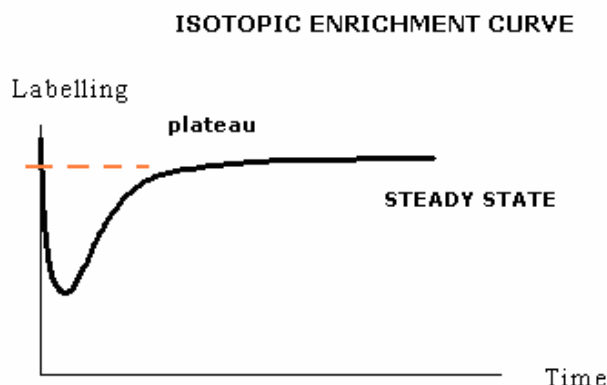
2.3.3.1 Isotopic enrichment determinations

Isotopic enrichment is the percentage of stable isotope measured in a sample above natural abundance over a specific time period. The time-course of stable isotopic enrichment can be assimilated into the time course of tracer to tracee ratio. Isotopic steady state implies the enrichment of the substance pool being constant over time. Several repeated samples are taken until the plateau is reached (Figure 9) and, subsequently, extensive processing of the blood samples is required to isolate each labelled protein before isotopic enrichment can be determined³²⁵.

The approaches available to assess *in vivo* lipoprotein metabolism from tracer data are based on measurements made at isotopic enrichment plateau and use of formulae given in terms of tracer to tracee ratios. The primed constant infusion approach is used to reach an early plateau so that the appearance of the introduced tracer into an accessible pool can be monitored. Assuming that the kinetics of the tracee and the tracer are identical enables the calculation to be made of specific tracee parameters such as production rate and catabolic rate^{333, 334}.

Frequently, an intracellular amino acid precursor pool in the liver, rather than a plasma pool itself, is taken as being the immediate precursor pool to protein synthesis²⁶⁷. Tracers are given until steady state is achieved, such that amino acid tracers enrich the amino acid tracee precursor pool (Figure 9)³³⁵. Enrichment at the end of the infusion period depends on the turnover rate of the protein-of-interest and any change in enrichment over time occurs as a consequence of dilution with unlabelled tracee³⁰⁴. For example, in the case of proteins with a high turnover rate, only a few hours of tracer infusion are required to reach the enrichment plateau^{290, 329}.

Figure 9. Tracer enrichment curve ³⁰⁴.



Isolated labelled proteins are analysed for tracer enrichment using gas chromatography-mass spectrometry (GC-MS) ^{290, 313, 329}. GC-MS is the most rapid, sensitive (from femtoM to nanoM sample size), specific and precise general analytical method available. Potentially, it can be applied to all biochemical compounds of interest (limited only by the need to derivatise or to modify molecules under study to make them volatile for detection). The specificity of this method is achieved by the combination of the resolving power of capillary GC separation and the resolving ability of MS to detect ions with very small mass differences. Usually, in kinetic studies, the use of multiple stable isotope labelled tracers in combination with GC-MS allows highly accurate measurements of minimal amounts of studied substance ^{262, 290, 291, 313, 329}.

2.3.3.2 Multi-compartmental analysis

The method of data analysis used depends on the data obtained. Multi-compartmental analysis is the most appropriate approach to analyse lipoprotein kinetics ^{257, 309, 318, 319}.

Multi-compartmental modelling approaches the natural system using models, the structures of which are developed from relationships between experimental data and natural metabolic systems to be modelled. Structures of models consist of compartments and their interconnections, and which represent the hypothesis on how the system functions under a given set of circumstances. Compartments reflect a specific physiological entity; either different metabolic sites or different events occurring at the same location. Thus, multi-compartmental modelling in steady state has a physiological basis but also reflects how the system works (Figure 10) ^{284, 287, 290, 317, 319, 322, 325, 331, 334}. The movement of material between compartments

reflect transport from one location to another and biochemical conversions (e.g. appearance rate of the substance into a compartment and its disappearance rate)³⁰⁴. Figure 10A shows the specific apo B-100 multi-compartmental model used in this project.

When using multi-compartmental modelling, enrichment curves are better fitted by multi-exponential functions. Usually, function number is directly related to compartment number in the model with individual equations describing the change in enrichment in each compartment^{304, 334}. As such, these functions describe the flux of material between compartments.

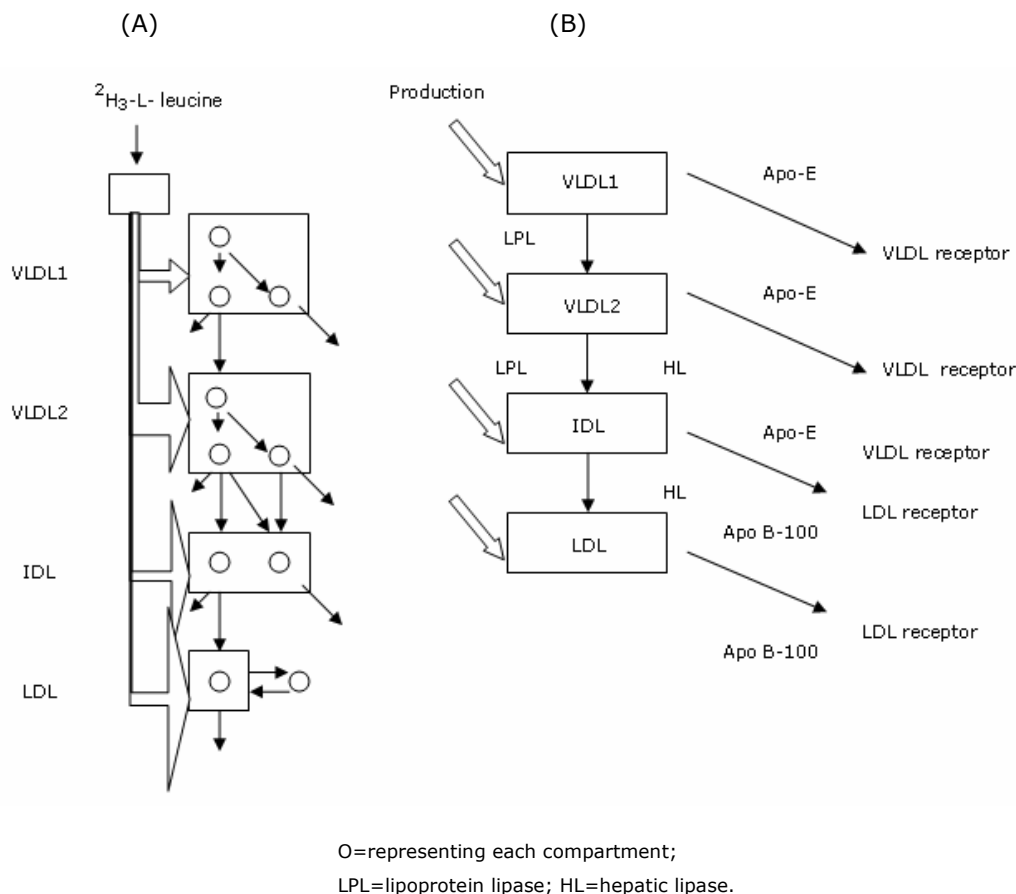
Also, hypotheses describing the system's models have to be tested. Validation of a model involves: numerical and statistical evaluation of the accuracy of calculated parameters, estimations and assessment of how well the model corresponds to known physiological characteristics of tracee. The identification of proper models structure requires knowledge of how the different compartments interact and comparison of model-calculated results with observed results. Thus, validation may be viewed as an effort to support a model or conclusions through alternative, independent approaches. Confidence in a model increases when results from alternative methods remain consistent with it^{304, 331}.

Several multi-compartmental models have been published and applied to study lipoprotein metabolism. The group of Packard et al had already described an apo B-100 multi-compartmental model (Demant, Packard et al, 1996)²⁶⁴. For the present project, they developed a new apoA-I and A-II model (Packard and Caslake, 2006). Both models are fully described in the Methodology section and, especially, in the Results section.

Multi-compartmental model for apo B-100 metabolism structure includes a site for ²H₃-L-leucine entrance to the system via plasma and, from which, tracer is distributed to body protein pools, and an intracellular compartment. This intracellular compartment acts as the precursor pool for liver apo B-100 synthesis. After a delay of 0.5h related to apo B-100 synthesis, tracer appears through the delipidation cascade in VLDL₁, VLDL₂, IDL and LDL compartments. The model also considers direct liver synthesis of apo B-100 appearing in these lipoprotein fractions and in remnants formation (Figure 10)^{267, 284}.

Multi-compartmental model for apoA-I and A-II metabolism is simpler and includes a site for ²H₃-L-leucine entrance to the system via plasma and, from which, tracer is distributed to body protein pools, and an intracellular compartment²⁶⁴. This intracellular compartment acts as the precursor pool for apoA-I and A-II synthesis. After a delay of 0.5h related to apoA-I and A-II synthesis, tracer appears in HDL compartment for direct synthesis. Without specifying to which study design the model is applied, apoA-I production can be distinguished as being from the liver or of intestinal origin.

Figure 10. Model structure design: multi-compartmental model for apo B-100-rich lipoproteins metabolism used for analysis of stable isotope data (A) and diagram showing its physiological basis (B) ²⁸⁴.



2.3.3.3 Data acquisition: simulation, analysis and modelling SAAM II program

Simulation, Analysis and Modelling Software II or SAAM II (SAAM Institute, Seattle, WA, US) is a program that has been used frequently to aid in the design and analysis of tracer data using multi-compartmental models ³³⁶.

Modelling is a simplified representation of the biochemical world. Starting from mathematical models, the application of computer simulators enables the prediction, or estimation, of kinetic parameters. Computer tools such as the SAAM II have been developed to aid in kinetic studies. The software is used to:

- * simulate the model and the experimental protocols.
- * fit the model to the observed tracer data by a weighted least squares approach to find the best fit.

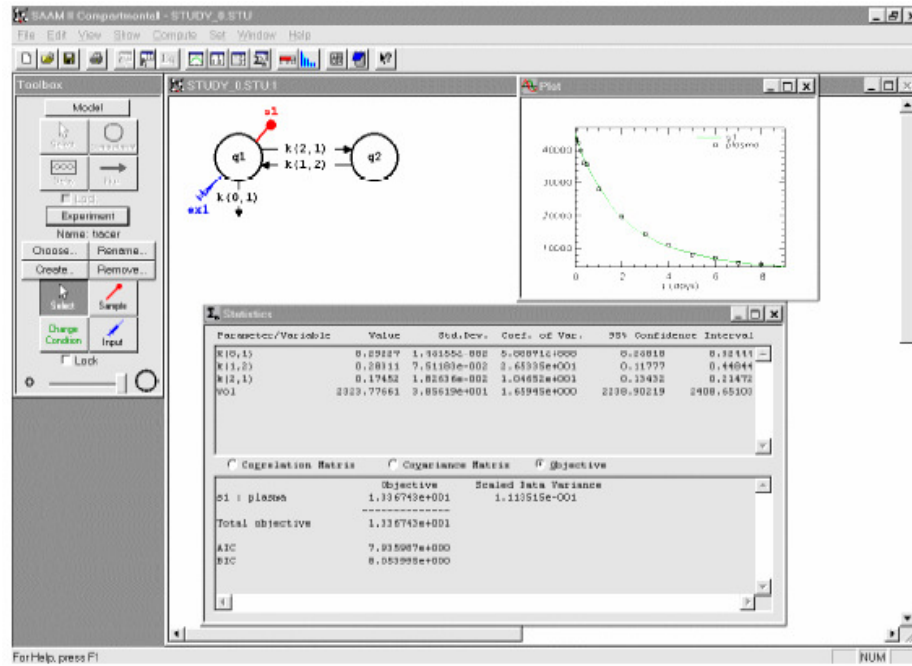
- * provide kinetic parameters given the input data of tracer enrichment curves.
- * estimate confidence limits for parameters.
- * display results in graphic and character modes.

Briefly, the SAAM II program conducts the following steps. Once the multi-compartmental model is constructed, the SAAM II automatically generates the system of multi-exponential equations from model structure. Additional user-defined equations or limitations can be incorporated into the model. In this way, quantitative or predictive information on the dynamics of the system can be obtained ^{290, 331, 336-338}. The next process involves specifying the experiment that is performed. This includes specifying the sites and protocol of tracer administration, compartments that are sampled or associated with experimental data. Then, using model structure and experimental data, model kinetic parameters are estimated by adjusting parameter values in an "optimisation" process. This process fits values of the adjustable parameters to obtain a "best fit" between model-calculated values and experimental data. A linear regression analysis, the weighted least squares approach, is used to fit the data ²⁶¹. Fitting provides the best estimates of calculated kinetic parameters, together with a measure of their precision. From these data, the following metabolic parameters can be estimated:

- * fractional catabolic rate (FCR) (mg/day); the fraction of tracee irreversibly removed from a compartment per unit of time.
- * pool size, the mass of tracee (mg) in a specific compartment.
- * secretion or production rate (PR) (mg/day); the rate at which the tracee enters the pool. PR can be expressed corrected for body weight (mg/kg/day).
- * conversion or transfer rate (TR) (mg/day); the fraction of tracee transferred from one compartment to another.

Finally, the SAAM II displays all these results and model diagrams in graphic as well as character mode. This enables all designed and analysed lipoprotein tracer kinetic studies to be easily visualised (Figure 11).

Figure 11. SAAM II program main window ³³⁶.



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JUSTIFICATION

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Justification

The mechanisms underlying the protective effects of HDL are of considerable interest: low plasma concentrations of HDLC and the major HDL apo, apoA-I, are independent risk factors for CVD. ApoA-I kinetic studies with stable isotopes can be used to study HDL metabolism *in vivo* in humans.

Evidence suggests high-MUFA diets increase, or maintain, HDLC plasma concentrations. Little is known about the basic kinetic mechanisms by which MUFAs increase, or preserve, HDLC concentrations. This is of special interest when comparing dietary recommendations provided by scientific societies for the prevention and treatment of CVD. Hence, to evaluate the role of high-MUFA diets in HDL metabolism regulation has a special interest. The Mediterranean diet has a high MUFA content derived from virgin olive oil, but also the minor components of the oil (e.g. polyphenols and vitamin E) are of interest because of their antioxidant properties. Kinetic studies make possible a more quantitative approach in the study of the protective effects of HDL against CVD since HDL plasma concentration increase can be the result of an increase in production rate or a decrease in catabolic rate, or both. Specifically, the majority of published kinetic studies suggest that production rate is the main determinant of HDLC concentrations in the case of dietary studies. The effect of MUFAs on kinetic parameters are of critical importance.

To our knowledge, no kinetic studies have focused on the effects on HDL particles of a high-MUFA Mediterranean diet to-date. In the present study, we compared the effects of a Mediterranean diet and a low-fat STEP II diet (prescribed by the AHA for individuals for high CVD risk) in middle-aged, moderate hypercholesterolaemic volunteers in whom a dietary recommendation is a key option.

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HYPOTHESIS

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Hypothesis

A Mediterranean diet rich in MUFAs from virgin olive oil, compared to a STEP II diet, increases, or preserves, HDLC concentrations due to an increase in apoA-I production, and not to a decrease in apoA-I catabolism.

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OBJECTIVES

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Objectives

1. General objective

To implement the necessary methodology for the *in vivo* measurement of the kinetic parameters of apo B-100 and, especially, of apoA-I and A-II in volunteers using stable isotope-labelled protein kinetic studies.

2. Specific objectives

1. To analyse the overall effects of a Mediterranean diet rich in MUFAs from virgin olive oil, and a STEP II diet on anthropometric parameters, plasma concentrations of lipids, lipoproteins and apos and, as well, on the composition of each lipoprotein fraction.
2. To apply the implemented methodology to compare the effects of both diets on HDL kinetics as reflected in the rates of apoA-I and A-II production and catabolism.
3. To apply the implemented methodology to compare the effects of both diets on VLDL₁, VLDL₂, IDL and LDL kinetics as reflected in the rates of apo B-100 production and catabolism.
4. To assess the effects of both diets on other CVD risk factors such as inflammation and oxidation markers.

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METHODOLOGY

UNIVERSITAT ROVIRA I VIRGILI
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1. Methodology of the general objective: implementation of the methodology of lipoprotein kinetic studies using stable isotopes

1.1 Description

Towards the end of the 1980's, our Research Unit was the first to introduce into Spain the study of lipoprotein kinetics to study human metabolic pathologies using radioactive isotopes ³³⁹⁻³⁴². Having at our disposal almost all the required infrastructure, our Research Unit wished to carry our investigations a step forward by implementing the methodology for the study of lipoprotein kinetics using stable isotopes. The methodology is quite complex and arduous and requires a high level of technical expertise, both when analysing the blood samples as well as in the interpretation of the kinetic data. These technical difficulties probably explain why there are not more laboratories involved in the quantification of lipoprotein metabolic pathways. We relied on the technical support and collaboration of Dr. Caslake and Professor Packard (Vascular Biochemistry Section, Division of Cardiovascular & Medical Sciences, Royal Infirmary, Glasgow, Scotland) in setting-up our system of laboratory analyses and the computational interpretation of the data generated. The Glasgow group has extensive experience in the field of apo B-100-rich lipoprotein kinetic studies and have internationally-recognised prestige.

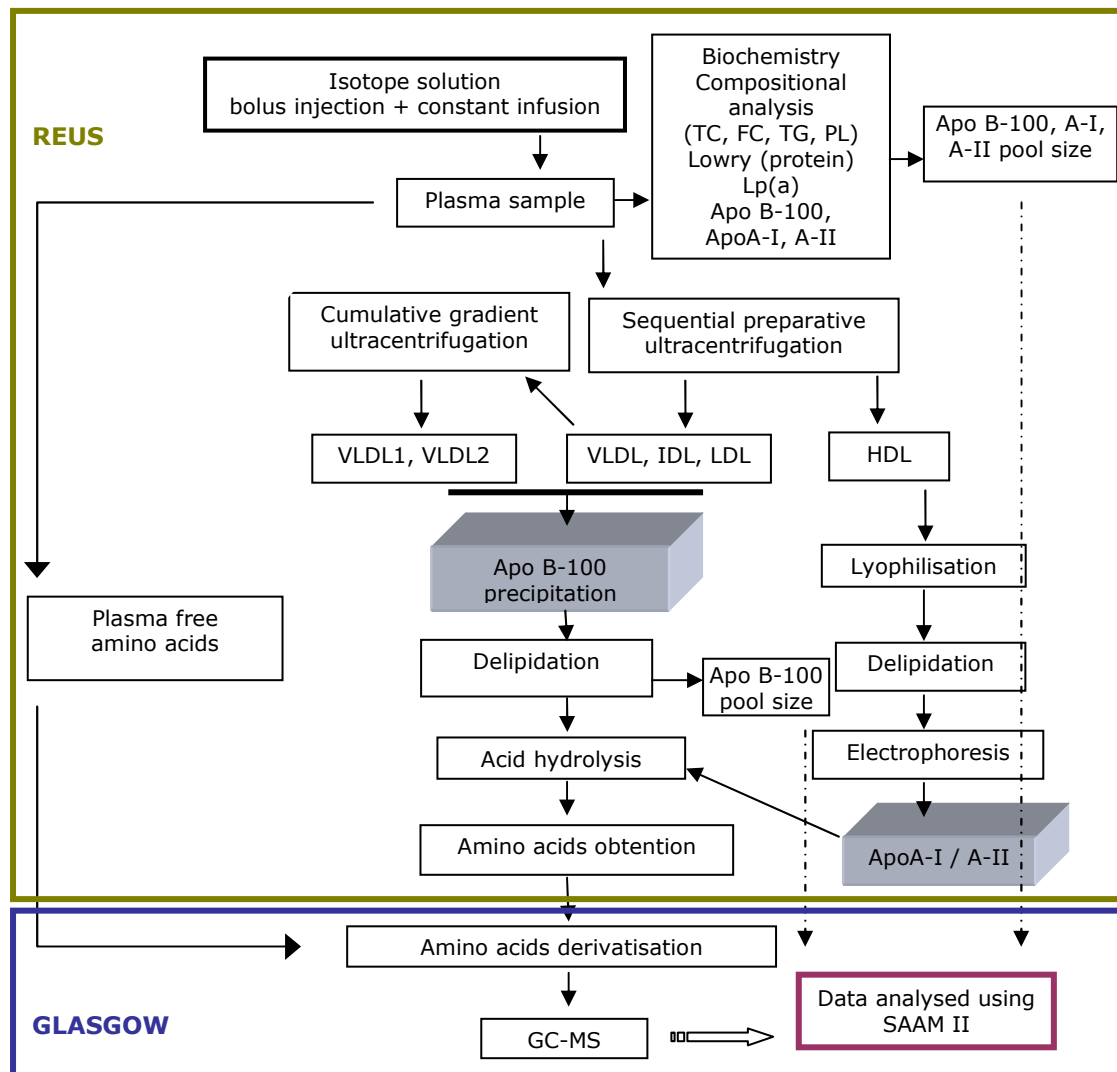
Once the methodology for the measurement of apo B-100 kinetic studies had been acquired following a 6-month stay in Glasgow, the system was set-up in our Unit in Reus and was applied in the measurement of apoA-I and A-II kinetics. Sample analyses on a GC-MS and acquisition of kinetic data were then interpreted using the SAAM II program in collaboration with the Glasgow Unit.

1.1.1 A brief outline of lipoprotein kinetics methodology

The methodology can be summarised briefly as (Figure 12, Annexe I):

- * obtention of lipoprotein fractions: VLDL₁, VLDL₂, IDL, LDL and HDL.
- * obtention of apo B-100 from VLDL₁, VLDL₂, IDL and LDL.
- * obtention of apoA-I and A-II from HDL.
- * apo B-100, AI and A-II acid hydrolysis to obtain their constituent amino acids.
- * obtention of plasma free amino acids.
- * amino acid derivatisation and the analysis using GC-MS.
- * obtention of kinetic data using the SAAM II program.

Figure 12. Flow diagram to illustrate lipoprotein kinetics methodology.



TC=total cholesterol; FC=free cholesterol; PL=phospholipids;
 GC-MS=gas chromatography-mass spectography.

1.1.2 Fine-tuning of the new methodologies: standardised work processes and laboratory accreditation

There were several new methodologies, laboratory techniques and major instruments used during the implementation of lipoprotein kinetic studies, and which form the basis of the present thesis. Hence, new standardised work processes (SWP) needed to be written. The purpose of implementing these SWP was to achieve International Standardisation Organisation (ISO) accreditation for our laboratory, as well as to improve local working skills ^{343, 344}.

The ISO is the world's leading monitor of standards. It consists of a network of standardisation institutes from 156 countries. It develops technical standards (e.g. ISO 9000, focused on "quality management") that are a worldwide reference.

SWP are defined by the Spanish government's *Instituto Nacional de Seguridad e Higiene del Ministerio de Trabajo y Asuntos Sociales* as: "written documents that describe the specific sequence of operations and methodologies that need to be applied to a particular objective. SWP indicate a unique manner within which the operations should be conducted each time they are performed in the laboratory. They are documents that complement the Quality Manual the aim of which is to establish who, how and when a planned activity is performed in a certain place". SWP were written for 16 new techniques and major equipment used in lipoprotein kinetic studies (see Annexes on CD). These include:

- * general kinetic study protocol.
- * new instrumentation used: swing-out rotor, freeze-dryer, vacuum centrifugal evaporator and vacuum manifold.
- * liquid density calculation: refractometers and picnometers.
- * cumulative gradient ultracentrifugation.
- * apo B-100 isolation by precipitation.
- * apo B-100 delipidation.
- * desalting HDL.
- * HDL lyophilisation.
- * HDL delipidation.
- * apoA-I and A-II isolation.
- * obtaining free amino acids from plasma.
- * protein acid hydrolysis.
- * amino acid purification.
- * protein determination using the method of Lowry.

The rest of the SWP were already present in our Unit and in Glasgow.

1.2 Methodological procedures for sample analysis

1.2.1 Obtaining different lipoprotein fractions

The different lipoprotein fractions VLDL₁, VLDL₂, IDL, LDL and HDL were obtained from plasma samples. Isolation was by ultracentrifugation ³⁴⁵⁻³⁵⁰.

1.2.1.1 Obtaining VLDL, IDL, LDL and HDL lipoprotein fractions

Lipoprotein isolation (VLDL, IDL, LDL, HDL) from plasma was performed using sequential preparative ultracentrifugation.

Sequential preparative (or flotation) ultracentrifugation is one of the techniques most used to separate lipoproteins. The technique exploits the lower hydration densities of lipoproteins compared with other plasma proteins. The separated lipoprotein fraction concentrates as a layer on the surface of the ultracentrifuge tubes containing solutions at the background hydrated density of the desired lipoprotein, while the remaining components of plasma remain at the bottom of the tube. The method depends only on the density of particles to be separated; the particle density being a measure of the mass per unit volume ^{348, 349}.

In our Research Unit, separation of the different lipoprotein fractions from plasma samples was performed using a Kontron TFT 45.6 fixed angle rotor and a Centricon 75 ultracentrifuge (Kontron Instruments, Ltd). The lipoprotein fractions isolated were: VLDL ($d < 1.006$ g/ml), IDL ($d = 1.006 - 1.019$ g/ml), LDL ($d = 1.019 - 1.063$ g/ml) and HDL ($d = 1.063 - 1.21$ g/ml). For specific methodological conditions see Tables 10, 11 and Annexes II, III.

One of the advantages of this technique is that a great number of different samples (up to 40) can be processed simultaneously using a high-capacity rotor.

The technique has some disadvantages such as, for example, with large numbers of lipoprotein samples there could be errors of misidentification. Also, the ultracentrifugation can cause apo components to become dislodged from the parent lipoprotein. There are also lipoprotein fraction losses in the recovery process. Peroxidation of lipid components can also happen if times of ultracentrifugation are protracted, but this can be overcome by the use of antioxidant or chelating agents (e.g. butylated hydroxyanisole, BHT or ethylenediaminetetraacetic acid, EDTA).

There are other aspects to take into account when using this technique since they can be limiting factors in obtaining correct separation of lipoprotein fractions. These factors include the correct adjustment of background density solutions and appropriate centrifugation times and speeds.

1.2.1.2 Obtaining VLDL₁ and VLDL₂ lipoprotein fractions

Isolation of VLDL₁ and VLDL₂ lipoprotein fractions from total VLDL was performed using cumulative gradient ultracentrifugation.

The cumulative gradient ultracentrifugation technique separates different lipoprotein fractions based on their densities and sedimentation, or Svedberg coefficients (S_f). Sedimentation velocity is a measure of the rate at which a particle moves in response to the centrifugal (gravitational) force applied to it. The sedimentation coefficient

depends on protein weight and structure. VLDL₁ (S_f 60-400), VLDL₂ (S_f 20-60), IDL (S_f 12-20) and LDL (S_f 0-12) can be isolated using this system^{346, 350}.

This technique uses consecutive density solutions in an ascending density gradient (layered from the heaviest to the lightest). The different solutions are prepared using different proportions of NaBr and NaCl. A pendular (or swing-out) rotor enables the formation of a continuous and curvilinear final density gradient. This continuous density gradient enables the separation of different lipoproteins along the length of the centrifuge tube so that the individual fractions can be successively and quantitatively aspirated from ultracentrifuge tube's surface³⁴⁹.

Isolation of VLDL₁ and VLDL₂ from total VLDL was performed using a Kontron TST 41.14 pendular (swing-out) rotor in a Centricon 75 (Kontron Instruments, Ltd) ultracentrifuge. For specific methodological conditions see Table 12 and Annexes II, III, IV.

When using this technique it is recommended to use zero deceleration (i.e. brake off) so as to minimise the disruption of lipoprotein particles that have been separated. Temperature is also a key aspect. When the particles have high TG levels, an aggregation/condensation of the lipoprotein can form on the surface at low temperatures.

A disadvantage of this technique is the low number of samples that can be processed at any one time (n=6).

Table 10.

Sequential preparative ultracentrifugation (Annexe III) ^{348, 349}

Obtaining VLDL

- add 2 ml of plasma and overlayer 3 ml of density solution 1.006 g/ml in an ultracentrifuge tube (Beckman, Inc)
- close and tighten tube caps and place in a fixed-angle rotor
- ultracentrifuge at 37,000, 16h, 10°C
- collect VLDL from the surface layer by aspiration up to the level of a 2 ml volumetric flask

Obtaining IDL

- add to the remaining 3 ml of the tube 1 ml of density solution 1.071 g/ml and 1 ml of density solution 1.006 g/ml; mix
- close and tighten tube caps and place in a fixed-angle rotor
- ultracentrifuge at 37,000 rpm, 20h, 10°C
- collect IDL from the surface layer by aspiration up to the level of a 2 ml volumetric flask

Obtaining LDL

- add to the remaining 3 ml of the tube 1 ml of density solution 1.019 g/ml and 1 ml of density solution 1.239 g/ml; mix
- close and tighten tube caps and place in a fixed rotor
- ultracentrifuge at 37,000 rpm, 20h, 10°C
- collect LDL from the surface layer by aspiration up to the level of a 2 ml volumetric flask

Obtaining HDL

- add to the remaining 3 ml of the tube 1 ml of density solution 1.063 g/ml and 1.1 gr NaBr; mix
- transfer the mix to a volumetric flask and make-up the level to 5 ml with density solution 1.063 g/ml
- close and tighten tube caps and place in a fixed-angle rotor
- ultracentrifuge at 37,000 rpm, 40h, 10°C
- collect HDL from the surface layer by aspiration up to the level of a 2 ml volumetric flask

NOTE: change damaged tubes to avoid breakage and loss of sample

Table 11.

Density solutions used in sequential preparative ultracentrifugation (Annexe II)

- Density solution 1.006 g/ml: 22.8 g NaCl, 0.2 g EDTANa₂, 2 ml 1N NaOH, 100 mg chloramphenicol, 1 g sodium azide, 2 l distilled water (pH 7.4)
- Density solution 1.239 g/ml: 328 g NaBr, 1 l density solution 1.006 g/ml
- Density solution 1.071 g/ml: mix solution 1.239 g/ml and solution 1.006 g/ml in the proportion of 12:31
- Density solution 1.019 g/ml: mix solution 1.006 g/ml and solution 1.071 g/ml in the proportion of 4:1
- Density solution 1.063 g/ml: mix solution 1.019 g/ml and solution 1.239 g/ml in the proportion of 4:1

NOTE: adjust density with a picnometer or a refractometer

Table 12.

Cumulative gradient ultracentrifugation (Annexe III, IV) ^{346, 349, 350}

Preparing samples

- adjust density of samples (2 ml VLDL) adding 0.341 g NaCl
- overlayer add the different density solutions (beginning with the most dense) and sample in ultracentrifuge tubes (Beckman, Inc)
- close and tighten tubes' cap and place in a swing-out rotor

Obtaining VLDL₁

- ultracentrifuge at 39,000 rpm, 1.38h , 23°C and brake off
- collect 1 ml VLDL₁ from the surface layer by aspiration up to the level of a 1 ml volumetric flask

Obtaining VLDL₂

- overlayer 1 ml of density solution 1.0588 g/ml
- close and tighten tubes' cap and place in a swing-out rotor
- ultracentrifuge at the required conditions (see Annexe)
- collect 0.5 ml VLDL₂ from the surface layer by aspiration up to the level of a 0.5 ml volumetric flask

NOTE: change damaged tubes to avoid breakage and loss of sample

1.2.2 Obtaining apo B-100

Apo B-100 was isolated using isopropanol precipitation followed by ethanol:ether delipidation.

1.2.2.1 Apo B-100 precipitation

Apo B-100 from VLDL₁, VLDL₂, IDL and LDL was isolated using the isopropanol precipitation method of Egusa (Table 13, Annexe V).

Apo B-100 has a high molecular weight and insolubility in aqueous medium, which makes it a difficult molecule to manipulate. Isopropanol precipitation technique of Egusa is effective in isolating apo B-100³⁵¹. Isopropanol selectively precipitates apo B-100, it is relatively cheap, can be highly purified, does not cause interference in colorimetric determinations and can solubilise apo B-100 very well. The method (and others) were developed because of the disadvantage associated with the original tetramethylurea (TMU) precipitation technique^{352, 353}. TMU had high costs, was difficult to obtain in a purified form and had considerable interference with colorimetric determination of proteins (e.g. the Lowry method).

To improve apo B-100 precipitation, the precipitation is conducted at low temperature (4°C) which reduces the strong ionic forces of interaction of apo B-100 with lipoprotein particles and helps to separate apo B-100.

Table 13.

Apo B-100 isopropanol precipitation using the method of Egusa (Annexe V)³⁵¹

- add isopropanol (room temperature) in a 1:1 proportion to lipoprotein samples
- close and tighten tube caps
- vortex mix
- let the mixture rest overnight at 4°C
- centrifuge at 3,000 rpm, 30 minutes, 4°C
- remove the isopropanol supernatant without disturbing the precipitated apo B-100

1.2.2.2 Apo B-100 delipidation

VLDL₁, VLDL₂, IDL and LDL apo B-100 delipidation was performed with the classical method of delipidation using organics solvents (ethanol:ether 3:1 mix), and repeated until completed apo B-100 delipidation was accomplished (the residue taking-on a shade of white). The result apo B-100 was sufficiently "clean" for later analysis (Table 14, Annexe V) ³⁵⁴.

The classic delipidation method is based on the use of organic solvents (e.g. ethanol, ether) ³⁵⁵⁻³⁵⁷. Delipidation using organic solvents is a better approach to obtain apo B-100. It is easier and faster and removes all lipids from apo B-100. There are more complex techniques such as solid phase extraction (SPE) using solid phase chromatography or techniques based on surfactants. However, delipidation using surfactants such as sodium dodecyle sulphate (SDS), sodium deoxycholate, or Triton® X-100 is an arduous technique and not very suitable. The disadvantages are that apo B-100-surfactant aggregates are formed, high volumes of reagents are needed, delipidation is not always complete, and the biological characteristics of the apos can be changed.

Use of ethanol:ether at low temperatures (4°C) increases the efficiency of delipidation. Scattering the apo B-100 precipitate also increases the efficiency of delipidation because it increases the contact of the apo B-100 with the solvent. Complete delipidation enables easier subsequent solubilisation of the precipitated apo B-100, and a better electrophoretic analyses. Eliminating the isopropanol supernatant has to be performed carefully so as to avoid loss of precipitated apo B-100.

Some disadvantages of this techniques are due to conformational changes of the proteins, or difficulties in subsequent protein recovery.

All these aspects are important when analysing apo B-100, since this apo is difficult to resolubilise ³⁴⁹.

Table 14.

Apo B-100 ethanol: ether delipidation (Annexe V) ^{349, 355}

- add 3 ml of ethanol:ether (3:1) at 4°C to precipitated apo B-100
- close and tighten tube caps; vortex mix; leave overnight at 4°C
- centrifuge at 3,000 rpm, 30 minutes, 4°C
- remove supernatant gently without disturbing the precipitated apo B-100
- repeat this process as many times as needed (precipitated apo B-100 should be completely delipidated, and taking-on a shade of white)
- add 3 ml of ether (4°C) to the precipitated apo B-100
- close and tighten tube caps; vortex mix; leave overnight at 4°C
- centrifuge at 3,000 rpm, 30 minutes, 4°C
- remove supernatant without disturbing the precipitated apo B-100
- evaporate-off any remaining solvent into air

1.2.3 Obtaining apoA-I and A-II

ApoA-I and A-II, the major HDL apolipoproteins, were isolated using PAGE (polyacrylamide gel electrophoresis) technique. Prior to this, the HDL were desalted using gel filtration chromatography, lyophilised using a freeze-dryer and delipidated using a chloroform:methanol mix. Total protein concentration was measured using the method of Bradford and a specific protein quantity was loaded on to the gels for electrophoresis.

1.2.3.1 Desalting HDL

HDL were obtained from plasma using sequential preparative ultracentrifugation, as previously described. HDL were desalted using gel filtration chromatography with PD-10 columns (Table 15, Annexe VI).

Gel filtration chromatography (or molecular exclusion gel chromatography) is a technique for the separation of compounds based on the molecular weights when passed through a stationary phase matrix which consists of porous beads with well-defined range of pore sizes. The pore size enables the selective exclusion of compounds when transported in a mobile phase solution. Proteins that are small enough can fit inside all pores and are said to be "totally included". Small proteins have access to mobile phase within the beads, as well as mobile phase between the beads, and have a delayed elution. Large proteins do not fit inside any pores and are "totally excluded". Large proteins have access only to mobile phase between beads and elute early. Proteins of intermediate size are "partially included" and elute intermediate between the large and the small proteins. This technique is also used to eliminate low molecular weight compounds (e.g. salts or detergents); the protein thus purified is used for subsequent structural and functional analyses³⁵⁸.

PD-10 columns (Amersham Pharmacia Biotech, Inc) are disposable desalting columns, prepacked with SephadexTMG-25 beads and equilibrated with distilled H₂O. These columns separate substance groups from high (>5000 daltons) to low (<1000 daltons) molecular weight by buffer exchange. PD-10 columns are easy and quick to use. They are used to eliminate salt solutions used in the ultracentrifugation process to isolate HDL particles. Desalting HDL is an important aspect because residual salts can interfere with later protein processing, such as in HDL delipidation.

Table 15.

Desalting HDL with gel filtration chromatography (Annexe VI)³⁵⁸

- equilibrate PD-10 columns prepacked with Sephadex™G-25 beads (Amersham Pharmacia Biotech, Inc) with 25 ml distilled H₂O
- add 3 ml phosphate buffered saline (PBS)
- add 2 ml sample (HDL)
- add 1 ml PBS
- add 2 ml PBS to elute the sample (collect in a tube and store at 4°C)

1.2.3.2 HDL protein determination

After desalting HDL with PD-10 columns, calculation of HDL protein content was necessary for later apoA-I and A-II isolation using electrophoretic gels (Table 16). Thus, initially approximately 800 µg of total HDL protein were lyophilised and subsequently 100 µg loaded onto the gels.

The method of Bradford is a protein assay used in determining total protein concentration³⁵⁹⁻³⁶². The method is very fast and uses about the same amount of protein as the method of Lowry (the other more-generally used protein assay). The method of Bradford is recommended for general use, especially in assessing protein concentrations for gel electrophoresis. It is fairly accurate and quick, such that any samples that are out-of-range can be retested within minutes. The assay is relatively sensitive and measures proteins within the range of 5-200 µg.

The method of Bradford is based on the shift of maximum absorbance for an acidic solution of Coomassie Brilliant Blue G-250 from 465 to 595 nm when binding to proteins, and on the equilibrium between different forms of Coomassie Blue dye (Figure 13). Under strongly acid conditions, the dye is stable as a doubly-protonated or cationic red colour. However, upon binding to proteins, it is stable as an unprotonated or anionic blue colour.

The dye reagent reacts primarily with arginine residues, and less so with histidine, lysine, tyrosine, tryptophan and phenylalanine residues. Thus, assay response is protein concentration dependent and varies with amino acid composition of the protein. The assay is less accurate for basic or acidic proteins. Immunoglobulin gamma (IgG) is the preferred protein standard, although bovine serum albumin (BSA) is more-commonly used.

Many substances can cause interference with the method of Bradford, particularly detergents such as SDS, ethyl trimethyl ammonium bromide, Triton® X-100, cholate, CHAPS.

Figure 13. Equilibrium forms of Coomassie Brilliant Blue G-250 ³⁵⁹⁻³⁶².

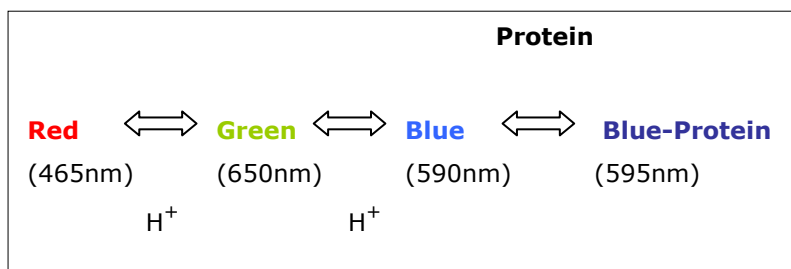


Table 16.

Protein determination using the method of Bradford ³⁵⁹⁻³⁶²

- add reactants with the sample volume in eppendorf tubes and mix by inversion
- measure absorbance at λ 595 nm (GeneQuant, Amersham Pharmacia Biotech)
- use a calibration curve to calculate samples concentrations ($\mu\text{g}/\mu\text{l}$) according to absorbance

Preparing calibration curve

- BSA protein standard solution \rightarrow 7.35 μg BSA in 10 μl volume
- use PBS as buffer
- Bradford reagent (Bio-Rad Laboratories, Inc)

Final prot concentration (μg)	BSA volume (μl)	PBS volume (μl)	Bradford reagent volumen (μl)
0		800	200
1.837	2.5	797.5	200
3.67	5	795	200
5.14	7	793	200
7.35	10	790	200
8.82	12	788	200

Preparing HDL samples

Sample volume (μl)	PBS volume (μl)	Bradford reagent volumen (μl)
2 (or less)	798	200

1.2.3.3 HDL lyophilisation

800 µg of total HDL protein samples were lyophilised after desalting HDL with gel filtration chromatography and measuring the protein content (Table 17, Annexe VII).

Lyophilisation or freeze-drying is a specific type of sublimation under vacuum. Lyophilisation is performed at low temperatures (<0°C) and low pressure (vacuum). Evaporation of water vapour directly from solid state (ice) is termed sublimation. Prior to lyophilisation process, samples are frozen (-70°C) at normal pressure and then freeze-dried at -29°C. The technique enables the separation of water from materials sensitive to higher temperatures. Lyophilisation is especially useful for biological samples because their biological, physiological and therapeutic properties remain unchanged at low temperatures (i.e. sample quality would be damaged using classical drying methods at higher temperatures) ^{349, 363}.

Lyophilisation simplifies and improves HDL delipidation. Lyophilised materials are highly soluble due to the porosity and large surface area and the final resolubilised sample volume can be reduced. This is an advantage because delipidation processes require large solvent volumes. It also increases the insolubility of proteins in the organic phase and weakens the non-polar lipid-protein interactions.

The only inconvenience of this technique is the protracted time needed to lyophilise the samples (about 24h or more). It is also important to avoid defrosting of samples during lyophilisation since this would involve loss of sample, or some water retention.

Table 17.

HDL lyophilisation (Annexe VII) ^{349, 363}

- estimate HDL volume corresponding to 800 µg total protein (estimate with the method of Bradford)
- transfer to tubes and close taps
- freeze HDL at -70° C
- open tubes and cover them with parafilm (make holes in the film cover to allow water evaporation)
- lyophilise samples (-29°C, vacuum) over 24-48h (Freeze-Dryer ALPHA 1-4, Martin Christ, GmbH)
- close and tighten tube caps and store at -70°C

1.2.3.4 HDL delipidation

After lyophilisation, HDL were delipidated using a chloroform:methanol 3:1 mix (Table 18, Annexe VIII).

HDL delipidation is similar to the one for the apo B-100-rich lipoprotein fractions, albeit with different solvents ^{349, 355}. When delipidating, it is important to take into account the different physico-chemical properties of lipoprotein fractions. HDL delipidation employs a chloroform:methanol 3:1 organic solvent mix.

Complete HDL delipidation facilitates the protein solubilisation and analysis.

Table 18.

HDL chloroform:methanol delipidation (Annexe VIII) ^{349, 355}

- add 2 ml chloroform:methanol (3:1) at 4°C to samples
- vortex mix and allow to stand for 1h at 4°C
- add 5 ml methanol at 4°C
- vortex mix and allow to stand for 30 minutes at 4°C
- centrifuge at 2,500 rpm, 10 minutes, 4°C
- remove supernatant without disturbing the precipitated proteins
- dry under N₂ (g)
- store at -70°C

1.2.3.5 ApoA-I and A-II isolation

ApoA-I and A-II were separated from the rest of the HDL apos using NuPAGE®Novex gels, a considerable improvement over handmade gels (Tables 19, 20, Annexe IX).

Electrophoretic techniques enable the separation of different proteins, identification of their subunits and evaluation of homogeneity. Proteins, when separated using polyacrylamide gel electrophoresis, move through the gel matrix pores in response to an applied electric current. Protein movement and separation depends on a combination of gel-pore size, the charge on the proteins and the molecular weight. The isolated proteins are visualised with specific staining techniques using dyes (e.g. Coomassie Blue or silver dye). It is a simple and rapid method to obtain the desired proteins ³⁵⁹.

The traditional electrophoretic method of Laemmli is based on discontinuous gels³⁶⁴. Discontinuous gels have a region used for compacting the protein or "stacking gel" and a protein separation region or "running gel". These gels are prepared under denaturing conditions with SDS^{364, 365}. Protein separation is based on SDS which is a denaturing detergent that binds proteins and confers a homogeneous negative charge. When needed, reducing agents (e.g. 2- β -mercaptoethanol, dithiotreitol) are added to reduce the protein disulfide bridges to obtain the subunits. When using SDS, proteins are separated with electrophoretic techniques with respect to the molecular weight, and not to the charge.

Several new variants have been developed from this general methodology. They include NuPAGE®Novex Bis-Tris electrophoretic gels (Invitrogen, Corp) used extensively in the present studies³⁶⁶.

NuPAGE®Novex system is simple, easy and rapid for the isolation and sensitive detection of proteins. The technique is based on precast mini gels which are discontinuous and have neutral pH. This improves the protein and gel matrix stability and allows better protein band resolution with high reproducibility. Another characteristic of the gels is that they do not contain SDS, although they can be used to separate protein under denaturing conditions. This characteristic allows addition of SDS into the sample if necessary. Post-separation visualisation of protein bands uses commercially available specific protein-staining reagents which are based on Coomassie Blue dye and which is a fast-staining procedure. Dyes bind to basic and hydrophobic amino acids and the resultant proteins develop blue banding patterns.

Reducing agents are preferably excluded during HDL sample manipulations in isolating apoA-II. Reduced apoA-II splits into 2 monomeric subunits, almost indistinguishable from apo-C's isoforms which have similar molecular weights. ApoA-II can be easily isolated as a dimer using NuPAGE®Novex gels. ApoA-I, as a monomer, can also be isolated using NuPAGE®Novex gels under non-reducing conditions. Both apos appear as clear gel bands that can be easily excised and processed.

Protein samples loaded on duplicate on gels enable sufficient sample to be detected later by GC-MS

Table 19.

ApoA-I and A-II isolation using NuPAGE®Novex gels (Annexe IX) ³⁶⁶

Preparing samples

- place delipidated samples on ice
- add 100 µl Sample Buffer and shake
- place samples in a water bath at 95°C, 5 minutes
- place samples on ice
- pulse centrifuge samples at 4°C
- recover samples dissolved in Sample Buffer and transfer to eppendorf tubes
- store at 4°C

Preparing Sample Buffer

SAMPLE BUFFER (8 ml total)		
0.5M Tris-HCl	1 ml →	62.5 mM Tris-HCl
Glycerol	800 µl →	10% Glycerol
10% SDS	1.6 ml →	2% SDS
Bromophenol Blue	light, swift tap with spatula	
Distilled H ₂ O	up to 8 ml	

NOTE: do NOT use reducing agent 2-β-mercaptoethanol

Table 20.

ApoA-I and A-II isolation using NuPAGE®Novex gels (Annexe IX) ³⁶⁶

Preparing polyacrylamide gels

- use 10% NuPAGE® Bis-Tris electrophoretic gels (Invitrogen, Corp)
- prepare gels and place them in an electrophoretic tank (Invitrogen, Corp)
- prepare SDS Running Buffer (Invitrogen, Corp) to fill the tank
- load 100 µg total protein in gels (estimate HDL volume corresponding to 100 µg total protein using the method of Bradford)
- load the same sample in duplicate on gels
- set electrophoretic running conditions of 80 V, 3.30h
- remove gels and stain for 1h or more at room temperature with GelCode Blue Stain Reagent (Pierce Biotechnology, Inc)
- destain gels with distilled H₂O until proteins bands are seen clearly

NOTE: use a molecular weight marker to identify proteins of interest (Bio-Rad Laboratories, Inc)

1.2.4 Acid hydrolysis of samples

Isolated VLDL₁, VLDL₂, IDL and LDL apo B-100 samples and HDL apoA-I and A-II samples were hydrolysed with hydrochloric acid to obtain their amino acids content (Tables 21, 22, Annexe X) ^{367, 368}.

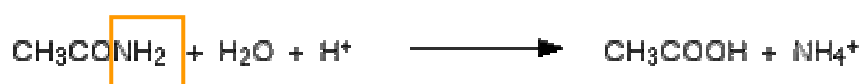
The first step to analyse amino acids is to isolate them from proteins following hydrolysis with hydrochloric acid (HCl). Protein peptide bonds are amide bonds. The carbon-nitrogen union of the amide group is ruptured during the hydrolysis with HCl and a mixture of positive-charged amino acids is obtained (Figure 14).

Duration of hydrolysis is normally fixed at 24h since longer hydrolysis times have the disadvantage of hydrolysis of amino acid side chains. When reacting with hydrogen ions, some amino acids can be adversely affected under these conditions. Asparagine and serine can be irreversibly modified to aspartic and glutamic acid, threonine and serine can be eliminated, and tryptophan may not be recoverable. It is also important that the samples are free from possible contaminants. This minimises interferences during hydrolysis which could cause undesired amino acid destruction or derivitisation processes. It also minimises interferences in subsequent amino acid analyses.

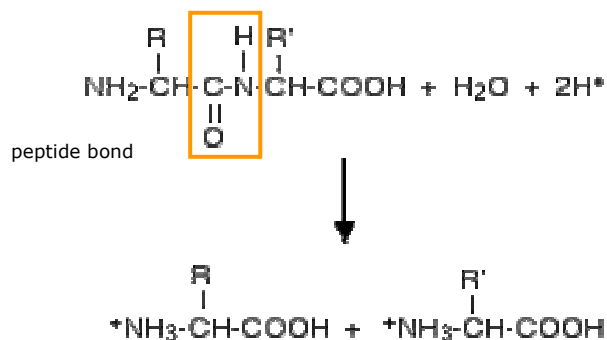
As explained later, the acid hydrolysis of apoA-I and apoA-II was followed by an amino acid purification process with ion exchange chromatography.

Figure 14. Proteins acid hydrolysis with HCl ³⁶⁷.

A) An amide acid hydrolysis



B) A peptide acid hydrolysis



1.2.4.1 Apo B-100 acid hydrolysis

Apo B-100 from VLDL₁, VLDL₂, IDL and LDL lipoprotein fractions was isolated with isopropanol precipitation and delipidated and the apo B-100 obtained was pure enough not to require further purification with ion exchange chromatography.

Apo B-100 samples were hydrolysed with HCl to obtain the constituent amino acids (Table 21, Annexe X).

Table 21.

Apo B-100 acid hydrolysis (Annexe X) ^{367, 368}

- add 1 ml 6N HCl to samples
- vortex mix and allow to stand for 24h in a heating block at 110°C

1.2.4.2 ApoA-I and A-II acid hydrolysis

ApoA-I and A-II from HDL fraction were isolated in electrophoretic gels as clean bands that were excised. Samples bands were hydrolysed with HCl to obtain the constituent amino acids (Table 22, Annexe X).

The amino acids so obtained were purified from polyacrylamide contaminants using ion exchange chromatography and this minimised interference in subsequent amino acid analysis (Table 23, Annexe XI).

Table 22.

ApoA-I and A-II acid hydrolysis (Annexe X) ^{367, 368}

- cut apoA-I and AII bands from the gels and place in tubes
- add 1 ml 6N HCl
- vortex mix and allow to stand for 24h in a heating block at 110°C
- place samples for 20 minutes at -20°C
- centrifuge at 3,000 rpm, 5 minutes, 4°C
- recover supernatant and store at 4°C until subsequent purification with ion exchange chromatography

1.2.4.3 Amino acids purification

After apoA-I and A-II acid hydrolysis, their amino acids were purified with ion exchange chromatography (Table 23, Annexes XI, XII).

Ion exchange chromatography separates molecules according to their charge and it is used to purify proteins, peptides and amino acids ^{359, 362, 368, 369}. The technique is based on interactions of charged groups of molecules with those of opposite charge immobilised on a stationary phase matrix. The net charge of a compound depends on the pH of the environment. When the net charge is zero, the pH is known as the isoelectric point (pI). Molecules have a negative net charge if the pH of the environmental is higher than the pI. The molecules interact with positive-charged groups, or anionic exchange groups. Molecules have a positive net charge if the pH of the environmental is lower than the pI. The molecules interact with negative charged groups, or cationic exchange groups. Molecules interacting with these stationary-phase groups are eluted using high concentration saline solutions, the pH of which is near to the pI of the molecule-of-interest which then separates from stationary phase, or "eluted". Solutions of reducing pH are used to elute molecules from anionic exchange groups. Solutions of increasing pH are used to elute molecules from cationic exchange groups (Figure 15).

Amino acids are purified using columns packed with cationic exchange resins such as AG®50W-X8 (BioRad Laboratories, Inc). The functional groups are sulfonic acids bound to a polymeric matrix of styrene divinylbenzene. Positively-charged amino acids (e.g. leucine) are separated with this type of exchange columns. Amino acids interacting with the matrix are eluted using ammonium hydroxide (NH₄OH) (Figure 15).

Figure 15. Cationic exchange resins ^{362, 369}.

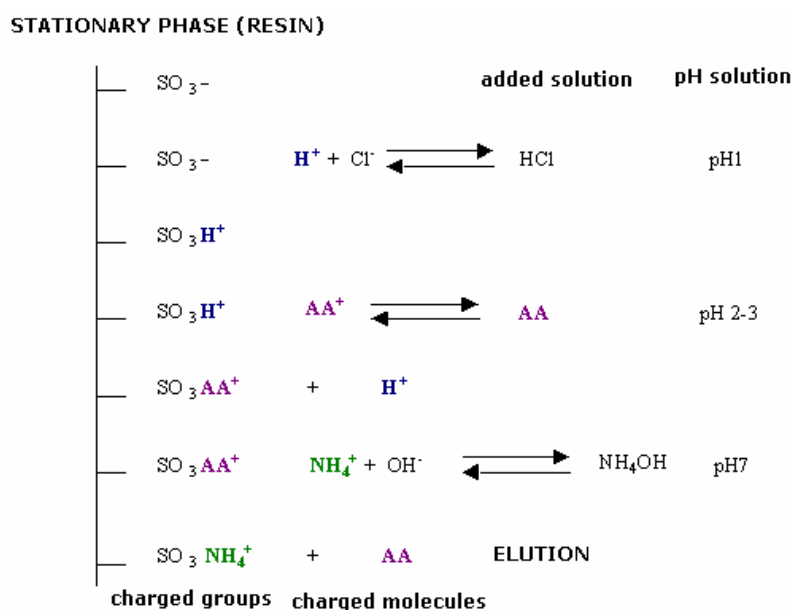


Table 23.

Amino acids purification using ion exchange chromatography (Annexes XI, XII) ^{359, 362, 368}

Preparing resin

- mix resin (AG®50W-X8, BioRad Laboratories, Inc) with distilled H₂O
- wash resin (filter until neutral pH)
- dehydrate resin to prepare columns (Vac-Elut™, Analytichem International, Inc)

Preparing columns

- place a filter in each column
- mix resin with distilled H₂O and shake
- fill columns with 2 ml resin
- add a second filter and allow to compact under gravity
- let distilled H₂O elute from columns
- add 2 ml 1N HCl (pH 1)
- wash columns with distilled H₂O until neutral pH
- fill columns with distilled H₂O to avoid resin dehydration
- cover columns and store at room temperature

Processing samples

- add hydrolysed samples to columns
- wash columns x3 with distilled H₂O
- place columns in a vacuum manifold (Vac-Elut™, Analytichem International, Inc)
- elute amino acids with 3 ml 4M NH₄OH and collect in tubes
- store at 4°C

1.2.5 Obtaining plasma free amino acids

Plasma free amino acids were obtained following plasma protein precipitation, also termed plasma method (Table 24, Annexes XI, XII, XIII)³⁶⁸. Plasma free amino acids needed a purification stage with ion exchange chromatography.

Molecular polarity is a key point when precipitating plasma free amino acids. Hydrogen and carbon atoms are very electronegative and can form hydrogen bonds between them (Figure 16). Hydrogen bonds between proteins and water molecules enable proteins to remain in suspension in aqueous solutions. Proteins precipitate when a compound causes the water molecules to separate from the protein polar groups. Plasma protein precipitation enables the plasma free amino acids to be separated in the supernatant. Precipitation techniques using acid compounds such as trichloroacetic acid (TCA) are easier and faster and, hence, more frequently used than organic solvents such as a hydrochloric acid-acetone mixture^{370, 371}.

Figure 16. Hydrogen bonds between water molecules^{72, 370}.

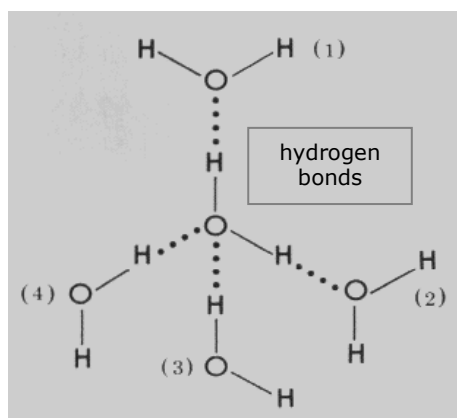


Table 24.

Plasma method (Annexes XI, XII, XIII) ^{368, 370, 371}

Deproteinization of samples

- mix 1 ml plasma with 1 ml 10% TCA
- vortex mix and allow to stand 15-30 minutes at room temperature
- set a centrifuge at 3,000 rpm, 30 minutes, 4°C
- collect amino acids supernatant

Purification of samples

- prepare ion exchange columns (Table 14)
- add amino acids supernatants to the columns
- wash columns x3 with distilled H₂O
- place columns in a vacuum manifold (Vac-Elut™, Analytichem International, Inc)
- elute amino acids with 3 ml 4M NH₄OH and collect in tubes
- store at 4°C

1.2.6 Vacuum centrifugal evaporation

To proceed to the next stage of sample processing, HCl from apo B-100 hydrolysed samples was subjected to vacuum centrifugal evaporation, as was NH₄OH from apoA-I, apoA-II and plasma amino acid purified samples (Table 25, Annexes XIV, XV) ³⁶⁸.

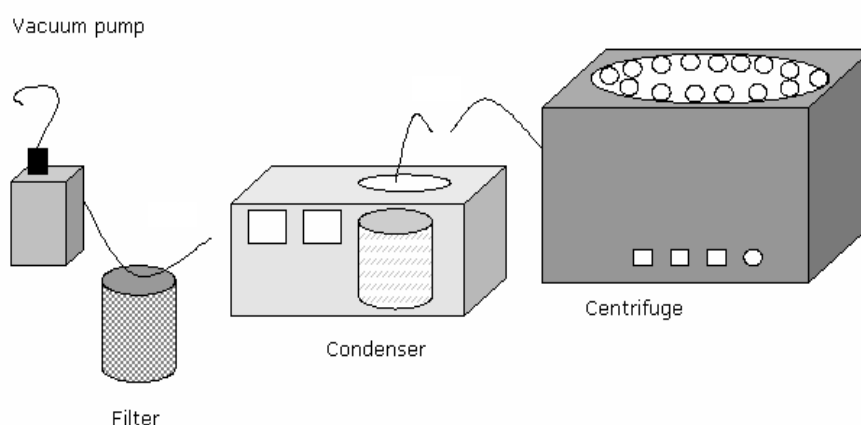
Vacuum centrifuges are a fast and efficient way to remove solvents from samples. The system includes a set of efficient vacuum pumps, very low temperature condensers, and acid or base filters to avoid corrosion in the system (Figure 17).

Table 25.

Vacuum centrifugal evaporation (Annexes XIV, XV) ^{368, 372}

- place sample tubes in a vacuum centrifugal evaporator (Jouan, Thermo Electron Corp)
- evaporate until a final sample volume of 1-0.5 ml
- transfer contents to vials (Chromacol, Supelco-Sigma Aldrich Inc)
- continue evaporation until a precipitated is formed
- cover and store samples at room temperature until later GC-MS analysis

Figure 17. Diagram of a vacuum centrifugal evaporator ³⁷².



1.2.7 Amino acid derivatisation

When solvent evaporation was completed, amino acid samples were stored until derivatisation for GC-MS analysis.

The amino acid samples are derivatised prior to GC-MS analysis; a system which is one of the most sensitive methods used in the detection of ²H₃-L-leucine isotopic enrichment. Amino acid samples react with N-(t-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) and acetonitrile to generate tertiary butyldimethylsilyl (t-BDMS) derivatives. The derivatives are easily detected in a GC-MS since MTBSTFA produces more volatile derivatives which improves their separation in a GC (Table 26, Figure 18) ^{304, 373-376}.

Table 26.

Amino acids derivatisation ^{304, 373-376}

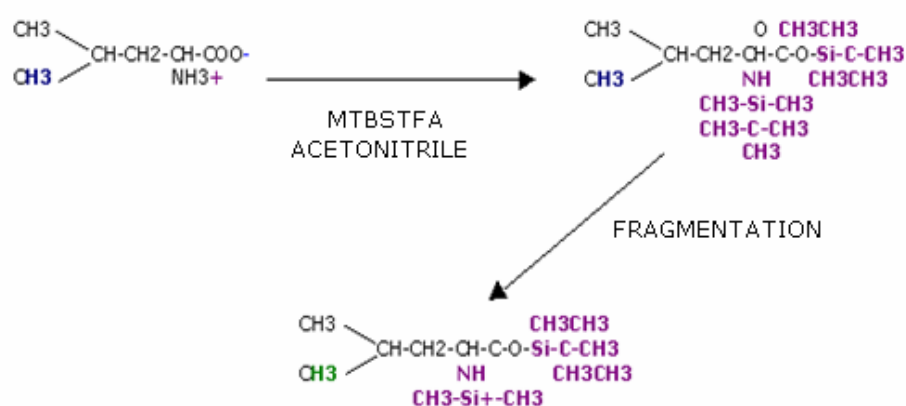
- choose 2 samples randomly from each lipoprotein fraction for analysis
- derivatisation conditions tested with these samples (analyse samples in a GC-MS to confirm)
- add to samples in vials (Chromacol, Supelco-Sigma Inc) the chosen reaction volume (in a proportion 1:1, depending of sample volume)

Examples of reactants volumes (less reactants volumes for less amino acids enriched samples to avoid excessive dilution which would make GC-MS detection more difficult)

Sample	Volume MTBSTFA (μ l)	Volume Acetonitrile (μ l)
Apo B-100 VLDL1	10	10
Apo B-100 VLDL2	10	10
Apo B-100 IDL	15	15
Apo B-100 LDL	150	150
ApoA-I HDL	10	10
ApoA-II HDL	10	10
Plasma	20	20

- seal vials
- incubate 1h at 70°C
- vortex mix to resuspend the samples
- place samples for their analysis in a GC-MS and check signal (appearance and quality)
- if quality is acceptable, proceed to process all sample derivatisations using the same conditions

Figure 18. Amino acid $^2\text{H}_3$ -L-leucine derivatisation and its t-MTBSTFA derivative ³⁷⁴⁻³⁷⁶.



1.2.8 Derivatized amino acid analysis in a GC-MS

Derivatized amino acid samples were analysed in a GC-MS to assess isotopic enrichment.

1.2.8.1 GC-MS isotopic enrichment determination: technical aspects

1.2.8.1.1 GC-MS

GC is a separation technique while MS is a detection technique. Combining both techniques makes GC-MS a potent quantitative tool that enables the detection of picogram (10^{-12}) to femtogram (10^{-21}) amounts of sample ³⁷⁷⁻³⁷⁹. Using GC-MS, sample ions in a gaseous phase are separated and detected depending on their mass-to-charge ratio (m/z ratio) which is the product of mass number of an ion divided by its charge number.

1.2.8.1.2 Mass spectra

Mass spectra represent data obtained from MS and are presented as intensity peaks. The peaks are characterised by their height (ionic abundance or number of ions of the sample) and width (m/z detected interval) (Figure 19) ³⁷⁷⁻³⁷⁹. Mass spectra provide information on structure and molecular weight of the molecules under study. For example, leucine has a $m/z=131$ (the sum of each element mass number) and

commercially-available leucine (labelled with 3 deuteriums) has a $m/z=134$ (Figure 20).

Figure 19. A GC-MS mass spectra ³⁷⁷.

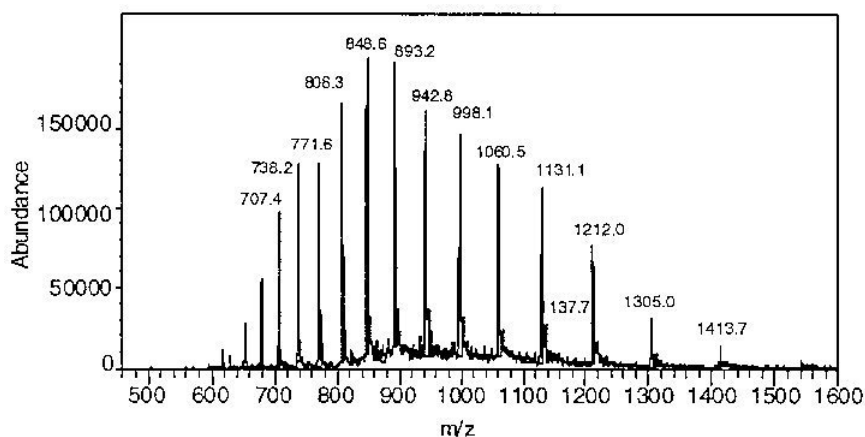
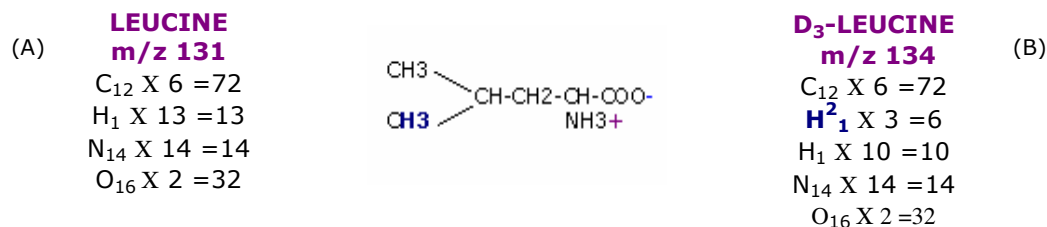


Figure 20. Leucine (A) and ²H₃-L-leucine (B) structure and m/z ratios ³⁷⁴⁻³⁷⁶.



1.2.8.1.3 Equipment characteristics of GC-MS

GC enables a selective separation of sample compounds depending on their volatility. This is achieved by samples moving in a mobile phase (an inert gas not reacting with samples, such as helium) through a stationary phase or column. Sample separation depends on the physico-chemical properties which determine the time of interaction with the stationary phase. Compounds-of-interest are separated and concentrated at the end of this process and are ready to be analysed in the MS ³⁷⁷⁻³⁷⁹.

The GC equipment has 3 basic parts (Figure 21): a sample injector, a column oven and a column ^{377, 378}. Samples, as volatile organic solutions, are injected (0.1-10 µl volume) using a micro-syringe. Injection can be performed manually or automatically. System temperature is a key point in the separation process and it needs to be controlled. The injector is normally maintained at a high temperature (300°C). The column oven, the external part of the equipment, can reach high temperatures of between 40°C to 320°C. The column is a long tube (about 30 m) with a narrow diameter, located inside the column oven. Compounds are separated using two types of columns: packed or capillary. Capillary columns are more efficient and more widely used than packed columns (Figure 22). Capillary column walls are coated with the stationary phase. Packed columns contain an inert, solid support material coated with the stationary phase.

Figure 21. GC-MS and its component characteristics ^{377, 378}.

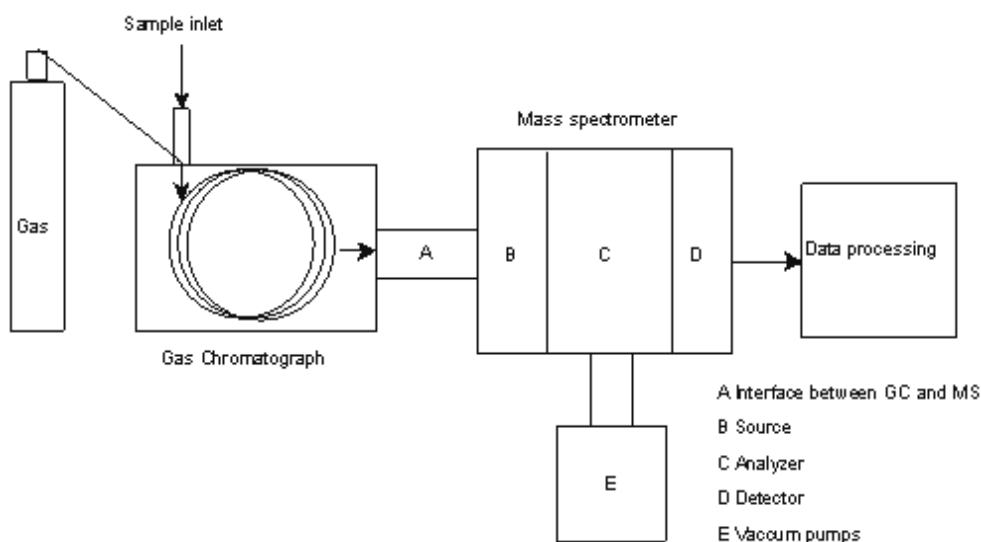
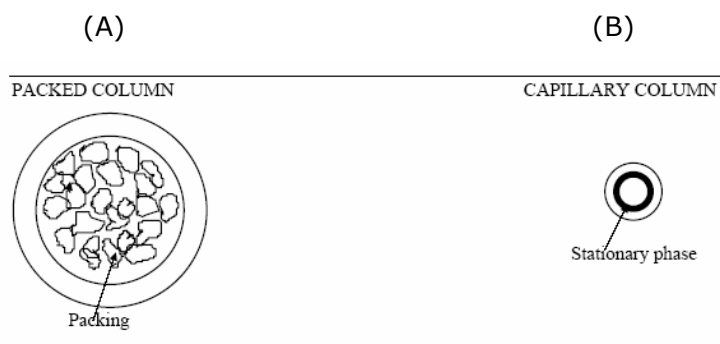


Figure 22. Columns used in GC: packed (A) and capillary (B) columns ^{377, 378}.



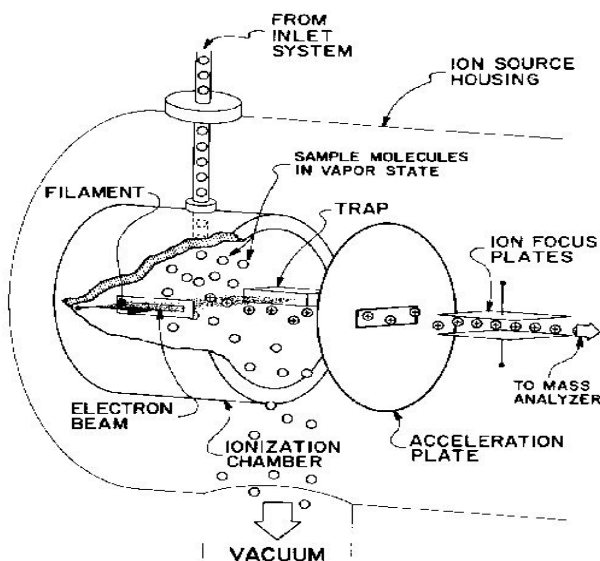
When analysing samples in a GC-MS, the minimum possible sample quantity injected has to be taken into account ^{377, 378}. The quantity of sample introduced into the GC-MS depends on the type of injection, splitless or split mode. It is preferable to use split mode and to choose the amount of sample reaching the detector. Split mode solves one of the inconveniences of GC-MS technique i.e. the non-recoverability of analysed sample. When the injector is operated in split mode, sample amount reaching the column is reduced to prevent column overloading and/or detector degradation. Hence, very narrow initial peak widths are obtained whereas when the injector is operated in splitless mode, all injected sample reaches the column and which facilitates maximum sensitivity of measurement.

In the present study, GC conditions chosen for the analysis of derivatised amino acid samples were (Table 27):

- * split injection mode.
- * injector temperature of 275°C.
- * inert gas helium pressure of 110 kPa.
- * capillary column at a temperature of 150°C and a temperature ramp for amino acid analyses of 110°C for 1 minute and 20°C/minute until 280°C is reached.

MS for the analysis of GC-separated compounds is composed of 4 basic parts: a sample injector, an ion source, a mass analyser and an ion detector (Figure 21, 23) ^{377, 378}. All these parts work under vacuum conditions because ions are very reactive and short-lived and their formation and manipulation must be conducted under vacuum conditions. The injector is the part where the GC-separated molecules arrive. The high temperature vaporises the molecules without them becoming degraded before detection and analysis. In the ion source, molecules are bombarded by electrons. The electron beam hits sample molecules in the gas phase and the atoms are ionised by having electrons knocked-off. The process generates positive ions, or molecular ions, M^+ (some of these as ion fragments). Ions and fragments, all of which have the same kinetic energy, are separated and accelerated into the mass analyser. Ions are then deflected by a magnetic field, the amount of deflection depending on the mass (the lighter the ions, the more they are deflected) and the number of positive charges. Hence, depending on the m/z ratios, the ions cross the mass analyser to the detector that converts the ion impact into electronic and digital signals. Finally, the signals are analysed using specific computer software and the data are presented as mass spectra.

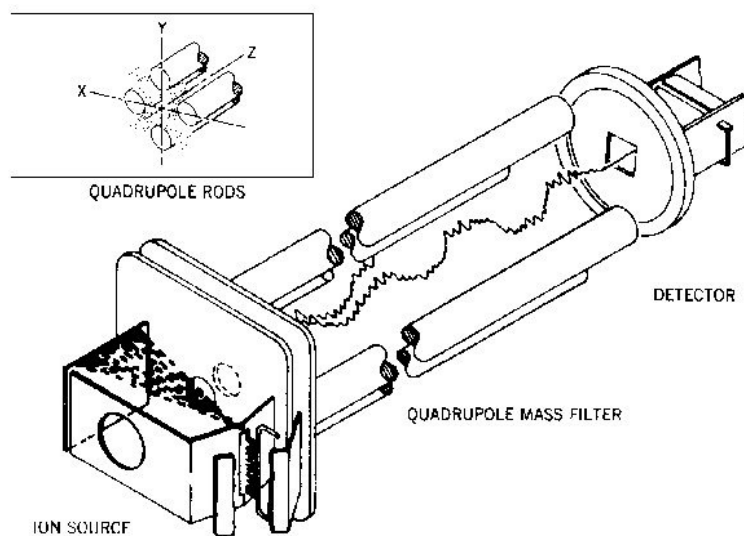
Figure 23. GC-MS injector and ion source ^{377, 378}.



When analysing samples in a GC-MS, the sample ionisation technique used needs to be taken into account ^{377, 378}. There are 5 ionisation techniques or sources: electron ionisation (EI), chemical ionisation (CI), field ionisation (FI), photo-ionisation (PI) and surface ionisation (SI). EI is the most frequently used technique since it enables comparisons to be made with a large number of mass spectra contained in various commercial databases and, as well, provides more information because EI produces more ion fragments. EI is used for volatile compounds.

There are 5 types of MS analysers: magnetic-sector and double-focusing instrument MS, transmission quadrupole MS, quadrupole ion-trap MS, time-of-flight MS (MS-TOF) and Fourier transform ion cyclotron resonance MS (MS-FTICR or MS-FTMS) ^{377, 378}. MS used for lipoprotein kinetic studies is of the quadrupole analyser type with a quadrupole magnetic field that enables ions with a unique m/z ratio to cross to the detector (Figure 24). The MS has smaller physical dimensions and is easier to operate. Quadrupole MS refers to the four parallel rods. Opposing rod pairs are connected together electrically and a voltage is generated between them; the ions travelling through the quadrupole between the rods. Only ions of a certain m/z reach the detector for a given ratio of voltages, and the rest have unstable oscillations and collide with the rods. This enables selection or scanning of a particular ion by varying the voltages.

Figure 24. Representation of a quadrupole mass analyser ^{377, 378}.



The particular manner in which the data are generated and analysed needs to be taken into account when analysing samples in a GC-MS ^{377, 378}. Quadrupole GC-MS mass spectra are generated at high speed and this requires computer support for processing. There are different approaches that facilitate data collection, storage and manipulation. MS ion detectors operate in 2 different modes: scanning (SCAN) and selected ion monitoring (SIM) also termed selected ion recording (SIR) modes. SIM, or SIR, is a much more sensitive technique and is the preferred method used in lipoprotein kinetic studies. Only a few ions and/or analogues are detected, depending on their specific m/z ratio, and sensitivity is increased at the expense of specificity. SCAN mode, on the other hand, generates different mass spectra while providing a reproducible mass spectra fragmentation pattern or "fingerprint". Such fingerprints can be compared with those stored in databases, or mass spectral libraries, to identify the compounds under test.

In the present study, MS conditions chosen to analysed derivatised amino acids samples were (Table 27):

- * quadrupole MS.
- * EI mode.
- * SIR mode.

Table 27.

GC-MS conditions for sample analysis ^{316, 372, 380}

- derivatise amino acid samples
- prepare a Worksheet with MassLab Program (GC-MS Fison Instruments MD 800, Finnigan Ltd) for samples, adding calibration curve samples at the beginning and at the end of the run
- check programed GC-MS conditions with the MassLab Program to be correct
 - GC injector temperature → 275°C
 - inert gas (helium) pressure → 110 kPa
 - capillary column → 30 m x 0.25 mm x 0.25 µm DB5-MS (Agilent Technologies)
 - column temperature → 150°C
 - temperature ramp used for amino acid analysis
 - 110°C, 1 minute
 - 20°C/minute until reaching 280°C
 - split injection mode (using a split or sample:discard proportion of 1:10)
→ 1-2 µl derivatised sample
 - EI mode
 - SIR mode
 - ions $m/z=277$, $m/z=276$ and $m/z=274$

1.2.8.1.4 GC-MS isotopic enrichment analysis

Samples $^2\text{H}_3$ -L-leucine specific enrichment was determined in free plasma amino acid samples and hydrolysed amino acids from apo B-100 VLDL₁, VLDL₂, IDL and LDL, and from apoA-I and A-II HDL. Additionally, calibration curve samples were also analysed in a GC-MS.

Calibration curve samples were prepared from a solution with similar characteristics to plasma, termed "Mock Plasma Solution". The solutions was prepared by adding known quantities of $^2\text{H}_3$ -L-leucine to commercial amino acids (Table 28, 29) ^{264, 316, 380, 381}. Apo B-100, apoA-I and A-II and calibration curve samples were derivatised to obtain their t-MTBSTFA derivatives for subsequent analysis in GC-MS: leucine derivatives had a $m/z=274$ and $^2\text{H}_3$ -L-leucine derivatives had a $m/z=277$ (Table 30, 31, Figure 25) ^{316, 380-382}. Finally, all derivatised samples were analysed in a quadrupole GC-MS, using a capillary column and working in split injection, EI and SIR

modes. Temperature, instructions, injected sample quantity and other conditions were fixed prior to sample analysis using the GC-MS computer software (Table 27).

Table 28.

Preparing $^2\text{H}_3$ -L-leucine calibration curve ³⁸¹

- combine 15 amino acids to prepare Mock Plasma Solution
 - prepare a 0.1M HCl solution
 - weight amino acids (LAA-21-L amino acids, Sigma Inc) (Table 29)
 - mix each amino acid with 0.1M HCl until desired concentration is reached (similar to that of human plasma)
 - combine the different amino acid solutions and mix well
- prepare calibration curve
 - prepare a 10 mg/ml $^2\text{H}_3$ -L-leucine solution
 - mix Mock plasma solution volumes with different volumes of $^2\text{H}_3$ -L-leucine solution to obtain calibration curve samples with different isotopic enrichments, or atomic percent excess (APE)

Samples	Volume Mock Plasma (ml)	Volume $^2\text{H}_3$ -L-leucine (μl)
MP (0) APE	155.94	-
RATIO	38.985	-
0.5 APE	77.97	240
1 APE	77.97	480
5 APE	77.97	2400

- prepare several aliquots for each sample in Chromacol vials (Sigma Aldrich Inc) for GC-MS analysis

Samples	Volume aliquot (μl)
MP (0) APE	491
RATIO	246
0.5 APE	493
1 APE	494
5 APE	507

- evaporate samples using vacuum evaporation centrifuge and store at 4°C

Table 29.

Mock plasma solution composition ³⁸¹

Amino acids	Equivalent in 1 ml plasma (nM)	Molecular weight (amu)	Weight (g)
Alanine	419	89.09	0.0713
Asparagine	49	133.10	0.0133
Cysteine	118	121.20	0.0364
Glutamine	645	147.10	0.1618
Glycine	236	75.07	0.0375
Hidroxyproline	20	131.00	0.0131
Isoleucine	84	131.20	0.0262
Leucine	160	131.20	0.0394
Methionine	32	149.20	0.0149
Phenylalanine	65	165.20	0.0330
Proline	239	115.10	0.0576
Serine	114	105.10	0.0210
Threonine	146	119.10	0.0357
Valine	252	117.10	0.0586
Cyclöleucine	20	129.20	0.0129

NOTE: use an analytical balance for weighing

Figure 25. ²H₃-L-leucine derivatisation to form t-MTBSTFA derivative: *m/z* ratios ³⁷⁴⁻³⁷⁶.

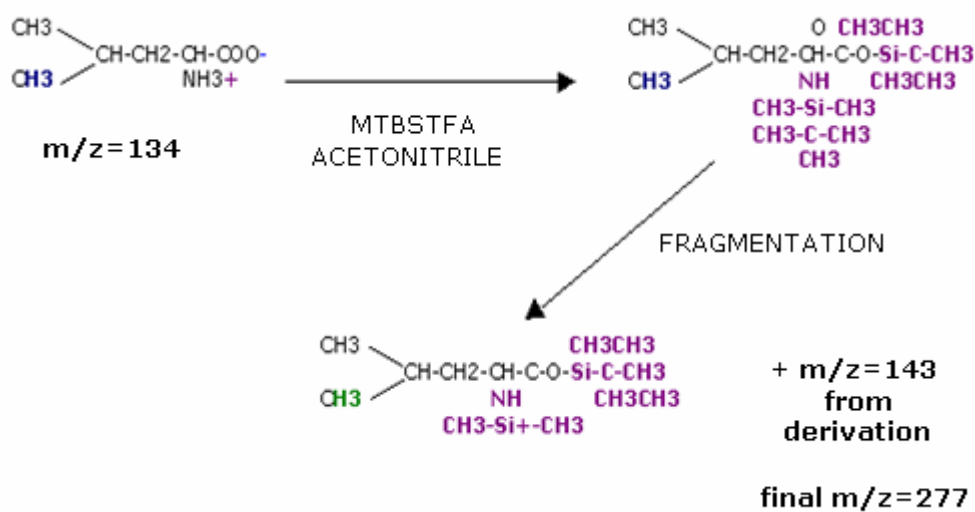


Table 30.

Derivatisation of calibration curve samples ^{304, 374-376}

- follow method described in Table 26

Samples	Volume MTBSTFA (μ l)	Volume Acetonitrile (μ l)
MP	60	60
Ratio	60	60
MP (0) APE	60	60
0.5 APE	60	60
1 APE	60	60
5 APE	60	60

- after derivatisation, dilute samples with acetonitrile

Samples	Volume sample (μ l)	Volume Acetonitrile (μ l)
MP	use this way	
Ratio	40	120
MP (0) APE	40	40
0.5 APE	40	40
1 APE	40	40
5 APE	40	40

- samples ready to be analysed in a GC-MS

Table 31.

Derivatisation of amino acid samples ^{304, 374-376}

- follow method described in Table 26

Samples	Volume MTBSTFA (μ l)	Volume Acetonitrile (μ l)	Injection Volume in GC-MS (μ l)
Apo B-100 VLDL1	10	10	2
Apo B-100 VLDL2	10	10	2
Apo B-100 IDL	15	15	1
Apo B-100 LDL	150	150	1
ApoA-I HDL	10	10	2
ApoA-II HDL	10	10	2
Plasma	20	20	1

NOTE: derivatisation and injection volumes can be modified if GC-MS requires more, or less, sample for detection

1.2.8.1.5 Theoretical considerations when analysing isotopic enrichment

Mass spectra provide information on isotopic enrichment (E) or atom percent excess (APE). Samples' isotopic enrichment were obtained by monitoring specific m/z ratios in SIR mode ^{264, 304, 304, 374-376, 380}.

When using ²H₃-L-leucine tracer in lipoprotein kinetic studies, m/z ratios or mass fragments registered in GC-MS are (Figure 25):

- * m/z ratio 274 which represents a fragment obtained from natural leucine.
- * m/z ratio 277 which represents a fragment (M+3) obtained from deuterated leucine.
- * m/z ratio 276 which represents a fragment (M+2) obtained from another derivative related to natural leucine.

E of a sample is calculated with the formula:

$$E = (R - R_0) / [(1 + R) \times (1 + R_0)]$$

where R is m/z 277 : m/z 274 (peak area ratio) from a sample and R_0 is m/z 277 : m/z 274 from deuterated leucine naturally present in the organism (natural abundance).

APE of a sample is calculated with the formula:

$$APE = [(IR_t - IR_0) / (IR_t - IR_0 + 1)] \times 100$$

where IR_t is the sample isotope content at specific time and IR_0 is the sample isotope content at $t = 0$ (before $^2\text{H}_3\text{-L-leucine}$ injection, and is related to natural abundance).

Tracer/tracee ratio (Z) of a sample is calculated with the formula:

$$Z = E / (E_t - E)$$

where $E_t = 0.998$ is the isotopic abundance of infused $^2\text{H}_3\text{-L-leucine}$ (related to its purity).

Instead of using the ratio m/z 277 : m/z 274, the ratio m/z 277 : m/z 276 is used to calculate E . When stable isotopic enrichment is very low, m/z 274 can be of higher order of magnitude compared to m/z 277. The m/z 277 and m/z 276 have similar magnitude of measurement. Detection of the ratio m/z 277 : m/z 276 is easier and more precise than the ratio m/z 277 : m/z 274. The ratio m/z 276 : m/z 274 is constant (0.1009 ± 0.0009). Thus, the ratio m/z 277 : m/z 276 has only to be multiplied by the constant value of the ratio m/z 276 : m/z 274 to get a ratio of m/z 277 : m/z 274 ²⁶⁴.

When calculating APE in lipoprotein kinetic studies, it is critical that the methodological precision in measuring specific isotopic enrichment falls within a range of $<0.2\%$ to $>10\%$ APE. Plasma amino acids usually have $>10\%$ APE and lipoprotein fractions (e.g. LDL) usually have $<0.2\%$ APE. This inconvenience is overcome when applying m/z 277 : m/z 276 ratios. The m/z 277 : m/z 276 ratio has a linear correlation with APE values ranging between 0 and 10%. Thus, monitoring these ratios enables low enrichment detection with sufficient precision ²⁶⁴.

Injected isotope dose has to allow enough isotopic enrichment in all lipoprotein fractions to be detected with GC-MS. As such, the labelled tracer requires a sizeable mass of isotope. Multiple labelled amino acid, such as leucine with 3 deuterium, are widely used (additional mass enables a better tracer peak to be differentiated and ensures appropriate and accurate detection of isotopic enrichment). Excessive doses are not recommended since they can cause temporary increase in plasma amino acid concentrations (up 10 times that of normal) and a disturbance in apo metabolism. No alterations of apo kinetics have been observed using isotope doses from 0.6 to 3 mg/kg body weight. So as not to perturb the system, our chosen $^2\text{H}_3\text{-L-leucine}$ doses

for our lipoprotein kinetic studies were 0.7 mg/kg body weight for bolus injection and 0.7 mg/kg/h for constant infusion ^{264, 382-384}.

1.2.9 Kinetic results obtained using the SAAM II program

As described in the Introduction section, we used the apo B-100 multi-compartmental model developed by Demant and Packard (1996) and a new apoA-I and A-II model developed by Packard and Caslake (2006) ²⁶⁴. They enabled the calculation of kinetic parameters using the SAAM II modelling program.

Kinetic parameters of samples were calculated with the SAAM II program using ^{313, 316, 334, 336-338, 385, 386}.

- * sample isotopic enrichment data.
- * apo pool size, or total mass.

Calculation of kinetic parameters involved determination of ³⁸⁶:

- * production rate i.e. *de novo* appearance which normally implies synthesis but also includes appearance of tracer from proteins that are turning over.
- * pool size i.e. total mass of studied compound in a particular compartment.
- * catabolic rate i.e. disappearance rate.
- * transfer i.e. flux of study compound from one compartment to another.

1.2.9.1 Applying multi-compartmental models to analyse kinetic data

Kinetic parameters can be calculated using different approaches such as non-compartmental models using algebraic or linear regression functions and mono-exponential functions to describe enrichment curves. However, these approaches provide limited quantitative information on the system under study ^{310, 312, 314-316, 317, 386}. Instead, multi-compartmental modelling was used since it appears to be the best and most-widely-used approach in obtaining data on lipoprotein kinetics and, thus, on their metabolism. It uses multi-exponential functions to describe enrichment curves and, despite its complexity, it has several advantages ^{330, 387}.

A multi-compartmental model is a schematic representation of the system using multi-exponential equations to describe the mass balance of tracee and tracer under steady state conditions. Compartments represent different aspects of lipoprotein metabolism *in vivo* (material transport, *de novo* synthesis, disappearance) in an attempt to explain the different physiological components involved in enrichment curves. As such, several physiological assumptions need to be made. Multi-compartmental models take into account kinetic heterogeneity of lipoprotein metabolism. Moreover, when using a multi-compartmental modelling approach, the

derived kinetic parameters are not influenced by the administration protocol of the tracer ^{261, 309, 331, 332, 388-394}.

Multi-compartmental models are described by several constants or kinetic factors ^{304, 395}. These include:

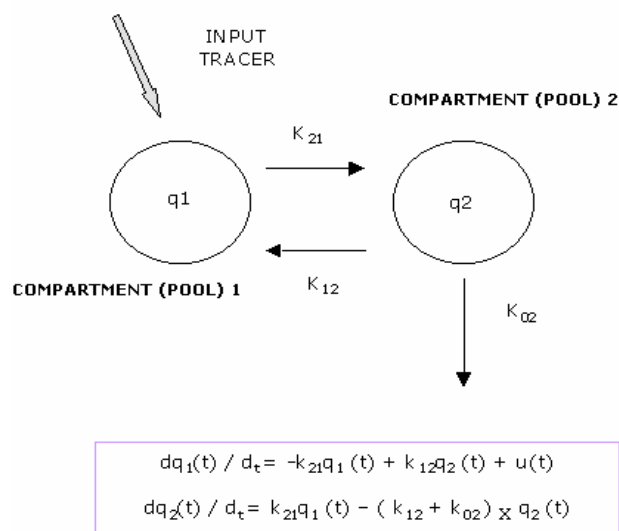
- * pool or compartment size (Q_i, q_i) i.e. the amount or mass of compound in each compartment where q_j is the tracer mass in compartment i and Q_j the tracee mass in compartment i .
- * fractional transfer coefficients or rates between compartments ($k_{i,j}$) i.e. the ratio of compound transferred from compartment j to compartment i per unit of time. Individual transfer rates k_{ij} provide rates of protein secretion, transfer and catabolism.
- * irreversible loss rates ($k_{0,j}$) i.e. irreversible loss of compound from compartment j per unit of time.
- * flux, i.e. the rate of movement in mass of tracer or tracee per unit time from compartment j to compartment i at time t .

$$\text{Flux (i,j)}=k(i,j) \times q_j \quad // \quad \text{Flux (i,j)}=k(i,j) \times Q_j$$

Multi-compartmental modelling generates an equation system corresponding to the number of compartments of the system. Each individual equation describes the enrichment curves of its corresponding compartment. An example of a two-compartment model is shown in Figure 26. Modelling enables the determination of the coefficients of differential equations characterising the model, calculation of transport rates, derivation of production and catabolic rates, pool sizes, flux and transfers between particles. With more equations, more $k_{i,j}$ values describe the model. This is a significant disadvantage and which potentially increases the possibility that the model will not be uniquely identifiable i.e. that the multi-compartmental model has no identifiability, or that it could not be determined by a unique set of values that solve the equations.

All these complex mathematical determinations are performed using multi-compartmental modelling programs such as the SAAM II program.

Figure 26. Diagram of a simple multi-compartmental model with two compartments, isotope injection site, and multi-exponential functions describing the kinetics ^{313, 334, 386}.



q=pool size; t=time; k=constant rates.

1.2.9.2 Complementary data used in lipoprotein kinetic studies

The SAAM II program determines lipoprotein kinetic parameters, a requirement for which isotopic enrichment data together with several data related to experimental protocol are needed ^{335, 336, 396-399}. These include:

- * mass or pool size of apo B-100, A-I and A-II lipoprotein fractions:

$$\text{apo pool (mg)} = \text{apo concentration (mg/dl)} \times \text{plasma volume (l)} \times 1 \text{ l} / 10 \text{ dl}$$

$$\text{plasma volume (l)} = 4.50\% \text{ of body weight (kg)}$$

- * leucine mass or pool size of apo B-100, A-I and A-II lipoprotein fractions (corrected by ± 10%):

$$\text{leucine (mg)} = \text{apo pool (mg)} \times \% \text{ leucine}$$

$$\% \text{ leucine in apo B-100} = 12.12$$

$$\% \text{ leucine in apo A-I} = 15.68$$

$$\% \text{ leucine in apo A-II} = 10.70$$

$$- 10\% \text{ leucine (mg)} = \text{leucine (mg)} \times 0.90$$

$$- 10\% = 90\%$$

$$\begin{aligned} + 10\% \text{ leucine (mg)} &= \text{leucine (mg)} \times 1.10 \\ + 10\% &= 110\% \end{aligned}$$

* mg of $^2\text{H}_3$ -L-leucine bolus injection:

$$\begin{aligned} ^2\text{H}_3\text{-L-leucine bolus (mg)} &= \text{body weight (kg)} \times \text{bolus } ^2\text{H}_3\text{-L-leucine concentration} \\ \text{bolus } ^2\text{H}_3\text{-L-leucine concentration (mg/kg)} &= 0.7 \end{aligned}$$

* mg of $^2\text{H}_3$ -L-leucine constant infusion:

$$\begin{aligned} ^2\text{H}_3\text{-L-leucine constant infusion (mg)} &= \\ [\text{body weight (kg)} \times \text{ } ^2\text{H}_3\text{-L-leucine constant infusion (mg/kg)} \times 16\text{h}] / \text{real-} \\ \text{time infusion (h)} & \\ \text{constant infusion } ^2\text{H}_3\text{-L-leucine concentration (mg/kg)} &= 0.7 \end{aligned}$$

* time of constant infusion:

$$\text{time (h)} = 16 \quad (\text{specifying exact hours and minutes})$$

* time of last collected blood sample during kinetic study:

$$\text{time (h)} = 48 \quad (\text{specifying exact hours and minutes})$$

Lipoprotein compositional analyses are also measured as data-of-interest when performing lipoprotein kinetic studies.

All these data are finally corrected for losses in ultracentrifugation processes and isolated lipoprotein fraction volumes recovered.

1.2.9.2.1 Apos' pool size determination

Apo B-100, apoA-I and A-II lipoprotein fractions' pool sizes were determined using the method of Lowry and/or an autoanalyser.

1.2.9.2.1.1 Apo B-100 pool size determination

The method of Lowry was used to calculate total mass or pool size of apo B-100 in VLDL₁, VLDL₂, IDL and LDL lipoprotein fractions ^{330, 400-405}.

The procedure for determining apo B-100 mass in each lipoprotein fraction was:

- * sample pools or plasma mixes were prepared in triplicate (with similar plasma volumes) from all plasma samples collected during the 48h kinetic study.
- * from pools 1, 2 and 3, VLDL₁, VLDL₂, IDL and LDL lipoprotein fractions were isolated using ultracentrifugation:
 - pool 1 (see page 107).
 - pools 2 and 3 were used to isolate apo B-100 after isopropanol precipitation and delipidation:
 - proteins present in isopropanol supernatant were determined using the method of Lowry.
 - precipitated and delipidated apo B-100 pellet was resuspended in NaOH and concentration determined using the method of Lowry.

The apo B-100 pool size of each lipoprotein fraction was calculated using two methods to improve the kinetic parameter measurements using the SAAM II program. Total apo B-100 in plasma was also measured for comparison with the total sum of apo B-100 in VLDL₁, VLDL₂, IDL and LDL. The procedure was as follows:

- * apo B-100 determined using the method of Lowry protein assay; the main method used to calculate apo B-100 pool size:

$$\text{apo B-100 concentration (mg/dl)} = \text{total protein pool 1 (mg/dl)} - [2 \times \text{isopropanol protein mean pools 2, 3 (mg/dl)}]$$

$$\begin{aligned} \text{apo B-100 pool size (mg)} = & \\ & \text{apo B-100 concentration (mg/dl)} \times \text{plasma volume (l)} \times 1 \text{ l/10 dl} \\ & \text{plasma volume (l)} = 4.5\% \text{ of body weight (kg)} \end{aligned}$$

Isopropanol supernatant provides information on the non-apo B-100 proteins present in lipoprotein fractions and of possible losses of apo B-100 during the precipitation process. To confirm the measurements and to ensure reliability of methodology, the resuspended apo B-100 pellet in NaOH was also measured using the method of Lowry. The specific methodology is described in the next two sections.

- * apo B-100 determined using an autoanalyser (Cobas Mira, Roche, Basel) and immunoturbidimetric commercial assay kits (Beckman Coulter Inc):

$$\begin{aligned} \text{apo B-100 pool size (mg)} = & \\ & \text{apo B-100 concentration (mg/dl)} \times \text{plasma volume (l)} \times 1 \text{ l/10 dl} \\ & \text{apo B-100 VLDL}_1, \text{ VLDL}_2, \text{ IDL and LDL concentration (mg/dl) from} \\ & \text{direct determination (pool 1)} \end{aligned}$$

plasma volume (l)=4.5% of body weight (kg)

This approach was used as an alternative to the previous one described above, and also as a comparative method to ensure reliability of the methodology.

1.2.9.2.1.1.1 Isopropanol extract determination

Pools 2 and 3 samples were processed to obtain apo B-100 VLDL₁, VLDL₂, IDL and LDL. After apo B-100 isopropanol precipitation, proteins present in the isopropanol supernatant were measured using the method of Lowry⁴⁰⁰⁻⁴⁰⁵ (Table 32, Annexe XVI).

The method of Lowry is an often-cited general-use protein assay. It is the method-of-choice for accurate protein determination, for cell content measurements, chromatography fractions and enzyme preparations. Compared to other protein assays such as the method of Bradford, it requires that several reagents be prepared and mixed during the assay. This is followed by protracted, and precisely-timed, incubations followed immediately by spectrophotometric absorbance measurements because the colour development is unstable. The assay is relatively sensitive and can measure proteins at 0.01-1 g/ml, or lower.

Protein determination using the method of Lowry is based on the reaction of protein peptide bonds with Cu⁺² ions, and relies on two different reactions:

- * the first reaction (Biuret reaction) forms a copper ion complex with protein amide bonds, thus reducing Cu⁺² to Cu⁺¹ in an alkaline environment.
- * the second reaction is a reduction of amino acid residues (mainly tyrosine and tryptophan) by the Folin-Ciocalteu reagent (phosphomolybdate and phosphotungstate). Reduced Folin-Ciocalteu reagent, or heteropolymolybdenum, is a blue colour and thus, detectable with a spectrophotometer covering the range of 500-750 nm.

The method of Lowry is sensitive to variations in the content of tyrosine and tryptophan residues. Many substances interfere with this assay (detergents, carbohydrates, glycerol, tricine, EDTA, Tris, potassium compounds, sulfhydryl compounds, disulfide compounds, magnesium and calcium). Most of these interfering substances are commonly found in buffers used in manipulating proteins.

Table 32.

Lowry method for the determination of proteins present in isopropanol extract (Annexe XVI) ⁴⁰⁰⁻⁴⁰⁵

- add 2 ml of alkaline solution (Na₂CO₃, NaOH, NaK Tartrate, CuSO₄ in a proportion 100:1:1) to calibration curve samples, standards and isopropanol supernatant samples to be analysed (total volume 200 µl)
- vortex mix and allow to stand for 10 minutes at room temperature
- add 200 µl of Folin solution diluted 1:1 with distilled H₂O
- vortex mix immediately
- allow all samples to stand for 30 minutes at room temperature
- measure absorbance at λ 750 nm (Kontron Instruments, Ltd) within 2h
- calculate samples' concentration (mg/dl) directly from calibration curve's absorbances

1.2.9.2.1.1.2 Precipitated apo B-100 determination

Pools 2 and 3 samples were processed to determine apo B-100 VLDL₁, VLDL₂, IDL and LDL. Following delipidation of apo B-100 pool samples with ethanol:ether, the precipitated apo B-100 pellet was determined for protein with the method of Lowry (Table 33, Annexe XVI) ⁴⁰⁰⁻⁴⁰⁵.

To determine the protein content of the precipitated samples, they need to be first dissolved in NaOH. This implies a simple modification of the general method of Lowry with addition of NaOH in the Biuret reagent. No other modifications are needed.

Table 33.

Lowry method for the determination of precipitated apo B-100 (Annexe XVI) ⁴⁰⁰⁻

405

- add 1 ml of 0.5N NaOH to precipitated apo B-100 samples and allow to dissolve at 4°C
- add 2 ml of alkaline solution (Na₂CO₃, NaOH, NaK Tartrate, CuSO₄ in a proportion 100:1:1) to calibration curve and standard samples (total 200 µl)
- add 968 µl of alkaline solution and 1032 µl of distilled H₂O to samples to be analysed (total volume 200 µl)
- vortex mix and allow to stand for 10 minutes at room temperature
- add 200 µl of *Folin* solution diluted 1:1 with distilled H₂O
- vortex mix immediately
- allow all samples to stand for 30 minutes at room temperature
- measure absorbance at λ 750 nm (Kontron Instruments, Ltd) within 2h
- calculate samples' concentrations (mg/dl) directly from standard curve's absorbances

1.2.9.2.1.2 ApoA-I and A-II pool size determination

Total mass, or pool size, of apoA-I and A-II HDL lipoprotein fraction were determined in an autoanalyser (Cobas Mira, Roche, Basel) using immunoturbidimetric commercial assay kits (Wako Ltd; Beckman Coulter Inc) (Annexe I) ⁴⁰⁶⁻⁴⁰⁸.

ApoA-I and A-II were determined in plasma samples since ≥90% of plasma apoA-I and A-II are associated with HDL and, as such, the plasma concentrations are considered equal to those of HDL ^{349, 310, 398, 399, 409}. ApoA-I and A-II pool sizes were calculated as:

apoA-I HDL concentration (mg/dl)=apoA-I plasma concentration (mg/dl)

apoA-II HDL concentration (mg/dl)=apoA-II plasma concentration (mg/dl)

apoA-I pool size (mg)=

apoA-I concentration (mg/dl) x plasma volume (l) x 1 l/10 dl

apoA-II pool size (mg)=

apoA-II concentration (mg/dl) x plasma volume (l) x 1 l/10 dl

plasma volume (l)=4.5% of body weight (kg)

1.2.9.2.2 Lipoprotein compositional analysis and other parameter determinations

Several measurements were made to determine the reliability of the methodology, as well as part of the lipoprotein kinetic study protocol (Annexe I) ^{407, 408}:

▪ plasma determinations on the day of the kinetic studies, at basal time (or t=0h) and before tracer primed constant infusion:

* plasma Lp(a) concentration was determined using an autoanalyser (Synchron LX®i725, Beckman Coulter Inc) and a nephelometric commercial kit (Beckman Coulter Inc). Lp(a) content is used for a subsequent correction for the apo B-100 pool size calculation. When isolating LDL using sequential ultracentrifugation, there is a slight contamination of LDL with Lp(a). However, any Lp(a) protein present would co-precipitate with apo B-100 LDL and, potentially, contaminate this fraction. Hence, Lp(a) measurement is used for recovery adjustment ³³⁰.

* plasma TC, FC, TG and PL concentrations were determined using an autoanalyser (Cobas Mira, Roche, Basel) and enzymatic commercial kits (Wako Ltd; Beckman Coulter Inc).

* plasma apo B-100, apoA-I and A-II concentrations were determined using an autoanalyser (Cobas Mira, Roche, Basel) and immunoturbidimetric commercial kits (Wako Ltd; Beckman Coulter Inc).

Quality Controls were used in all measurements (Virgil™ Serology Control, Synchron® Control Multi-Level and Apo Synchron LX® Control, Beckman Coulter Inc).

▪ from all plasma samples obtained during the 48h kinetic study, sample pools or plasma mixes were prepared in triplicate from 0.5 ml of plasma (up to 12.5 ml). VLDL₁, VLDL₂, IDL, LDL and HDL lipoprotein fractions were isolated from pools 1, 2 and 3 using ultracentrifugation. Pool 1 lipoprotein fractions were used to determine:

* lipoprotein composition:

- TC, FC, TG and PL concentrations were determined using an autoanalyser (Cobas Mira, Roche, Basel) and enzymatic commercial kits (Wako Ltd; Beckman Coulter Inc) (Annexe I) ^{407, 408}.

- total protein concentrations were determined using a modified method of Lowry (Annexe XVI). TG-rich lipoproteins (VLDL) interfere with UV absorbance of proteins and, hence, the general method of Lowry is modified using detergents such as SDS. No other modifications are needed (Table 34).

* apo B-100, apoA-I and A-II concentrations (to calculate apos pool size) were determined using an autoanalyser (Cobas Mira, Roche, Basel) and

immunospectrometric commercial kits (Wako Ltd; Beckman Coulter Inc)
(Annexe I) ^{407, 408}.

Quality controls were used in all measurements (Synchron[®]Control Multi-
Level and Apo Synchron LX[®]Control, Beckman Coulter Inc).

Table 34.

**Lowry method for the determination of total protein content in lipoprotein
fractions (Annexe XVI)** ⁴⁰⁰⁻⁴⁰⁵

- add 2 ml of alkaline solution (Na₂CO₃, NaOH, NaK Tartrate, CuSO₄ in a proportion 100:1:1) to calibration curve' samples, standards and samples to be analysed (total volume 200 µl)

Lipoprotein fraction	Volume (µl)
Cm	200
VLDL	200
IDL	50
LDL	20
HDL	20

NOTE: samples with high TG content (e.g. VLDL) need to be delipidated prior to protein determination (turbidity due to lipids alter the measurement). In this case, add 1 ml of 10% SDS to the alkaline solution (Na₂CO₃, NaOH, NaK Tartrate, CuSO₄ in a proportion 100:1:1) and continue sample processing as for standard Lowry method

- vortex mix and allow to stand for 10 minutes at room temperature
- add 200 µl of Folin solution diluted 1:1 with distilled H₂O
- vortex mix immediately
- allow all samples to stand for 30 minutes at room temperature
- measure absorbance at λ 750 nm (Kontron Instruments, Ltd) within 2h
- determine the samples' concentration (mg/dl) directly from standard curve's absorbances

2. Methodology of the specific objectives: applying lipoprotein kinetic studies using stable isotopes to compare the effects of two diets on lipoprotein metabolism

2.1 Subjects of study

Male volunteers (aged <75 years) diagnosed as having moderate hypercholesterolaemia, were selected to take part in the dietary intervention study. These adults with moderate C levels ($130 \text{ mg/dl} \leq \text{LDLC} \leq 189 \text{ mg/dl}$) were those in whom a dietary intervention may be prescribed ⁴¹⁰.

2.1.1. Selection criteria

Volunteers inclusion criteria were:

- * LDLC >130 mg/dl.
- * TG <250 mg/dl.
- * body mass index (BMI) <30 kg/m².

Exclusion criteria were:

- * smoking habit.
- * consumption of >40 g alcohol/day.
- * drugs affecting lipoprotein metabolism.
- * evidence of hepatic, cardiovascular, renal or endocrine disease.

Each volunteer was provided with detailed information on the study when first interviewed and all provided signed consent to participation in the study.

The study received approval from the Clinical Research Ethical Committee of the *Hospital Universitari de Sant Joan, Reus*.

2.2 Experimental design

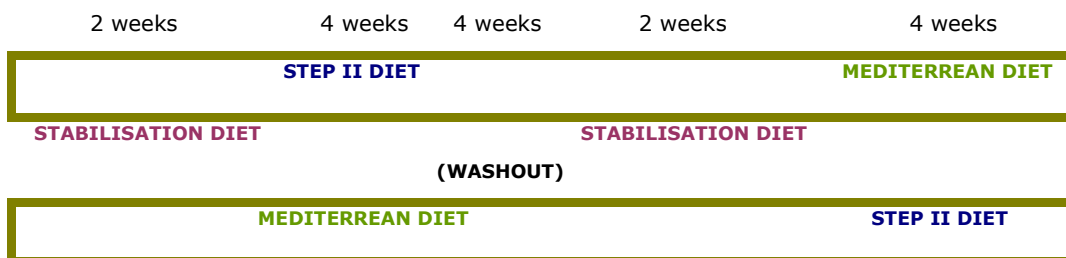
2.2.1 Dietary intervention protocol

2.2.1.1 Study design

Dietary intervention periods were designed as a randomised, dietary-controlled, crossover study that consisted of a 2-week stabilisation period and a 4-week dietary period which consisted of a Mediterranean-type diet rich in MUFAs from virgin olive oil (from now on referred to as Mediterranean diet) and a low-fat STEP II diet. At least a 4-week washout period was included between dietary periods in order to test for possible interactions between diets and to ensure no carry-over, or sequence, effect (Figure 27).

In this type of clinical trial, each volunteer acts as his own control.
 The kinetic parameters were determined at the end of each dietary intervention period.

Figure 27. Crossover study design: randomised dietary intervention periods.



2.2.1.2 Diets selection

The effects of a Mediterranean and a STEP II diets on lipoprotein metabolism were compared. Dieticians in our Unit ensured that the diets were isocaloric to avoid effects on lipoprotein metabolism resulting from changes in body weight ⁴¹¹.

Compositional characteristics of the diet in the stabilisation period were adjusted to the average consumption pattern of the Catalan population ^{412, 413}. This consumption pattern was used because:

- * it was the dietary pattern of volunteers' population.
- * it stabilised all volunteers before they entered the dietary intervention period.
- * it had a high lipid (SFAs and MUFAs) content that enabled changes of lipoprotein metabolism due to FAs to be evaluated when comparing dietary interventions.

Compositional characteristics of the Mediterranean diet were adjusted to the Spanish recommendations for prevention and treatment of CVD ⁴¹⁴. The compositional characteristics of STEP II diet were adjusted to the AHA recommendations for prevention and treatment of CVD (Table 35) ^{2, 60-69}. These patterns were used because:

- * the Mediterranean diet has a higher MUFAs content especially from virgin olive oil intake compared to

* the STEP II diet which has decreased total lipid content, mainly MUFAs.

The Mediterranean and STEP II diets composition had similar protein content (15%). Both had similar targets with respect to SFAs (<7%), PUFAs (<7%) and C contents (<200 mg/dl). The Mediterranean diet had less CH content than the STEP II diet (45-50% versus 60%, respectively) and a higher lipid content than the STEP II diet (35% versus 25%, respectively) especially from MUFAs (20% versus 12%, respectively). The stabilisation diet before each dietary intervention period had higher protein (17%) and lipid content (39%) especially from SFAs (13%), whereas the CH content was less (41%).

Table 35. Composition of diets used in the study ^{2, 60-69, 412-414}.

	PERCENTAGE TOTAL CALORIES						mg/dl
	PROT	CH	LIP	SFAs	MUFAs	PUFAs	C
STABILISATION DIET	17	41	39	13	20	<7	300
MEDITERRANEAN DIET	15	50	35	<7	20	<7	<200
STEP II DIET	15	60	25	<7	12	<7	<200

C=cholesterol; PROT=proteins; CH=carbohydrates; LIP=lipids;
 SFAs=saturated fatty acids; MUFAs=monounsaturated fatty acids;
 PUFAs=polyunsaturated fatty acids.

2.2.1.3 Diet control and evaluation

Meals were prepared from readily available foodstuffs so that it was easier for volunteers to adhere to the dietician's recommendations. On weekdays, lunches were prepared by the nutrition research kitchen of the *Hospital Universitari de Sant Joan* and consumed in the hospital's canteen, in the presence of the research dietician. All weekday breakfasts and dinners, together with all weekend meals, were prepared and consumed at home under instructions from the research dietician. Volunteers registered 3-day food records (which included 1 weekend day) after each stabilisation period as well as before and after each dietary intervention period (Figure 28).

Volunteers consumed ≤ 40 g alcohol/day and were supervised to ensure that they did not exceed this level.

Food records provide a picture of a subject's typical eating patterns. These records register intake of food and beverages over a 3-day period. To provide accurate intake information, foods are to be weighed or volume measured and the methods of food preparation (baked, sautéed, steamed) described in detail. Finally, diet composition is determined using nutritional tables, data-bases and specific dietary software programs ^{411, 415-418}.

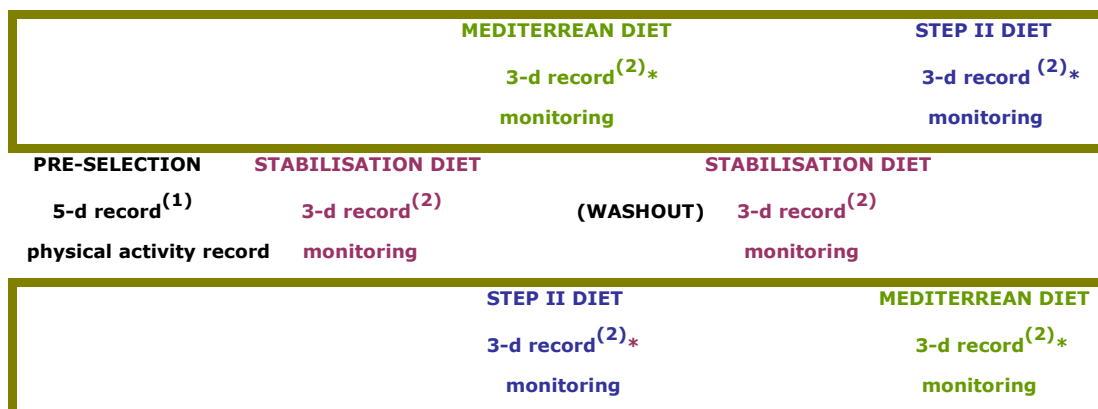
Each volunteer recorded a total of 7 food records during the study so that the dietician was able to monitor adherence to dietary recommendations during the study and, hence, the Mediterranean and the STEP II dietary compositions were controlled. To ensure that the diets were isocaloric, the volunteers recorded 5-day food records (including 2 weekend days) during the pre-selection period at the beginning of the study (Figure 28). Further, their metabolic requirements were calculated using the Harris-Benedict formula adapted for men and corrected for each volunteer's physical activity (light, moderate or heavy) ^{411, 416}:

$$66.5 + (13.7 \times \text{weight in kg}) + (5 \times \text{height in cm}) - (6.8 \times \text{age in years})$$

The nutrient composition of the diet was calculated using the *DATABASE: Répertoire Général Des Aliments* ⁴¹⁵.

The body weight of the volunteers was monitored every week in the *Hospital Universitari de Sant Joan* outpatient clinic.

Figure 28. The total 7 food records registered during the crossover study.



d=days; w=Wednesday; t=Thursday; f=Friday; s=Saturday; su=Sunday; (1)=w, t, f, s, su; (2)=t, f, s; * =analysed.

2.2.2 Kinetic study protocol

2.2.2.1 Preparing the $^2\text{H}_3\text{-L-leucine}$ solution

A stock solution of pyrogen-free deuterated leucine, $^2\text{H}_3\text{-L-leucine}$ (Sigma-Aldrich, Inc), was prepared to a final concentration of 10 mg/ml (Annexe I) ⁴¹⁹. An external laboratory (Fontlab2000 SL) checked for endotoxin content of this stock solution using the LAL assay.

The endotoxin test measures bacterial endotoxins (mainly, pyrogens). The Royal Spanish Pharmacopeia, together with other international institutions, recommend the *in vitro* LAL assay (Limulus Amebocyte Lysate test or lysate of amebocytes of *Limulus polyphemus* crab) to test for pyrogen content to ensure that the final product is pyrogen-free ⁴²⁰⁻⁴²³.

$^2\text{H}_3\text{-L-leucine}$ stock solution was used to prepare working solutions. A bolus injection and a constant infusion were based on the body weight of each individual. The bolus contained 0.7 mg $^2\text{H}_3\text{-L-leucine}$ /kg body weight and the constant infusion over a 16h period was at the rate of 0.7 mg $^2\text{H}_3\text{-L-leucine}$ /kg body weight/h.

2.2.2.2 Kinetic study design

Kinetic studies were performed at the end of each dietary intervention period (Figure 29).

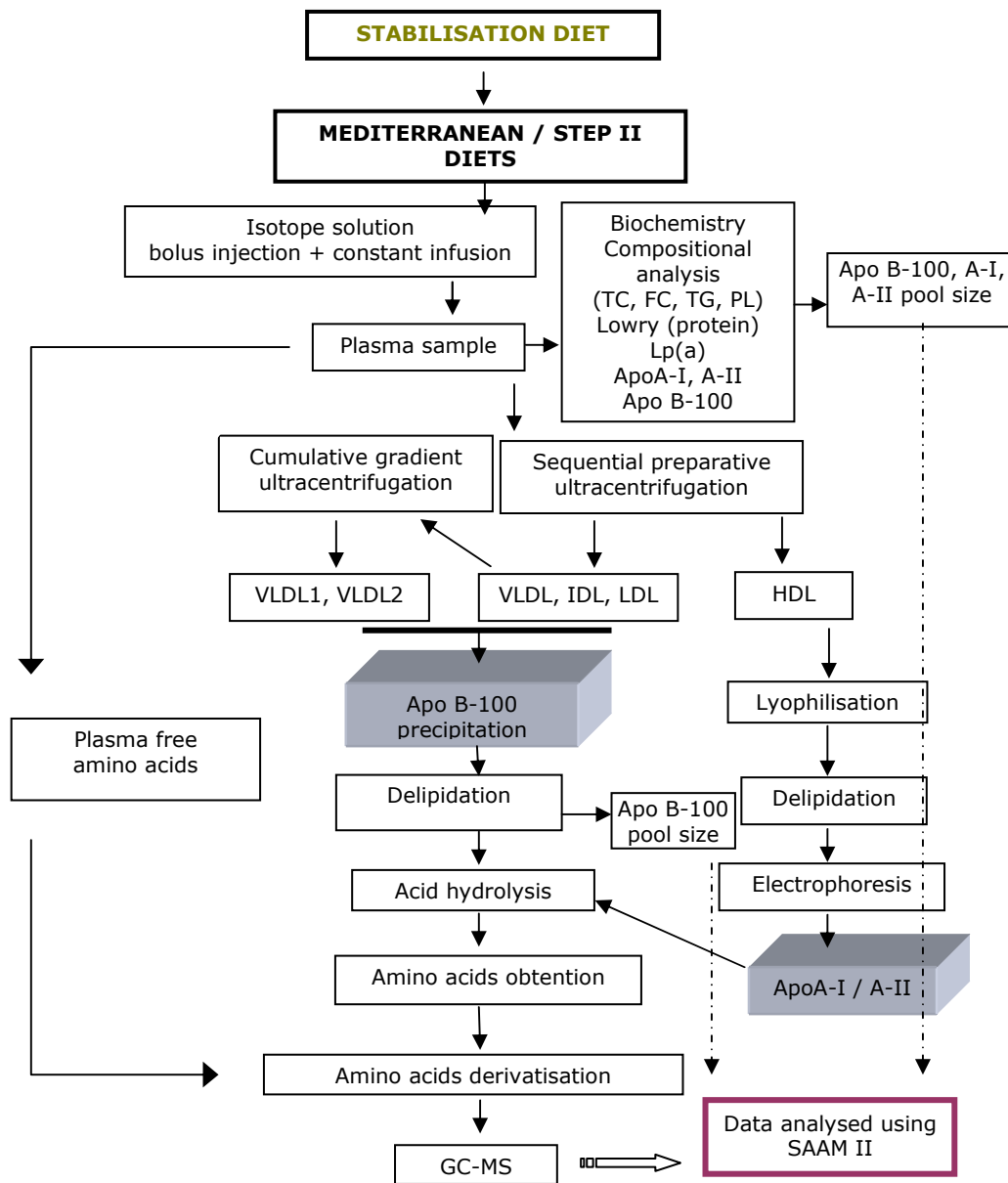
At 7:15 a.m., after a 10h overnight fast, volunteers were admitted to a private room in the hospital where they rested comfortably for the next 16h. Two blood samples of 15ml each were extracted into EDTA tubes at basal time ($t=0$). The $^2\text{H}_3\text{-L-leucine}$ bolus dose of 0.7 mg/kg was injected and was immediately followed by the constant infusion dose of 0.7 mg/kg/h over the next 16h.

To assure safety of the infusion, in addition to the LAL assay, sterile intravenous injection filters (Posidyne NEOfilter, Pall Biomedical) were attached to the intravenous catheter ^{424, 425}. The filters provide added protection from adverse effects of possible contaminants in the infusion (e.g. endotoxins that might cause inflammatory, pyrogenic and/or toxic reactions). Posidyne is a 0.2 μm nylon membrane that is positively charged and, by electrostatic interaction, retains endotoxins and/or gram negative bacteria.

The kinetic study proceeded over a 16h period of $^2\text{H}_3\text{-L-leucine}$ infusion. Blood samples, a total of 375 ml over the course of the study, were taken into EDTA tubes at multiple time points (0, 1, 2, 5, 10, 15, 20, 30 and 45 minutes, 1, 1.5, 2, 3, 3.5, 4, 5, 6, 8, 10, 11, 14, 16 hours after injection). Volunteers were resting comfortably throughout and were in a fasting state. Eight hours after commencement of the infusion, the volunteers were allowed a light low-fat meal consisting of a salad and a pot of skimmed yogurt.

After 16h of $^2\text{H}_3\text{-L-leucine}$ infusion, the volunteers went home and returned to the hospital in the morning (at 8:00 a.m.) in a fasting state for blood samples collection at 24 and 48h post infusion.

Figure 29. Flow diagram of stable isotope kinetic methodology used to study the effects of two diets on lipoprotein metabolism (Annexe I).



TC=total cholesterol; FC=free cholesterol; PL=phospholipids;
 GC-MS=gas chromatography-mass spectography.

2.2.3 Anthropometric, biochemical and isotopic enrichment determinations

Several anthropometric, biochemical and isotopic enrichment determinations were conducted for each volunteer during dietary intervention periods and in the course of the kinetic studies.

2.2.3.1 Determinations during dietary interventions

The following were determined before and after each dietary intervention (Figure 30):

- * anthropometric measurements ^{426, 427}:
 - weight (kg) and height (m) were measured with calibrated scales and wall-mounted stadiometer, respectively.
 - waist circumference (cm) was measured midway between the lowest rib and the iliac crest using an anthropometric tape.
 - trained personnel measured systolic and diastolic blood pressure (mmHg) in triplicate with a validated semi-automatic sphygmomanometer (Omron HEM 907-E, The Netherlands). The subjects were seated and rested for at least 5 min before blood pressure measurement.

- * biochemical measurements. Two blood samples (5 ml each) were taken after an 8h fast on 3 separate days to reduce intra-individual variations. Blood samples were taken into tubes with EDTA as anticoagulant, centrifuged immediately (2,500 rpm, 10 minutes, 4°C) and the plasma used to determine several plasma constituents ^{407, 408, 428-430}:
 - plasma lipid concentrations were determined using an autoanalyser (Synchron LX®i725, Beckman Coulter Inc) and enzymatic commercial assay kits (Beckman Coulter Inc). TC, LDLC (calculated using the formula proposed by Friedewald), HDLC (determined after precipitation of apo B-100-rich lipoproteins using dextran sulphate and magnesium) and TG (determined after hydrolysis to glycerol and FFAs by lipase, glycerol kinase, glycerol phosphate oxidase and acid peroxidase) concentrations were determined. A Quality Control was used throughout (Synchron®Control Multi-Level, Beckman Coulter Inc).All measurements were made on freshly-obtained plasma samples.
 - plasma apo B-100 and apoA-I apo concentrations were determined using an autoanalyser (Synchron LX®i725, Beckman Coulter Inc) and immunoturbidimetric commercial assay kits (Beckman Coulter Inc). A Quality Control was used throughout (Apo Synchron LX®, Beckman Coulter Inc).

All measurements were made on freshly-obtained plasma samples.

- plasma inflammation markers concentrations:

- high sensitivity C reactive protein (hs CRP) was determined using a highly sensitive Near Infrared Particle Immunoassay (NIPIA) with an autoanalyser (Synchron LX®i725, Beckman Coulter Inc) and an immunoturbidimetric commercial assay kit (Beckman Coulter Inc). A Quality Control was used throughout (Synchron®Systems CAL5 PLUS, Beckman Coulter Inc).

- interleukin 6 (IL-6), soluble intercellular adhesion molecule (sICAM-1) and vascular adhesion molecule (sVCAM-1) concentrations were determined using an ELISA analyser (Kinetic-QCL, BioWhittaker) and commercial immunoassay kits (Bender MedSystems Inc).

All samples were stored at -70°C until their later determination.

- plasma oxidation markers concentrations: oxidised LDL (ox LDL) and oxidised LDL antibodies (ox LDL-Ab) concentrations were determined using an ELISA analyser (Kinetic-QCL, BioWhittaker) and commercial immunoassay kits (MercodiaAB; IMTEC Immundiagnostika GmbH, respectively).

All samples were stored at -70°C until their later determination.

2.2.3.2 Determinations during kinetic studies

Several determinations were performed on the day of the kinetic studies, at baseline time ($t=0\text{h}$) (Figure 30)²⁶⁴:

* biochemical determinations (see page 107). Blood samples were taken into EDTA tubes, centrifuged at 2,500 rpm, 10 minutes, 4°C (Annexe I). The plasma were used to determine:

- Lp(a) concentrations.
- TC, FC, TG and PL concentrations.
- apo B-100, apoA-I and A-II concentrations.

Several determinations were performed after each kinetic study (Figure 30, Table 36):

* VLDL₁, VLDL₂, IDL, LDL and HDL pool samples. Blood samples were collected during the 48h kinetic study into EDTA tubes, centrifuged at 25,000 rpm, 10 minutes, 4°C . From all plasma samples obtained during the 48h kinetic study, sample pools or plasma mixes were prepared in triplicate from

0.5 ml of plasma (up to 12.5 ml). Several aliquots were prepared for each pool, depending on the requirements for subsequent measurements:

- 2 ml for ultracentrifugation(x3 pools).
- 2 ml for storage (x3 pools).

From pool 1, VLDL₁, VLDL₂, IDL, LDL and HDL lipoprotein fractions were separated by ultracentrifugation. Pool 1 was used to determine:

- lipoprotein compositional analyses:
 - TC, FC, TG and PL concentrations.
 - total protein concentration.
- apo determinations:
 - apo B-100, apoA-I and A-II concentrations.

From pools 2 and 3, VLDL₁, VLDL₂, IDL and LDL lipoprotein fractions were separated by ultracentrifugation. Pools 2 and 3 were used to determine:

- apo B-100-rich lipoproteins pool size using the method of Lowry.

* samples obtained for isotopic enrichment determination (Annexe I). Blood samples were taken into EDTA tubes at specific times over the course of the 16h ²H₃-L-leucine infusion, up until the end of kinetic studies at 48h post injection:

- samples were centrifuged at 2,500 rpm, 10 minutes, 4°C to obtain plasma.
- corresponding plasma aliquots were prepared for each time-point of the kinetic study. The aliquots were prepared depending on their later processing requirements:
 - 1ml for plasma method.
 - 2 ml for sequential preparative ultracentrifugation.
 - 2 ml for storage (for ultracentrifugation).
 - 1 ml for storage (for plasma method).

All these aliquots were prepared and stored at -70°C until their later processing.

When taking blood samples to obtain the different lipoprotein fractions, the blood is collected into tubes with EDTA, a crystalline acid that acts as a strong chelating agent (inhibiting proteolytic enzymes that degrade proteins). EDTA also forms a sodium salt that acts as an anticoagulant, as an antioxidant (chelating Cu⁺² ions) and as a bacteria phospholipase C inhibitor (which can physico-chemically modify lipoproteins). Other protective reagents are not used since these may affect the subsequent lipoprotein delipidation process ^{349, 368}.

Figure 30. Plasma determinations performed during dietary intervention periods and kinetic studies.

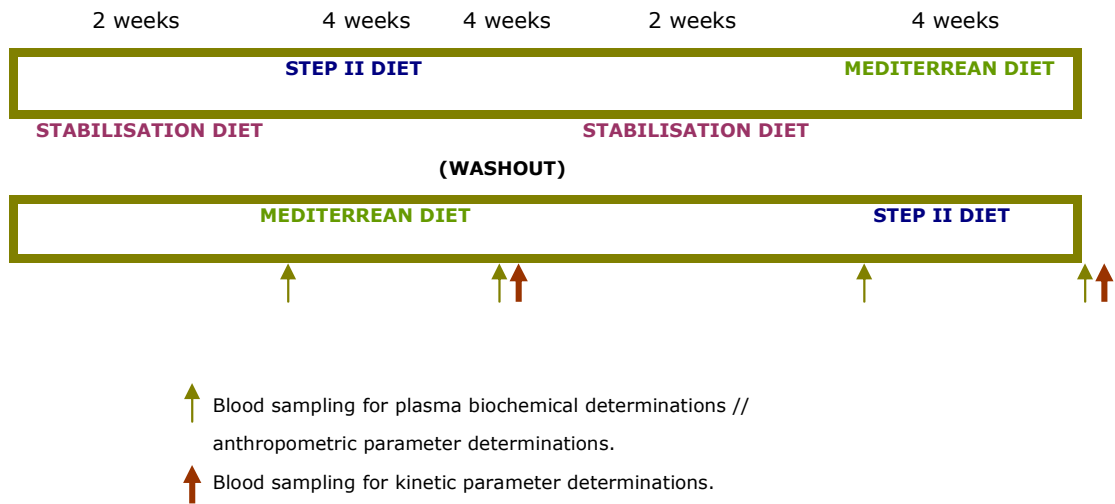
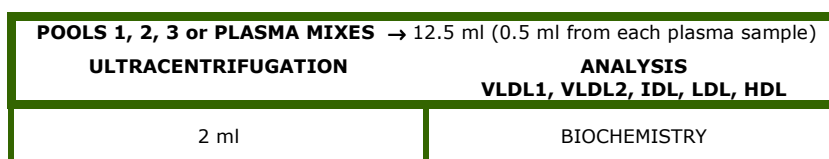


Table 36. Blood sampling and determinations during kinetic studies²⁶⁴.

TIMING (Time of day)	BLOOD VOLUME	PLASMA METHOD	ULTRACENTRIFUGATION	ANALYSIS OF VLDL1, VLDL2, IDL, LDL, HDL, PLASMA
0h (8:00)	30 ml	1 ml	2 X 2 ml	ISOTOPIC ENRICHMENT
1 min (8:01)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
2 min (8:02)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
5 min (8:05)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
10 min (8:10)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
15 min (8:15)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
20 min (8:20)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
30 min (8:30)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
45 min (8:45)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
1h (9:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
1.5h (9:30)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
2h (10:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
3h (11:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
3.5h (11:30)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
4h (12:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
5h (13:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
6h (14:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
8h (16:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
10h (18:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
11h (19:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
14h (22:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
16h (24:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
24h (8:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT
48h (8:00)	15 ml	1 ml	2 ml	ISOTOPIC ENRICHMENT



Total blood volume: 375 ml.

2.3 Statistical analysis

All lipid values represent the mean of two measurements conducted on 3 separate days.

Data were expressed as mean \pm standard deviation (SD). A 2-tailed *P* value of <0.05 was considered statistically significant.

The analyses were made according to crossover design.

The paired *t*-test is a parametric statistical approach used when variables of interest are normally distributed. This test is used for comparing the means of two situations i.e. the *t*-test compares the actual difference between two means taking into account the variation in the data ⁴³¹. The paired *t*-test was used to analyse the variables from all 10 volunteers, as well as the 7 kinetic studies completed to-date. The paired *t*-test was used to compare parameters before and after each dietary intervention period and to assess differences resulting from the diets.

The possible interaction between diets and the dietary sequence (carry-over effect) was also tested using the paired *t*-tests.

The Pearson's correlation coefficient measures the strength of the linear relationship between two variables ⁴³². This correlation was used to determined relationships between quantitative variables.

All statistical analyses were performed using SPSS 11.01® (SPSS Inc) ⁴³³.

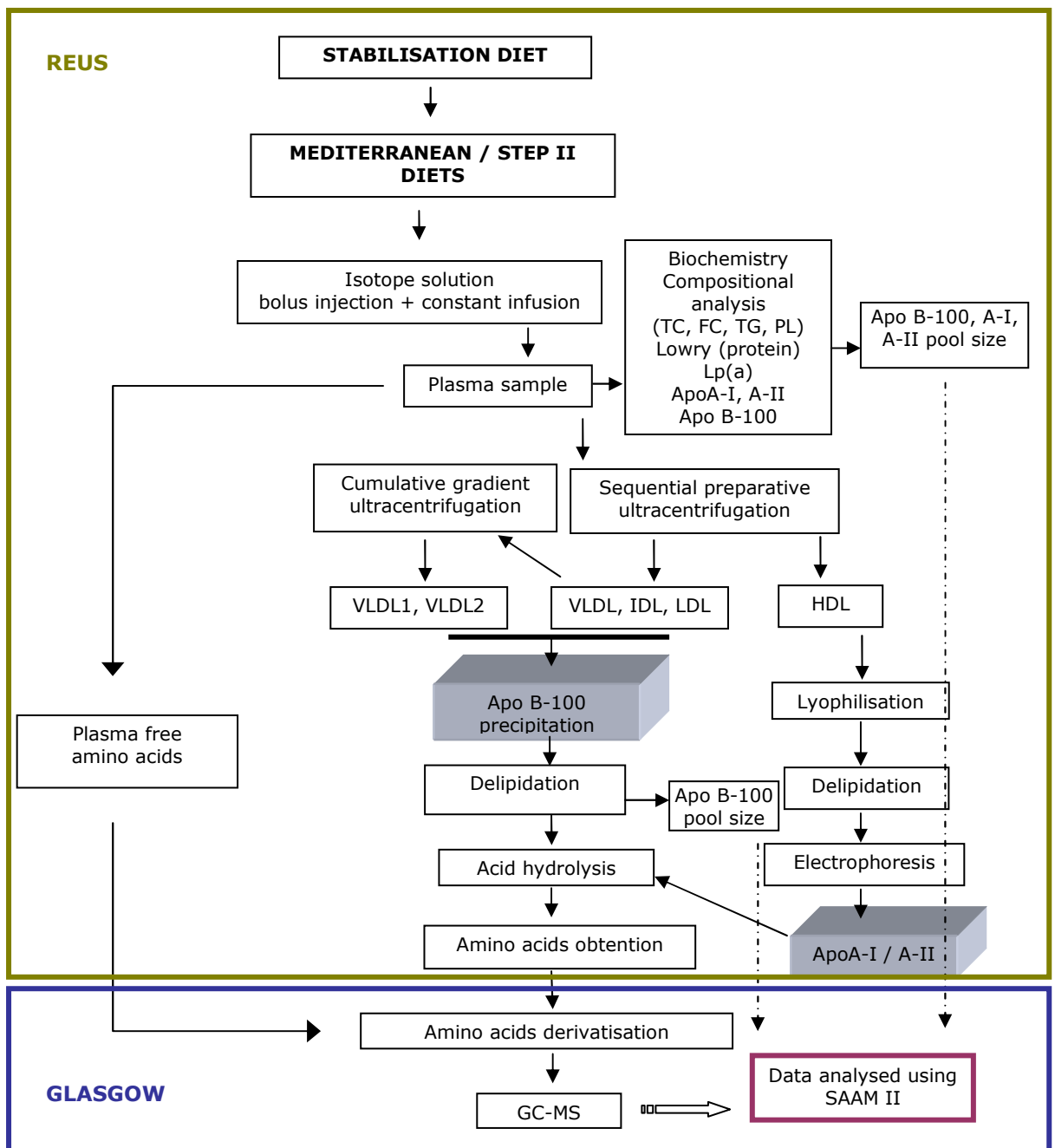
RESULTS

UNIVERSITAT ROVIRA I VIRGILI
IMPLEMENTATION OF STABLE ISOTOPES LIPOPROTEIN KINETIC STUDIES: EFFECTS ON HDL METABOLISM OF A MEDITERRANEAN TYPE DIET RICH
IN MUFAS FROM VIRGIN OLIVE OIL.
Autora: Katia Uliaque Cugat
ISBN: 978-84-690-6746-8 DL: T.1183-2007

1. Results of the general objective: implementation of the methodology of lipoprotein kinetic studies using stable isotopes

The flow diagram of the methodological results performed in each Unit are presented in Figure 31. The overall methodology was applied to assess the effects of two diets on lipoprotein metabolism; the results of which are shown in Results section 2.

Figure 31. Flow diagram indicating the involvement of each of the two Research Units; Reus (Catalunya) and Glasgow (Scotland).



1.1 Methodological results of stable isotope kinetics applied to apo B-100-rich lipoproteins

We implemented the methodology of stable isotope kinetics to study apo B-100-rich lipoproteins metabolism.

Apo B-100 multi-compartmental model was developed by Demant and Packard (1996) in Glasgow, using the SAAM II modelling program. It enabled apo B-100 kinetic parameters to be calculated²⁶⁴.

The model structure comprised 15 compartments and had the following features (Figure 32):

- * plasma leucine represented by compartment $q3$ where leucine tracer was injected as a primed constant infusion in rapid equilibrium with compartment $q4$.
- * an intracellular compartment $q4$, the immediate source of leucine for apo B-100 synthesis.
- * compartments $q1$ and $q2$ accounted for uptake and subsequent slow release of leucine by slowly turning over body protein pools.
- * compartment $d15$, a delay component set at 0.5h, represented an intrahepatic delay compartment that accounted for the time required for apo B-100 synthesis and secretion.
- * following this delay, direct output of apo B-100 occurred into VLDL₁ or compartment $q5$, VLDL₂ or compartment $q8$, IDL or compartment $q11$, and LDL or compartment $q13$. These four interconnected compartments formed a sequential delipidation chain.
- * compartments $q7$, $q10$ and $q12$ represented remnant lipoprotein particles (derived from the delipidation cascade).
- * compartment $q14$ represented an extravascular LDL exchange compartment.

These characteristics enabled the correct fitting of apo B-100 enrichment curves data to derive the kinetic parameters. The model had been constructed by introducing physiologically plausible parameter dependencies or constraints to reduce the number of unknowns to within the limit necessary for system identifiability. The dependent parameters were:

- * $k(4,3)=k(3,4)$
- * $k(7,5)=k(10,8)$
- * $k(0,7)=k(0,10)$
- * $k(0,12)=k(12,9)$
- * $k(2,1)=0.1 \times k(1,2)$
- * $k(13,14)=2.5 \times k(14,13)$
- * $k(5,15)=1- k(8,15) - k(11,15) - k(13,15)$

Other parameters determined were steady state production rates (mg/day) and transport rates (pools/day) of apo B-100 from one compartment to another, which were obtained applying the model to observed data:

* fractional transfer rates (FTR) (pools/day) were calculated:

- for VLDL₁ to VLDL₂, transport rate from compartment 6 to 8 was divided by VLDL₁ apo B-100 mass (combined masses of compartments 5, 6 and 7):

$$FTR_{VLDL_1, VLDL_2} = k(8,6) / (q_5 + q_6 + q_7)$$

- for VLDL₂ to IDL, transport rates from compartment 9 to 11 and 9 to 12 were combined and divided by VLDL₂ apo B-100 mass (combined masses of compartments 8, 9 and 10):

$$FTR_{VLDL_2, IDL} = [k(11,9) + k(12,9)] / (q_8 + q_9 + q_{10})$$

- for IDL to LDL, transport rate from compartment 11 to 13 was combined and divided by IDL apo B-100 mass (combined masses of compartments 11 and 12):

$$FTR_{IDL, LDL} = k(13,11) / (q_{11} + q_{12})$$

* fractional rates of direct catabolism (dirFCR) (pools/day) were similarly derived for each lipoprotein fraction:

- for VLDL₁, by dividing the sum of outputs from compartments 6 and 7 by VLDL₁ apo B-100 mass:

$$dirFCR_{VLDL_1} = [k(0,6) + k(0,7)] / (q_5 + q_6 + q_7)$$

- for VLDL₂, by dividing the sum of outputs from compartments 9 and 10 by VLDL₂ apo B-100 mass:

$$dirFCR_{VLDL_2} = [k(0,9) + k(0,10)] / (q_8 + q_9 + q_{10})$$

- for IDL, by dividing the sum of outputs from compartments 11 and 12 by IDL apo B-100 mass:

$$dirFCR_{IDL} = [k(0,11) + k(0,12)] / (q_{11} + q_{12})$$

- for LDL, by dividing the output from compartment 13 by LDL apo B-100 mass:

$$\text{dirFCR}_{\text{LDL}} = k(0,13) / q_{13}$$

* overall FCR (pools/day) were calculated:

- for VLDL₁, VLDL₂ and IDL apo B-100 was the sum of FTR and dirFCR
for each lipoprotein fraction:

$$\text{FCR} = \text{FTR} + \text{dirFCR}$$

* production rates (PR) (mg/day) were calculated as the product of the pool
size in a lipoprotein fraction and its overall FCR:

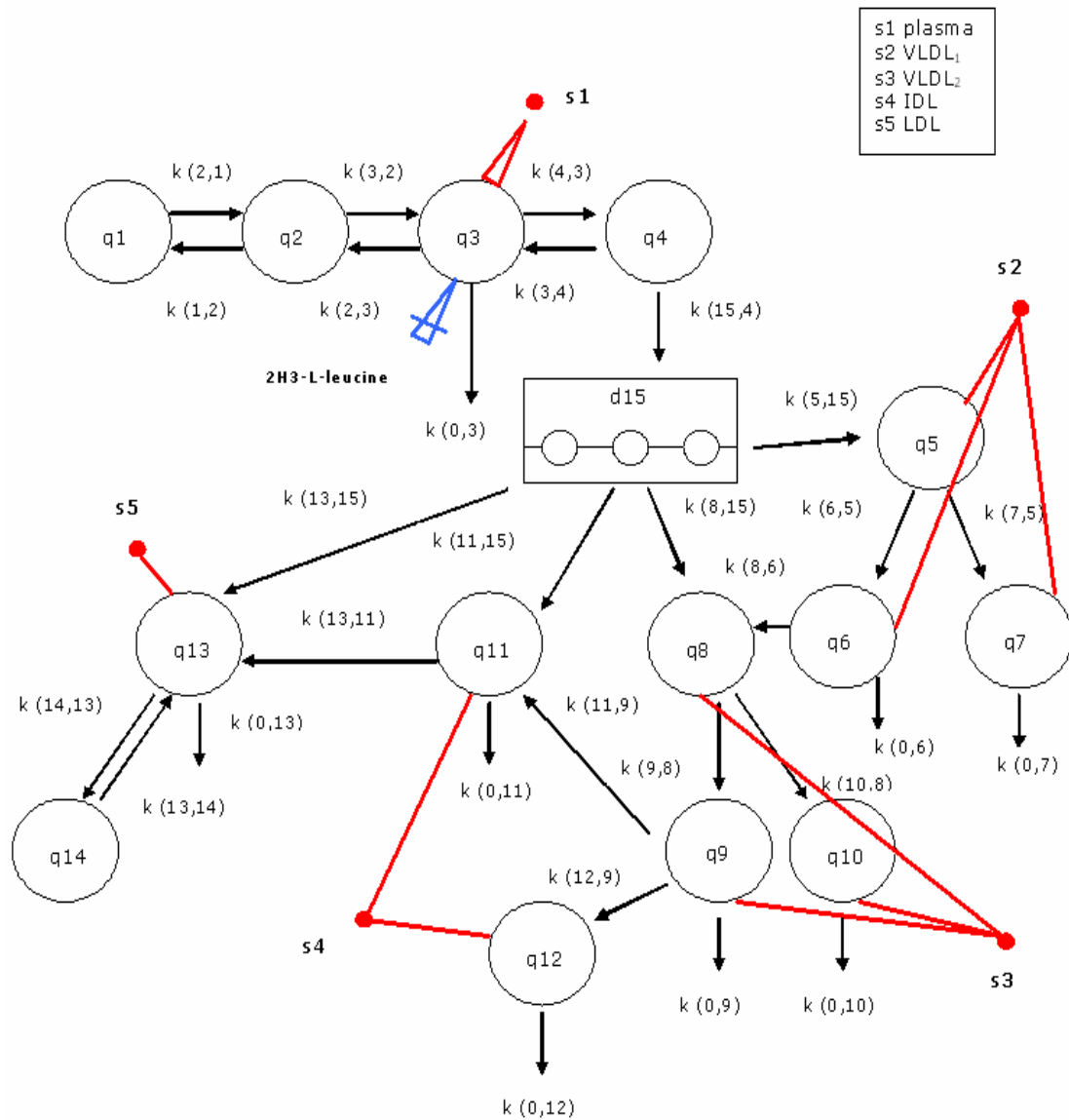
$$\text{PR} = \text{pool size (mg)} \times \text{FCR}$$

* residence time (RT) (days) for each lipoprotein fraction was calculated as
the reciprocal of the FCR for each lipoprotein fraction:

$$\text{RT} = 1 / \text{FCR}$$

The model had been tested and found to be identifiable (all rate constants
determined if data were sufficiently precise).

Figure 32. Multi-compartmental model for apo B-100-rich lipoproteins metabolism.



q=pool or compartment size; d=intrahepatic delay compartment;
 k=constant rates; s=site of determination;
 2H3-L-leucine=site for tracer injection and infusion.

1.2 Methodological results of stable isotope kinetics applied to apoA-I and A-II HDL

We implemented the new methodologies in our Research Unit, specifically applied to HDL.

The results for apoA-I and A-II PAGE isolation, as performed in our Unit using commercial NuPAGE®Novex gels (Invitrogen, Corp), are presented in Figure 33. This variation on the general PAGE technique made obtaining HDL apos easier and more reproducible than previous, handmade, gels.

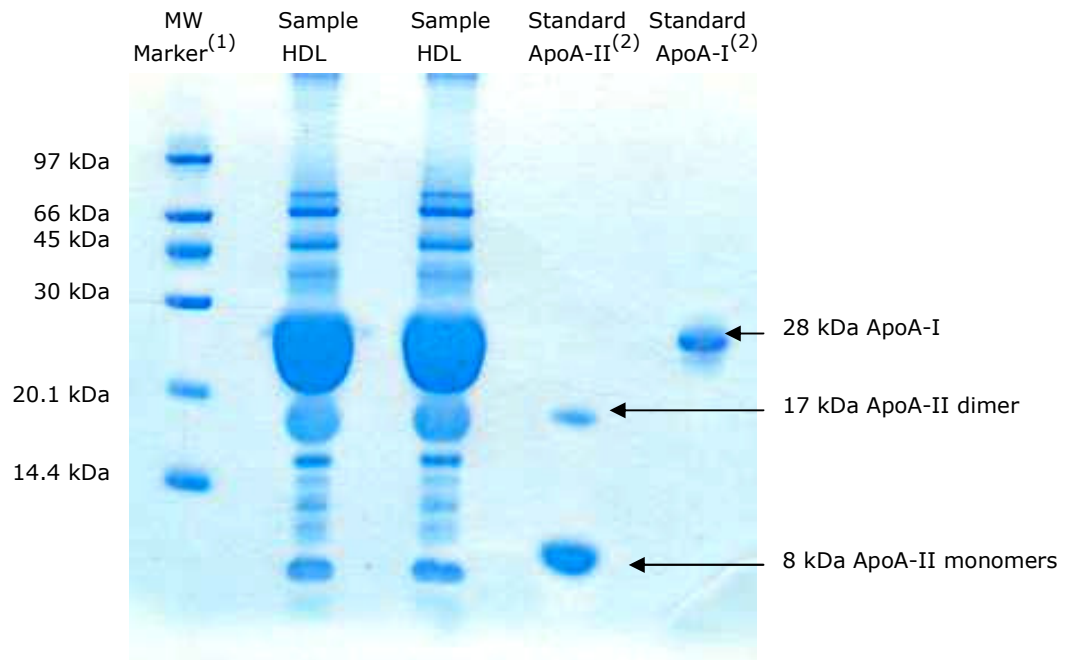
A new apoA-I and A-II model was developed by Packard and Caslake (2006) in Glasgow using the SAAM II modelling program. It enabled apoA-I and A-II kinetics parameters to be calculated.

The model structure comprised 7 compartments and had the following features (Figure 34):

- * plasma leucine represented by compartment $q3$ (where leucine tracer was injected as a primed constant infusion) in rapid equilibrium with compartment $q4$.
- * an intracellular compartment $q4$, which was the immediate source of leucine for apoA-I and A-II synthesis.
- * compartments $q1$ and $q2$ accounted for uptake and subsequent slow release of leucine by slowly turning over body protein pools.
- * compartment $d15$, a delay component set at 0.5h, represented an intrahepatic delay compartment that accounted for time required for apoA-I and A-II synthesis and secretion.
- * following this delay, direct output of apoA-I and A-II occurred into plasma HDL or compartment $q7$ and $q8$, respectively.

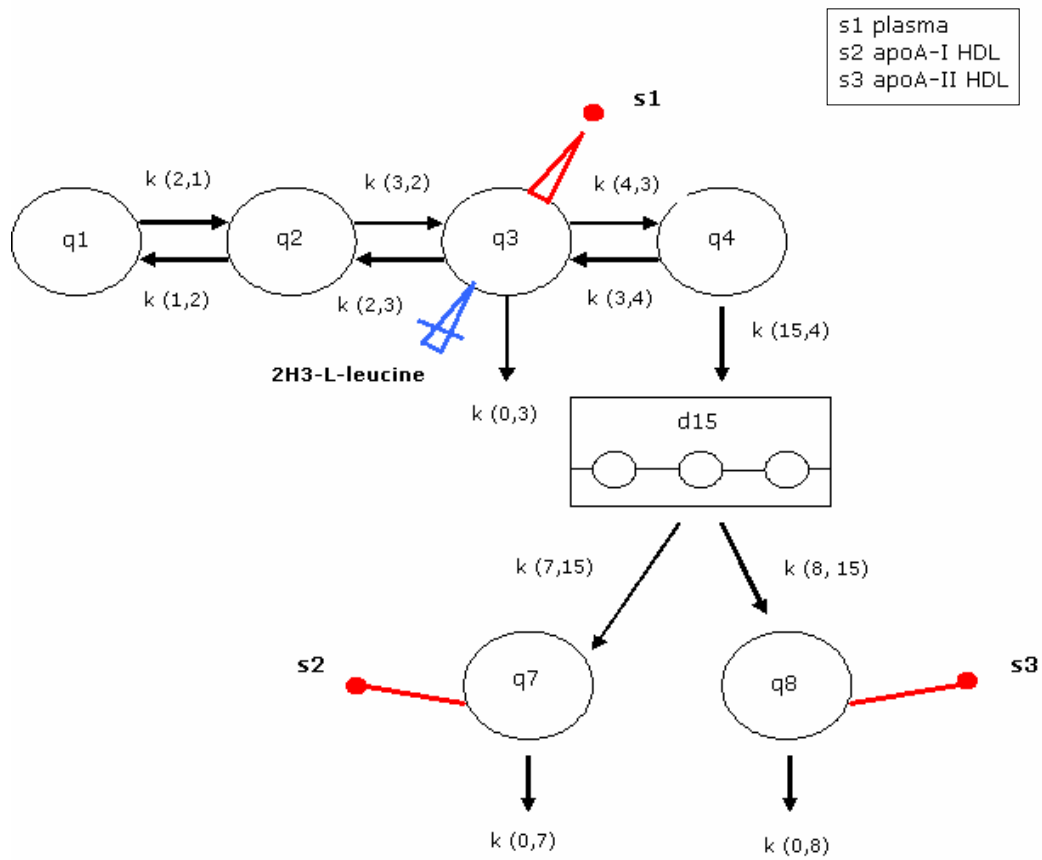
The new model characteristics, dependencies and the kinetic parameters determined were comparable to those for the apo B-100 model.

Figure 33. ApoA-I and A-II HDL isolation using NuPAGE®Novex gels.



MW=molecular weight; kDa=kilodaltons;
Sample=100 µg total protein of delipidated HDL;
(1) Bio-Rad Laboratories, Inc;
(2) Sigma-Aldrich Co.

Figure 34. Multi-compartmental model for apoA-I and A-II HDL metabolism.



q=pool or compartment size; d=intrahepatic delay compartment;
 k=constant rates; s=site of determination;
 2H3-L-leucine=site for tracer injection and infusion.

2. Results of the specific objectives: applying lipoprotein kinetic studies using stable isotopes to compare the effects of two diets on lipoprotein metabolism

2.1 Effects on lipoprotein metabolism

2.1.1 Subjects of study

The subjects studied were 10 volunteers, males ranging from 42 to 64 years, mean \pm standard deviation of 55.8 ± 8.6 . They were diagnosed as having moderate hypercholesterolaemia (plasma LDLC concentrations ranging from 130 to 170 mg/dl, mean \pm standard deviation of 154.45 ± 2.53). They were non-smokers and did not have diabetes or have evidence of any hepatic, cardiovascular, renal or endocrine chronic diseases. Baseline characteristics of the volunteers assigned to each dietary intervention period were comparable. There were no significant differences between groups at the start of the study (Table 37).

We had pre-selected a total of 13 volunteers. Two volunteers were withdrawn because they did not fulfil all of the inclusion criteria. In the first study period, 6 volunteers were randomly assigned to the Mediterranean diet, and 5 to the STEP II diet. The kinetic studies were performed after each dietary intervention period, with the exception of 1 volunteer who had to be withdrawn from the study because of technical difficulties during tracer infusion. Thus, a total of 10 volunteers continued the study. From these 10 volunteers, 2 volunteers completed only one dietary intervention and kinetic study and they chose not to continue with the second study period. Another volunteer completed both dietary intervention periods and kinetic studies but only one period was analysed because the volunteer was not compliant with STEP II diet and kinetic study was not analysed. A flow diagram of the volunteers who had completed the dietary interventions and the kinetic studies for the Mediterranean and STEP II diets in crossover are summarised in Figure 35.

Table 37. Baseline characteristics of volunteers assigned to each dietary intervention period.

BASELINE	MEDITERRANEAN DIET (N=10)	STEP II DIET (N=7)	MED vs. STEP II P
Weight (kg)	75.57 ± 6.85	75.18 ± 6.88	0.44
BMI (kg/m ²)	25.81 ± 1.72	25.67 ± 1.66	0.44
Waist circumference (cm)	91.86 ± 6.77	91.93 ± 7.18	0.94
Systolic blood pressure (mmHg)	124.00 ± 9.02	123.71 ± 7.43	0.93
Diastolic blood pressure (mmHg)	83.14 ± 6.91	83.71 ± 4.94	0.18
TC (mg/dl)	231.08 ± 24.52	228.28 ± 14.43	0.83
LDLC (mg/dl)	156.24 ± 19.72	152.66 ± 13.65	0.75
HDLC (mg/dl)	50.23 ± 8.78	50.65 ± 7.70	0.82
TG (mg/dl)	120.23 ± 28.66	121.96 ± 30.19	0.86
CT/LDL	1.48 ± 0.06	1.50 ± 0.05	0.55
CT/HDL	4.68 ± 0.65	4.59 ± 0.75	0.68
Apo B-100 (g/l)	1.14 ± 0.21	1.10 ± 0.23	0.25
ApoA-I (g/l)	1.48 ± 0.32	1.65 ± 0.29	0.21
Apo B100/apoA-I	0.78 ± 0.13	0.67 ± 0.11	0.20
IL-6 (pg/ml)	0.95 ± 1.04	0.42 ± 0.34	0.27
sVCAM-1 (ng/ml)	570.67 ± 154.97	600.33 ± 202.66	0.56
sICAM-1 (ng/ml)	250.14 ± 63.90	194.71 ± 47.94	0.09
hs CRP (mg/l)	2.26 ± 2.72	1.51 ± 1.27	0.54
ox LDL (U/l)	80.88 ± 16.23	80.85 ± 10.06	0.99
ox LDL-Ab (U/ml)	15.50 ± 2.97	14.96 ± 2.59	0.14

Results expressed as mean ± standard deviation (SD).

Significance $P < 0.05$.

MED=Mediterranean; vs.=versus; BMI=body mass index; TC=total cholesterol;

LDLC=LDL cholesterol; HDLC=HDL cholesterol; TG=triglycerides;

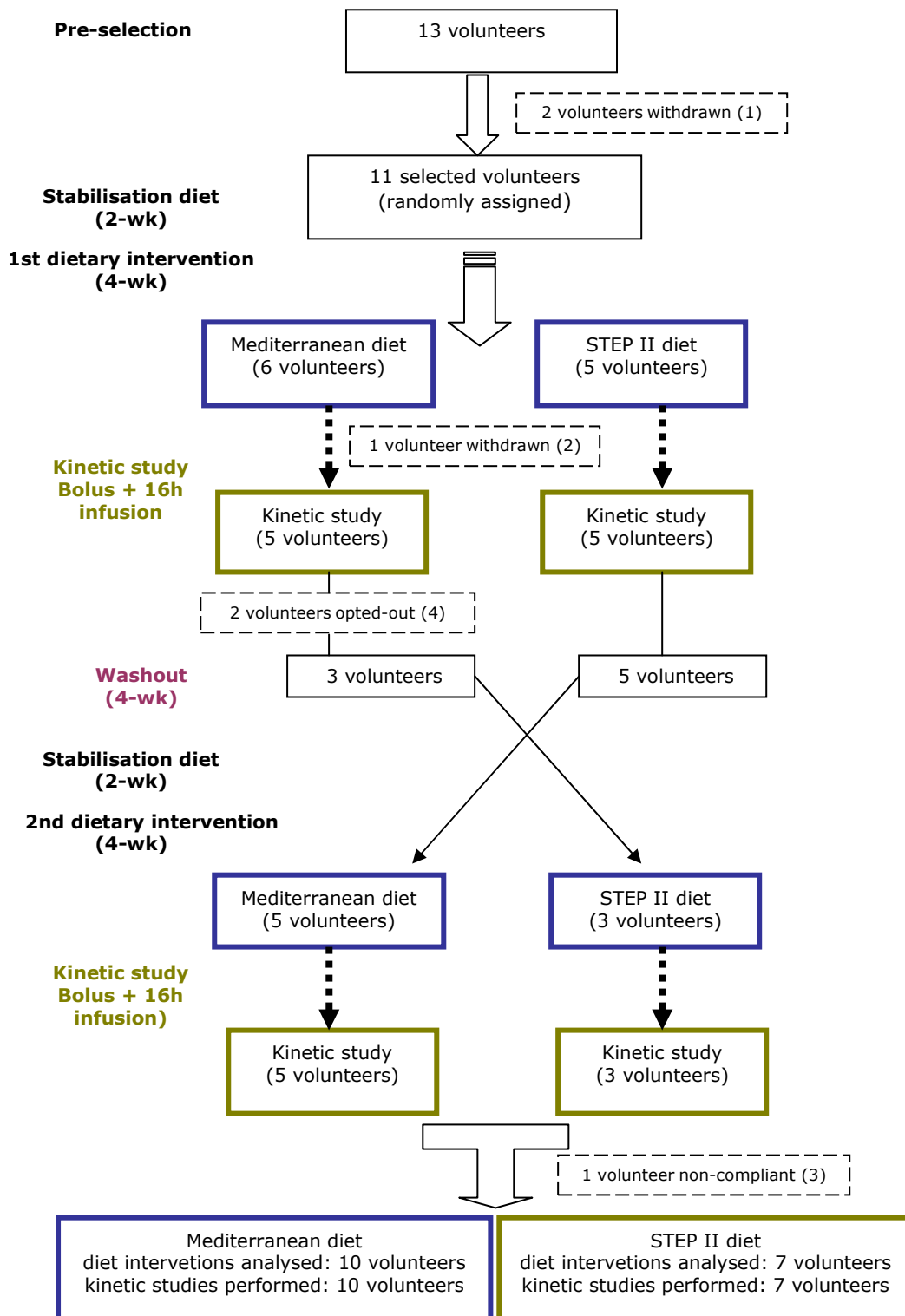
IL-6=interleukin 6; sVCAM-1=soluble vascular adhesion molecule 1;

sICAM-1=soluble intercellular adhesion molecule 1;

hs CRP=high sensitivity C reactive protein; ox LDL=oxidised LDL;

ox LDL- Ab=oxidised LDL antibodies.

Figure 35. Flow diagram of the crossover dietary intervention and kinetic study.



wk=week; (1) non-compliance with inclusion criteria; (2) no kinetic study due to difficulties during tracer infusion; (3) non-compliance with the STEP II diet and kinetic study not analysed; (4) did not continue onto the second study period.

2.1.2 Diets

The final composition of prescribed and achieved Mediterranean and STEP II diets are summarised in Table 38.

The dietary intervention outcomes comprised (Table 38):

- * the Stabilisation diet showed significant differences between prescribed and achieved composition in CH (41% versus 42.33%; $P=0.01$, respectively), lipids (39% versus 36.31%; $P=0.05$, respectively), MUFAs (20% versus 17.19%; $P=0.01$, respectively) and SFAs (13% versus 11.17%; $P=0.01$, respectively).

There were no significant differences between prescribed and achieved compositions for the Mediterranean and STEP II diets.

Comparing the composition of the achieved dietary interventions (Table 39):

- * the Mediterranean versus the STEP II diet induced significant differences in lipids (33.95% versus 25.01%; $P=0.02$, respectively), MUFAs (19.09% versus 11.89%; $P=0.01$, respectively). Both diets achieved a reduced content of SFAs; significantly higher in the Mediterranean than in the STEP II diet (7.29% versus 5.60%; $P=0.02$, respectively).

There was no significant carry-over effect.

Table 38. Diets: composition prescribed and achieved.

	STABILISATION DIET (N=10)			MEDITERRANEAN DIET (N=10)			STEP II DIET (N=7)		
	presc	achiev	presc vs. achiev P	presc	achiev	presc vs. achiev P	presc	achiev	presc vs. achiev P
Kcal	-	2582.53 ± 223.43	-	-	2232.72 ± 211.04	-	-	2008.88 ± 252.90	-
Percentage of total calories									
CH	41	42.33 ± 1.89	0.01	45-50	44.96 ± 5.09	0.24	60	52.23 ± 5.15	0.59
PROT	17	14.87 ± 0.42	0.15	15	14.20 ± 2.02	1.00	15	15.51 ± 2.35	0.65
LIP	39	36.31 ± 2.27	0.05	35	33.95 ± 2.82	0.27	25	25.01 ± 4.84	0.99
MUFAs	20	17.19 ± 1.26	0.01	19	19.09 ± 2.09	0.89	12	11.89 ± 2.39	0.94
SFAs	13	11.17 ± 1.08	0.01	<7	7.29 ± 0.90	0.34	<7	5.60 ± 1.21	1.00
PUFAs	7	7.00 ± 0.81	1.00	<7	4.20 ± 0.60	1.00	<7	4.36 ± 2.59	1.00
mg/day									
C	<200	192.00 ± 85.99	1.00	<200	219.90 ± 85.99	1.00	<200	171.89 ± 77.72	1.00

Results expressed as mean ± standard deviation (SD).

Significance $P < 0.05$.

vs.=versus; presc=prescribed; achiev=achieved; Kcal=kilocalories; CH=carbohydrates;

PROT=proteins; LIP=lipids, MUFAs=monounsaturated fatty acids;

SFAs=saturated fatty acids; PUFAs=polyunsaturated fatty acids; C=cholesterol.

Table 39. Comparing composition of achieved dietary interventions.

COMPARING DIETARY INTERVENTIONS			
MEDITERRANEAN (N=10)			
vs.			
STEP II DIETS (N=7)			
	MEDITERRANEAN achieved	STEP II achieved	P
Kcal	2232.72 ± 211.04	2008.88 ± 252.90	0.14
Percentage of total calories			
CH	44.96 ± 5.09	52.23 ± 5.15	0.07
PROT	14.20 ± 2.02	15.51 ± 2.35	0.09
LIP	33.95 ± 2.82	25.01 ± 4.84	0.02
MUFAs	19.09 ± 2.09	11.89 ± 2.39	0.01
SFAs	7.29 ± 0.90	5.60 ± 1.21	0.02
PUFAs	4.20 ± 0.60	4.36 ± 2.59	0.80
mg/day			
C	219.90 ± 85.99	171.89 ± 77.72	0.21

Results expressed as mean ± standard deviation (SD).

Significance $P < 0.05$.

vs.=versus; Kcal=kilocalories; CH=carbohydrates;

PROT=proteins; LIP=lipids;

MUFAs=monounsaturated fatty acids;

SFAs=saturated fatty acids;

PUFAs=polyunsaturated fatty acids; C=cholesterol.

2.1.3 Dietary intervention results

Several anthropometric and biochemical parameters were determined in the 10 volunteers that completed dietary intervention periods. Parameter values within and between diets were analysed.

2.1.3.1 Anthropometric and biochemical parameters

Anthropometric and biochemical characteristics of all volunteers, pre- and post-intervention, are summarised in the following tables.

The dietary intervention outcomes comprised:

* anthropometric parameters (Table 40, Figure 36). Comparing the anthropometric parameters pre- and post-intervention periods:

- the Mediterranean diet *per se* significantly decreased diastolic blood pressure by -3.71 ± 3.35 mm Hg ($P=0.03$).
- the STEP II diet *per se* significantly decreased body weight by -1.05 ± 1.11 kg ($P=0.05$) and BMI by -0.37 ± 0.38 kg/m² ($P=0.04$).

Comparing the Mediterranean versus the STEP II diet, the Mediterranean diet increased, albeit not significantly, body weight by $+0.74 \pm 0.07$ kg ($P=0.06$) and BMI by $+0.34 \pm 0.3$ kg/m² ($P=0.06$). As such, the volunteers' body weight variation in the course of the study was 1.01%.

* plasma lipid and apo parameters (Table 41A, 41B, Figure 37, 38).

Comparing the plasma parameters pre- and post-intervention periods:

- the Mediterranean diet *per se* slightly maintained the plasma concentrations of HDLC by $+4.97\% \pm 12.07$ ($P=0.44$). The plasma concentrations of apoA-I were similar to baseline values, without significant changes over the course of the dietary interventions ($+0.67\% \pm 4.62$; $P=0.91$). The Mediterranean diet decreased, albeit not significantly, the plasma concentrations of LDLC, total C and apo B-100, ($-5.57\% \pm 16.73$, $P=0.29$; $-3.13\% \pm 12.35$, $P=0.37$; $-5.55\% \pm 7.40$, $P=0.65$, respectively).
- the STEP II diet *per se* showed a tendency towards a decrease of the plasma concentrations of HDLC ($-5.90\% \pm 6.52$, $P=0.06$) and significantly decreased plasma concentrations of apoA-I ($-9.29\% \pm 5.08$, $P=0.01$). Further, the STEP II diet significantly decreased the plasma concentrations of total C ($-5.59\% \pm 6.25$; $P=0.05$) and decreased, albeit not significantly, the plasma concentrations of LDLC ($-4.51\% \pm 8.89$; $P=0.19$). The plasma concentrations of apo B-100 were not significantly altered following the STEP II diet ($-0.28\% \pm 19.01$; $P=0.71$).

Comparing the Mediterranean versus the STEP II diet, the Mediterranean diet increased, albeit not significantly, the plasma concentrations of HDLC (+10.87% ± 14.65; $P=0.06$), while significantly increased the plasma concentrations of apoA-I (+9.96% ± 1.70; $P=0.01$) and significantly decreased the apo B-100/apoA-I ratio (-14.97% ± 18.88; $P=0.05$). Further, the Mediterranean diet decreased, albeit not significantly, the plasma concentrations of apo B-100 (-5.26% ± 18.20; $P=0.47$) and LDLC (-1.06% ± 21.46; $P=0.90$).

* plasma inflammation and oxidation markers (Table 41C). Comparing the plasma parameters pre- and post-intervention periods:

- the Mediterranean diet *per se* did not induce significant differences in any of the measured parameters.
- the STEP II diet *per se* did not induce significant differences in any of the measured parameters.

Comparing the Mediterranean versus the STEP II diet, there were no significant differences in either the oxidation or the inflammation markers.

Table 40. Anthropometric parameters of volunteers.

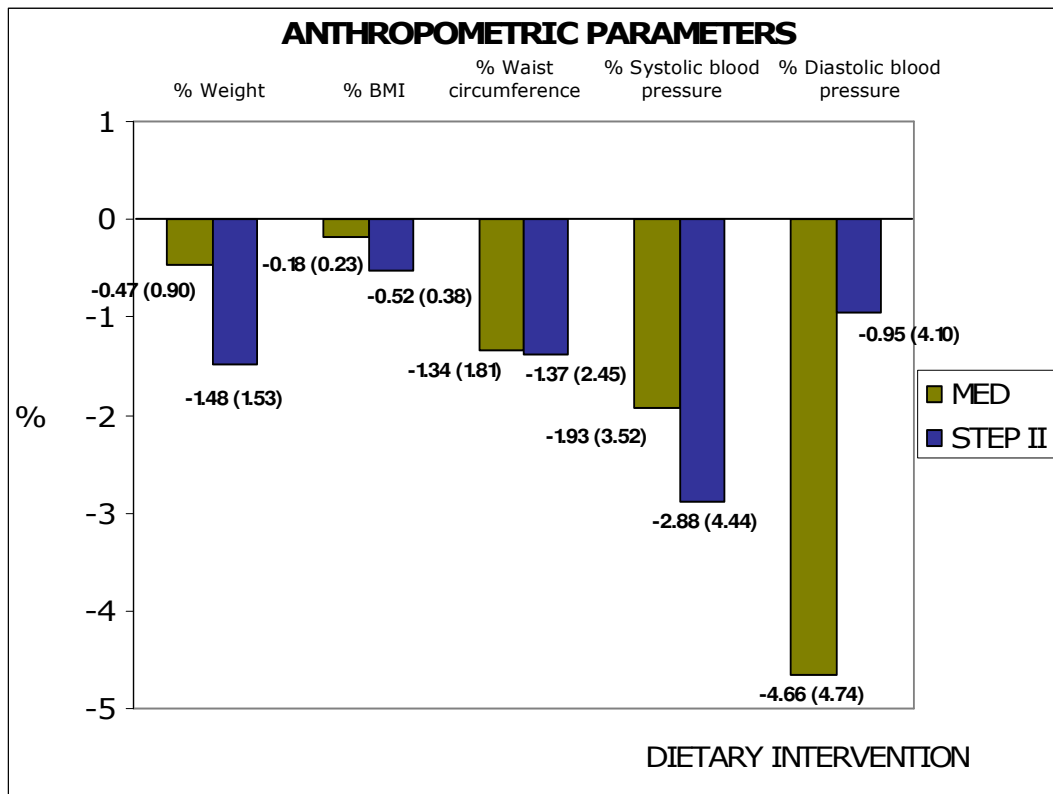
	MEDITERRANEAN DIET (N=10)				STEP II DIET (N=7)			MED vs. STEP II	P
	Pre-	Post-	Δ	Post- vs. Pre- P	Pre-	Post-	Δ	Post- vs. Pre- P	
Weight (kg)	75.57 ± 6.85	75.26 ± 7.35	-0.31 ± 0.67	0.26	75.18 ± 6.88	74.13 ± 7.66	-1.05 ± 1.11	0.05	0.06
BMI (kg/m ²)	25.81 ± 1.72	25.69 ± 1.74	-0.12 ± 0.23	0.22	25.67 ± 1.66	25.30 ± 1.88	-0.37 ± 0.38	0.04	0.06
Waist circumf. (cm)	91.86 ± 6.77	90.64 ± 7.18	-1.21 ± 1.63	0.10	91.93 ± 7.18	90.71 ± 8.08	-1.21 ± 2.16	0.19	1.00
Systolic blood pressure (mm Hg)	124.00 ± 9.02	121.43 ± 6.90	-2.57 ± 4.58	0.19	123.71 ± 7.43	120.00 ± 6.11	-3.71 ± 5.59	0.13	0.71
Diastolic Blood pressure (mm Hg)	83.14 ± 6.91	79.43 ± 9.00	-3.71 ± 3.35	0.03	83.71 ± 4.94	80.86 ± 5.52	-2.86 ± 4.60	0.53	0.15

Results expressed as mean ± standard deviation (SD).

Significance $P<0.05$.

MED=Mediterranean; vs.=versus; Δ=change; circum=circumference; BMI=body mass index.

Figure 36. Comparison of anthropometric parameters; percentage change between dietary interventions.



MED (Mediterranean diet) N=10 / STEP II diet N=7.
 Results expressed as mean (SD).
 BMI=body mass index.

Table 41. Concentrations of lipid (A), apo (B), and inflammation and oxidation parameters (C) in the volunteers.

(A)

	MEDITERRANEAN DIET (N=10)				STEP II DIET (N=7)			MED vs. STEP II	
	Pre-	Post-	Δ	Post- vs. Pre- P	Pre-	Post-	Δ	Post- vs. Pre- P	P
TC (mg/dl)	231.08 ± 24.52	221.82 ± 15.44	-9.26 ± 25.09	0.37	228.28 ± 14.43	214.87 ± 8.23	-13.41 ± 14.62	0.05	0.75
LDLC (mg/dl)	156.24 ± 19.72	145.39 ± 13.88	-10.86 ± 24.63	0.29	152.66 ± 13.65	144.96 ± 9.27	-7.70 ± 13.80	0.19	0.81
HDLC (mg/dl)	50.23 ± 8.78	52.02 ± 5.73	1.79 ± 5.80	0.44	50.65 ± 7.70	47.57 ± 7.20	-3.08 ± 3.45	0.06	0.06
TG (mg/dl)	120.23 ± 28.66	127.21 ± 37.89	6.99 ± 25.96	0.50	121.96 ± 30.19	113.30 ± 36.07	-8.66 ± 25.24	0.40	0.35
TC/LDLC	1.48 ± 0.06	1.53 ± 0.11	0.05 ± 0.10	0.25	1.50 ± 0.05	1.48 ± 0.05	-0.01 ± 0.05	0.48	0.24
TC/HDLC	4.68 ± 0.65	4.29 ± 0.38	-0.39 ± 0.47	0.07	4.59 ± 0.75	4.60 ± 0.67	0.01 ± 0.35	0.95	0.15

(B)

	MEDITERRANEAN DIET (N=10)				STEP II DIET (N=7)			MED vs. STEP II	
	Pre-	Post-	Δ	Post- vs. Pre- P	Pre-	Post-	Δ	Post- vs. Pre- P	P
Apo B-100 (g/l)	1.14 ± 0.21	1.11 ± 0.15	-0.03 ± 0.15	0.65	1.10 ± 0.23	1.08 ± 0.12	-0.03 ± 0.18	0.71	0.56
ApoA-I (g/l)	1.48 ± 0.32	1.49 ± 0.26	0.01 ± 0.09	0.91	1.65 ± 0.29	1.49 ± 0.20	-0.16 ± 0.11	0.01	0.01
Apo B-100/ apoA-I	0.78 ± 0.13	0.76 ± 0.11	-0.02 ± 0.07	0.48	0.67 ± 0.11	0.73 ± 0.06	0.06 ± 0.09	0.17	0.05

Results expressed as mean ± standard deviation (SD).

Significance $P < 0.05$.

MED=Mediterranean; vs=versus; Δ=change; TC=total cholesterol;

LDLC=LDL cholesterol; HDLC=HDL cholesterol; TG=triglycerides.

(C)

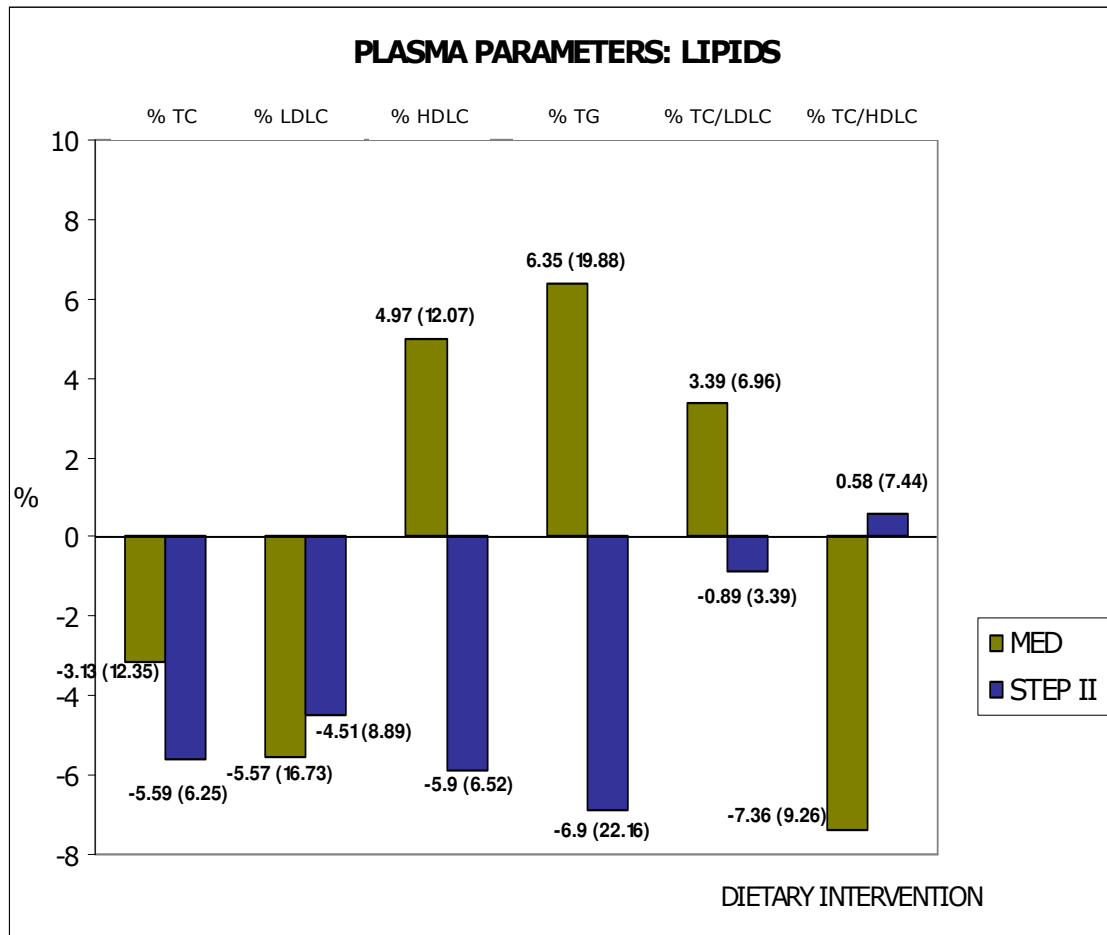
	MEDITERRANEAN DIET (N=10)				STEP II DIET (N=7)			MED vs. STEP II	
	Pre-	Post-	Δ	Post- vs. Pre- P	Pre-	Post-	Δ	Post- vs. Pre- P	P
IL-6 (pg/ml)	0.95 ± 1.04	0.60 ± 0.50	-0.35 ± 1.21	0.47	0.42 ± 0.34	0.56 ± 0.21	0.14 ± 0.20	0.12	0.37
sVCAM-1 (ng/ml)	570.67 ± 154.97	589.93 ± 206.47	19.17 ± 73.42	0.55	600.33 ± 202.66	591.17 ± 195.35	-9.17 ± 78.39	0.79	0.62
sICAM-1 (ng/ml)	250.14 ± 63.90	260.43 ± 57.68	10.29 ± 46.89	0.58	194.71 ± 47.94	202.14 ± 44.00	7.43 ± 66.84	0.78	0.90
hs CRP (mg/l)	2.26 ± 2.72	3.27 ± 4.17	1.01 ± 5.89	0.67	1.51 ± 1.27	1.28 ± 0.93	-0.23 ± 0.93	0.53	0.58
ox LDL (U/l)	80.88 ± 16.23	77.61 ± 10.65	-3.28 ± 9.25	0.38	80.85 ± 10.06	73.62 ± 8.23	-7.22 ± 8.16	0.06	0.25
ox LDL-Ab (U/ml)	15.50 ± 2.97	15.56 ± 2.86	0.06 ± 0.60	0.79	14.96 ± 2.59	15.73 ± 2.59	0.78 ± 0.91	0.06	0.16

Results expressed as mean ± standard deviation (SD).

Significance $P < 0.05$.

MED=Mediterranean; vs=versus; Δ=change; IL-6=interleukin 6; sVCAM-1=soluble vascular adhesion molecule 1; sICAM-1=soluble intercellular adhesion molecule 1; hs CRP=high sensitivity C reactive protein; ox LDL=oxidised LDL; ox LDL-Ab=oxidised LDL antibodies.

Figure 37. Comparison of plasma lipid parameters; percentage changes between dietary interventions.

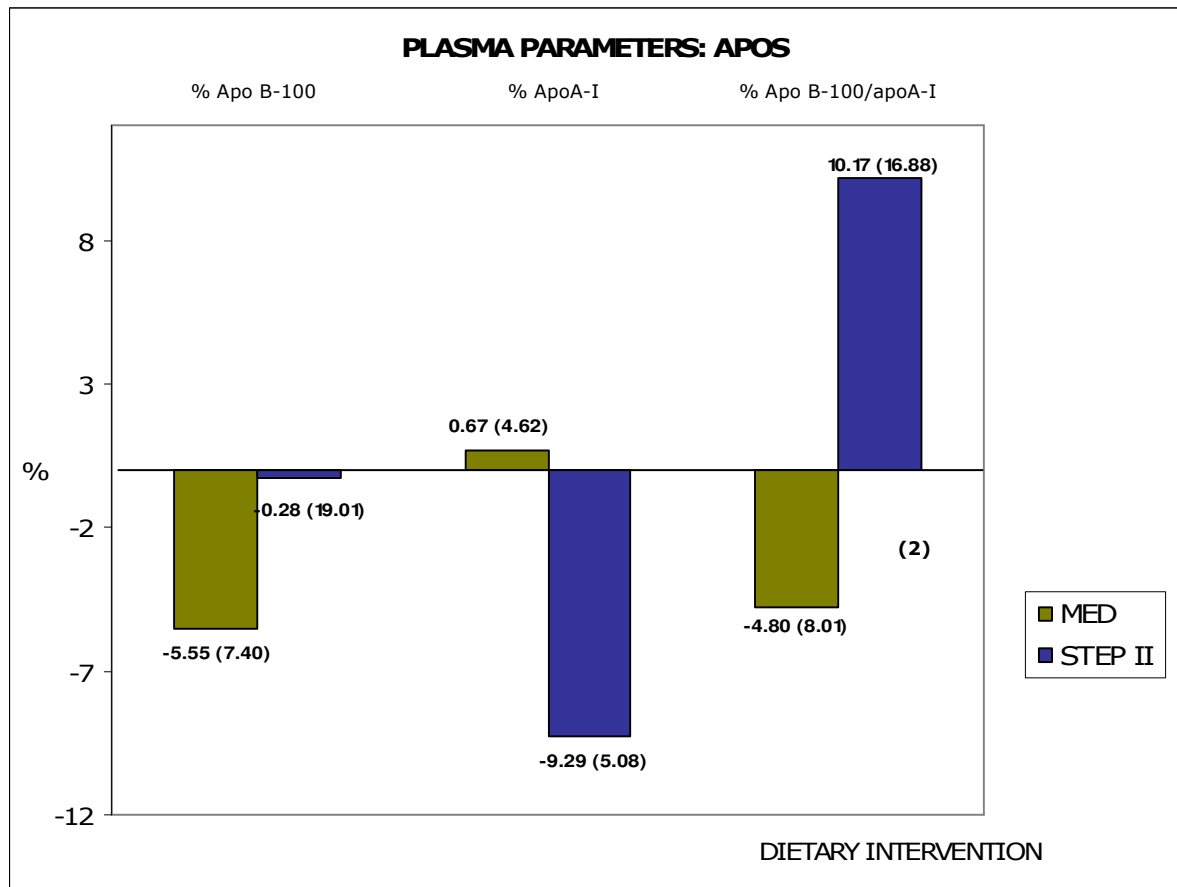


MED (Mediterranean diet) N=10 / STEP II diet N=7.

Results expressed as mean (SD).

TC=total cholesterol; TG=triglycerides; LDLC=LDL cholesterol; HDLC=HDL cholesterol.

Figure 38. Comparison of plasma apo parameters; percentage changes between dietary interventions.



MED (Mediterranean diet) N=10 / STEP II diet N=7.

Results expressed as mean (SD).

(1) $P=0.01$; (2) $P=0.05$.

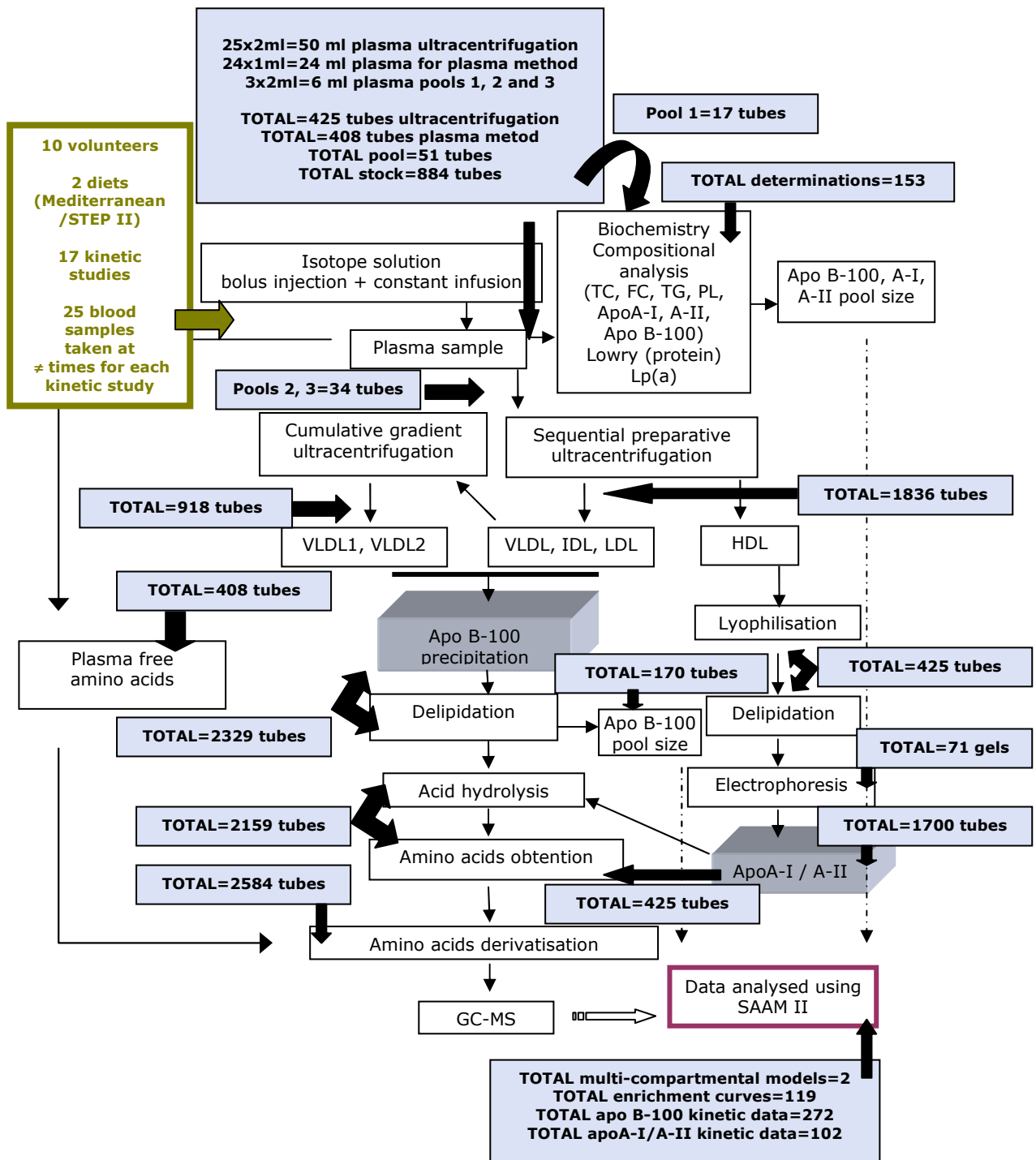
2.1.4 Preliminary results from kinetic studies

The outcomes of plasma and kinetic parameters were measured after each dietary intervention period.

The total samples generated during lipoprotein kinetic studies for all 10 volunteers are summarised in Figure 39. A total of 17 kinetic studies were completed and 2,584 samples were finally derivatised and analysed by GC-MS.

To-date, the plasma and kinetic parameters have been analysed in 4 volunteers. There have been 7 kinetic studies completed using the SAAM II program; 4 kinetic studies following the Mediterranean diet and 3 following the STEP II diet. The rest are to be completed.

Figure 39. Flow diagram indicating the total number of samples generated in the course of the lipoprotein kinetic studies in all 10 volunteers.



TC=total cholesterol; FC=free cholesterol; PL=phospholipids;
 GC-MS=gas chromatography-mass spectography.

2.1.4.1 Diets in the kinetic subset of volunteers

The dietary intervention outcomes in the subset of 4 volunteers comprised:

- * the Stabilisation, Mediterranean and STEP II diets induced no significant differences between prescribed and achieved compositions.

Comparing the achieved dietary intervention compositions in the subset of 4 volunteers:

- * the Mediterranean versus the STEP II diet induced the same differences in percentage content as observed in the 10 volunteers.

2.1.4.2 Anthropometric and biochemical parameters in the kinetic subset of volunteers

The anthropometric and biochemical characteristics, pre- and post-intervention, in the subset of 4 volunteers are summarised in the following tables.

The dietary intervention measures comprised:

- * anthropometric parameters (Table 42, Figure 40). Comparing the anthropometric parameters pre- and post-intervention periods:
 - the Mediterranean diet *per se* significantly decreased body weight by -0.58 ± 0.36 kg ($P=0.05$) and tended to decrease BMI by -0.21 ± 0.14 kg/m² ($P=0.06$).
 - the STEP II diet *per se* did not induce significant differences in any of the measured parameters.

Comparing the Mediterranean versus the STEP II diet, there were no significant differences in any of the measured anthropometric parameters.

- * plasma lipid and apo parameters (Table 43A, 43B, Figure 41, 42).

Comparing the plasma parameters pre- and post-intervention periods:

- the Mediterranean diet *per se* significantly increased the plasma concentrations of apoA-I ($+4.43\% \pm 0.29$; $P=0.04$) and tended to increase the plasma concentrations of HDLC ($+12.13\% \pm 11.31$; $P=0.08$). Further, the Mediterranean diet increased, albeit not significantly, the plasma concentrations of LDLC ($+2.66\% \pm 23.41$; $P=0.80$), total C ($+2.20\% \pm 19.29$; $P=0.76$) and apo B-100 ($+6.35\% \pm 0.41$; $P=0.67$).
- the STEP II diet *per se* significantly decreased the plasma concentrations of apoA-I ($-4.87\% \pm 0.40$; $P=0.05$) and tended to decrease the plasma concentrations of HDLC ($-9.80\% \pm 4.83$; $P=0.09$). Further, the STEP II diet decreased, albeit not significantly, the plasma concentrations of LDLC ($-6.28\% \pm 11.73$; $P=0.40$), total C

(-6.33% ± 7.70; *P*=0.27) and increased, albeit not significantly, the plasma concentrations of apo B-100 (+16.25% ± 26.09; *P*=0.55).

Comparing the Mediterranean versus the STEP II diet, the Mediterranean diet significantly increased the plasma concentrations of apoA-I (+9.30% ± 0.69; *P*=0.03).

* plasma inflammation and oxidation markers. There were no significant differences in any of the measured markers between diets as showed in the 10 volunteers; neither with respect to parameters pre- and post-intervention nor with respect to the Mediterranean versus the STEP II diet.

Table 42. Anthropometric parameters in the kinetic subset of volunteers.

	MEDITERRANEAN DIET (N=4)			Post- vs. Pre- <i>P</i>	STEP II DIET (N=3)			Post- vs. Pre- <i>P</i>	MED vs. STEP II <i>P</i>
	Pre-	Post-	Δ		Pre-	Post-	Δ		
Weight (kg)	73.33 ± 7.14	72.75 ± 7.21	-0.58 ± 0.36	0.05	70.25 ± 6.33	69.23 ± 4.24	-1.02 ± 1.10	0.25	0.42
BMI (kg/m ²)	26.19 ± 1.79	25.98 ± 1.75	-0.21 ± 0.14	0.06	25.48 ± 1.39	25.10 ± 0.99	-0.38 ± 0.40	0.24	0.40
Waist Circumf. (cm)	90.25 ± 6.80	88.88 ± 6.54	-1.38 ± 1.38	0.14	88.67 ± 7.02	86.67 ± 4.67	-2.00 ± 3.46	0.42	0.50
Systolic blood pressure (mm Hg)	120.00 ± 8.16	119.50 ± 5.74	-0.50 ± 2.52	0.72	123.33 ± 5.77	121.33 ± 1.33	-2.00 ± 7.21	0.68	0.82
Diastolic Blood pressure (mm Hg)	78.50 ± 6.61	75.50 ± 10.63	-3.00 ± 4.76	0.30	76.67 ± 6.11	78.67 ± 4.67	2.00 ± 2.00	0.23	0.12

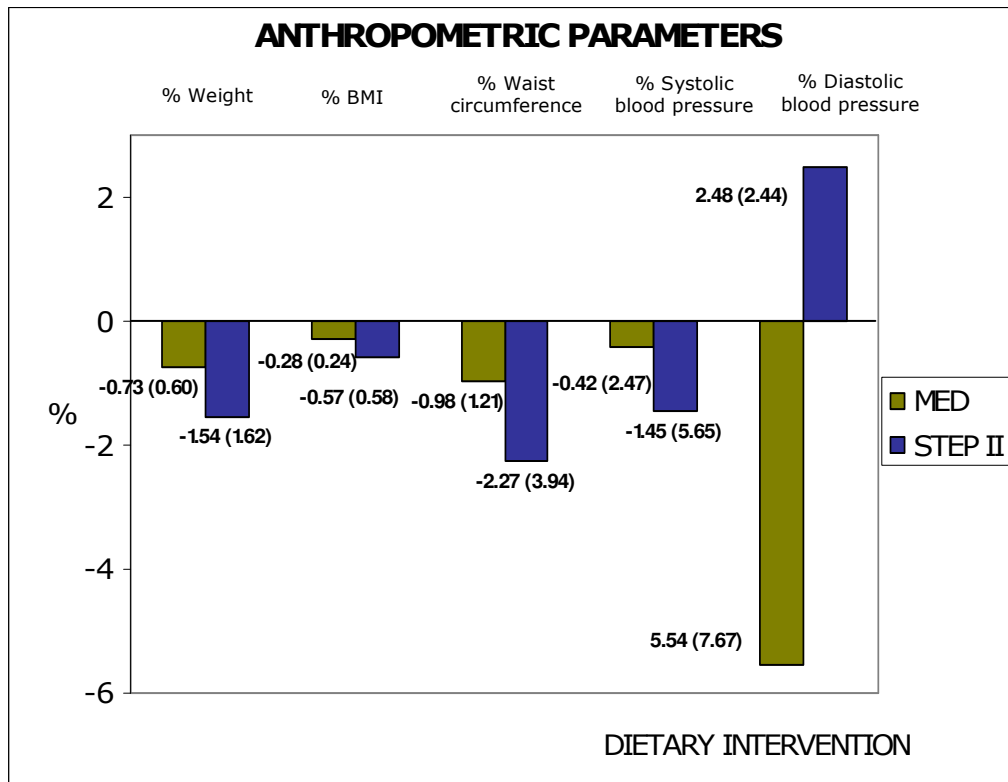
Results expressed as mean ± standard deviation (SD).

Significance *P*<0.05.

MED=Mediterranean; vs=versus; Δ=change;

circum=circumference; BMI=body mass index.

Figure 40. Comparison of anthropometric parameters; percentage changes in the kinetic subset of volunteers between dietary interventions.



MED (Mediterranean diet) N=4 / STEP II diet N=3.

Results expressed as mean (SD).

BMI=body mass index.

Table 43. Concentrations of lipid (A), apo (B), and inflammation and oxidation parameters (C) in the kinetic subset of volunteers.

(A)

	MEDITERRANEAN DIET (N=4)				STEP II DIET (N=3)			MED vs. STEP II	
	Pre-	Post-	Δ	Post- vs. Pre- P	Pre-	Post-	Δ	Post- vs. Pre- P	P
TC (mg/dl)	209.46 ± 21.24	214.85 ± 13.06	5.39 ± 32.44	0.76	225.75 ± 20.22	210.52 ± 4.63	-15.23 ± 14.62	0.27	0.62
LDLC (mg/dl)	142.11 ± 14.52	145.85 ± 13.89	3.74 ± 26.76	0.80	152.83 ± 20.56	141.88 ± 6.43	-10.95 ± 17.82	0.40	0.67
HDLC (mg/dl)	44.00 ± 5.17	48.86 ± 5.70	4.86 ± 3.64	0.08	45.27 ± 5.91	40.65 ± 2.03	-4.62 ± 2.63	0.09	0.14
TG (mg/dl)	111.40 ± 30.38	105.46 ± 24.66	-5.94 ± 10.20	0.33	130.57 ± 33.46	141.23 ± 16.43	10.67 ± 22.19	0.49	0.23
TC/LDLC	1.47 ± 0.02	1.48 ± 0.07	0.00 ± 0.06	0.92	1.48 ± 0.07	1.49 ± 0.03	0.00 ± 0.07	0.95	1.00
TC/HDLC	4.81 ± 0.69	4.43 ± 0.46	-0.37 ± 0.44	0.19	5.06 ± 0.92	5.20 ± 0.22	0.14 ± 0.55	0.70	0.31

(B)

	MEDITERRANEAN DIET (N=4)				STEP II DIET (N=3)			MED vs. STEP II	
	Pre-	Post-	Δ	Post- vs. Pre- P	Pre-	Post-	Δ	Post- vs. Pre- P	P
Apo B-100 (g/l)	0.94 ± 0.10	0.98 ± 0.05	0.03 ± 0.14	0.67	0.82 ± 0.12	0.94 ± 0.05	0.12 ± 0.19	0.55	0.42
ApoA-I (g/l)	1.29 ± 0.28	1.38 ± 0.31	0.10 ± 0.06	0.04	1.35 ± 0.25	1.28 ± 0.18	-0.06 ± 0.01	0.05	0.03
Apo B-100/ apoA-I	0.76 ± 0.19	0.73 ± 0.15	-0.03 ± 0.01	0.55	0.63 ± 0.21	0.74 ± 0.06	0.11 ± 0.12	0.42	0.28

Results expressed as mean ± standard deviation (SD).

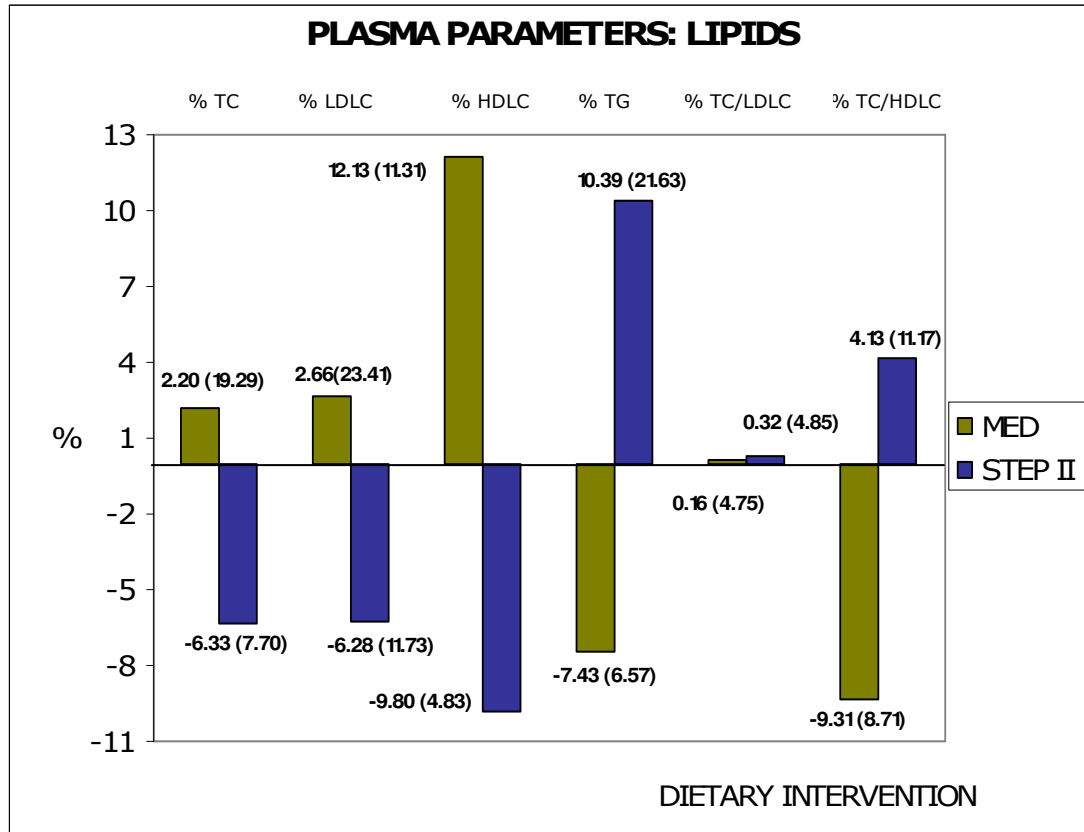
P<0.05

MED=Mediterranean; vs.=versus; Δ=change;

TC=total cholesterol; LDLC=LDL cholesterol;

HDLC=HDL cholesterol; TG=triglycerides.

Figure 41. Comparison of plasma lipid parameters; percentage changes in the kinetic subset of volunteers between dietary interventions.



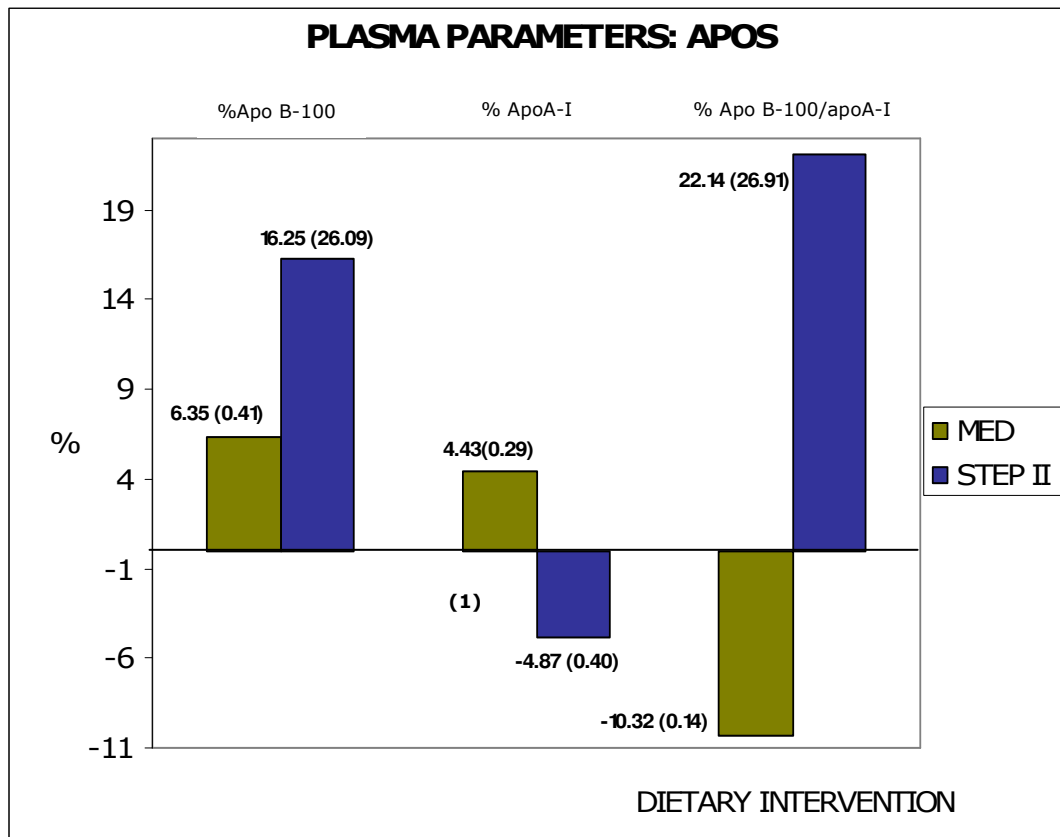
MED (Mediterranean diet) N=4 / STEP II diet N=3.

Results expressed as mean (SD).

TC=total cholesterol; TG=triglycerides; LDLC=LDL cholesterol;

HDLC=HDL cholesterol.

Figure 42. Comparison of plasma apo parameters; percentage changes in the kinetic subset of volunteers between dietary interventions.



MED (Mediterranean diet) N=4 / STEP II diet N=3.

Results expressed as mean (SD).

(1) $P=0.03$.

2.1.4.3 Lipoprotein kinetic studies in the kinetic subset of volunteers

2.1.4.3.1 Biochemical and lipoprotein parameters in the kinetic subset of volunteers

Plasma parameters in the subset of 4 volunteers were analysed on the day of the kinetic study following dietary intervention periods.

Comparing the Mediterranean versus the STEP II diet:

* plasma biochemical parameters (Table 44):

- lipid and apo concentrations. The Mediterranean diet significantly increased the plasma concentrations of total C (231.44 versus 223.64 mg/dl; $P=0.01$) and apoA-I (1.37 versus 1.23 g/l; $P=0.05$). Further, compared to the STEP II diet, the Mediterranean diet tended towards an increase of the plasma concentrations of apoA-II (0.32 versus 0.30 g/l; $P=0.07$).

* lipoprotein parameters (Table 45, 46, 47, Figure 43):

- apo content of lipoprotein fractions. There were no significant differences in the apo B-100 content in VLDL₁, VLDL₂, IDL and LDL between diets. The Mediterranean diet significantly increased apoA-I content in HDL fraction (1.37 versus 1.23 g/l) and caused a trend, albeit not significantly, towards an increase of the apoA-II content (0.32 versus 0.30 g/l; $P=0.07$). The apoA-I and A-II concentrations were determined in plasma samples since $\geq 90\%$ of plasma apoA-I and A-II are associated with HDL.

- compositional analysis of lipoprotein fractions. The Mediterranean diet changed apo B-100-rich lipoproteins composition, especially PL content; the content of PL was significantly increased in VLDL₂ (18.56% versus 8.57%; $P=0.03$, respectively) and in LDL (21.17% versus 19.76%; $P=0.05$, respectively).

- composition of core-surface lipoprotein particles. The Mediterranean diet significantly decreased VLDL₁ FC:PL ratio (0.54 versus 0.58; $P=0.04$).

- plasma concentrations of lipoprotein fractions (derived from compositional analysis). There were no significant differences in plasma lipoprotein fractions between diets.

Table 44. Plasma lipid and apo parameters in the kinetic subset of volunteers (day of the kinetic study).

	MEDITERRANEAN DIET (KINETICS) (N=4)	STEP II DIET (KINETICS) (N=3)	MED vs. STEP II P
Weight (kg)	72.88 ± 7.77	69.60 ± 7.29	0.51
TC (mg/dl)	231.44 ± 21.12	223.64 ± 26.24	0.01
LDLC (mg/dl)	184.17 ± 30.75	169.36 ± 69.09	0.45
HDLC (mg/dl)	55.08 ± 15.78	52.93 ± 23.61	0.95
TG (mg/dl)	129.94 ± 58.45	126.08 ± 22.24	0.44
Apo B-100 (g/l)	1.04 ± 0.10	1.06 ± 0.08	0.69
ApoA-I (g/l)	1.37 ± 0.15	1.23 ± 0.12	0.05
ApoA-II (g/l)	0.33 ± 0.03	0.30 ± 0.03	0.07
Lp(a) (g/l)	0.06 ± 0.03	0.08 ± 0.03	0.41

Results expressed as mean ± standard deviation (SD).

Significance $P < 0.05$.

MED=Mediterranean; vs.=versus; TC=total cholesterol;

LDLC=LDL cholesterol; HDLC=HDL cholesterol; TG=triglycerides.

Table 45. Apo B-100 (A), apoA-I and A-II (B) content of lipoprotein fractions in the kinetic subset of volunteers (day of the kinetic study).

(A)

	MEDITERRANEAN DIET (KINETICS) (N=4)	STEP II DIET (KINETICS) (N=3)	MED vs. STEP II P
Apo B-100 content in			
VLDL1 (g/l)	0.04 ± 0.03	0.04 ± 0.03	0.93
VLDL2 (g/l)	0.02 ± 0.02	0.02 ± 0.02	0.95
IDL (g/l)	0.04 ± 0.01	0.03 ± 0.04	0.77
LDL (g/l)	0.75 ± 0.08	0.74 ± 0.35	0.84

MED=Mediterranean; vs.=versus.

Results expressed as mean ± standard deviation (SD).

(B)

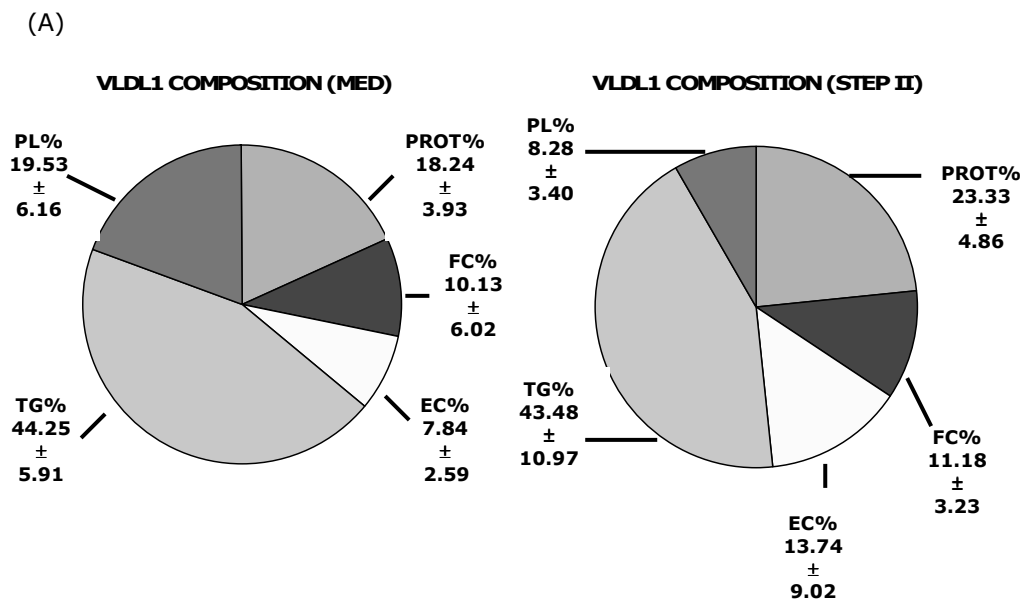
	MEDITERRANEAN DIET (KINETICS) (N=4)	STEP II DIET (KINETICS) (N=3)	MED vs. STEP II P
ApoA-I content in HDL (g/l)	1.37 ± 0.15	1.23 ± 0.12	0.05
ApoA-II content in HDL (g/l)	0.33 ± 0.03	0.30 ± 0.03	0.07

MED=Mediterranean; vs.=versus.

Results expressed as mean ± standard deviation (SD).

Significance $P < 0.05$.

Figure 43. Compositional analysis of lipoprotein fractions VLDL₁ (A), VLDL₂ (B), IDL (C), LDL (D) and HDL (E) in the kinetic subset of volunteers (day of the kinetic study).



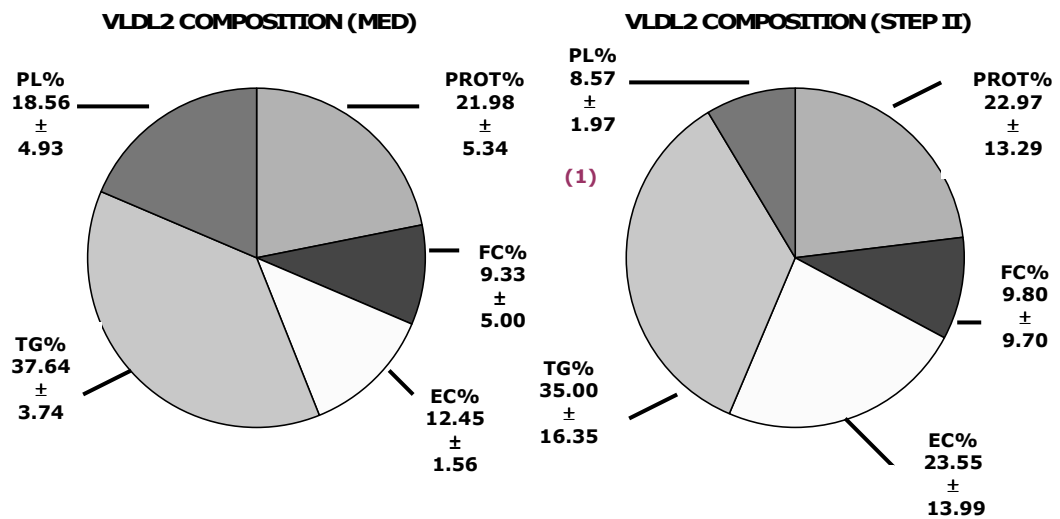
MED (Mediterranean diet) N=4 / STEP II diet N=3.

Results expressed as mean ± standard deviation (SD).

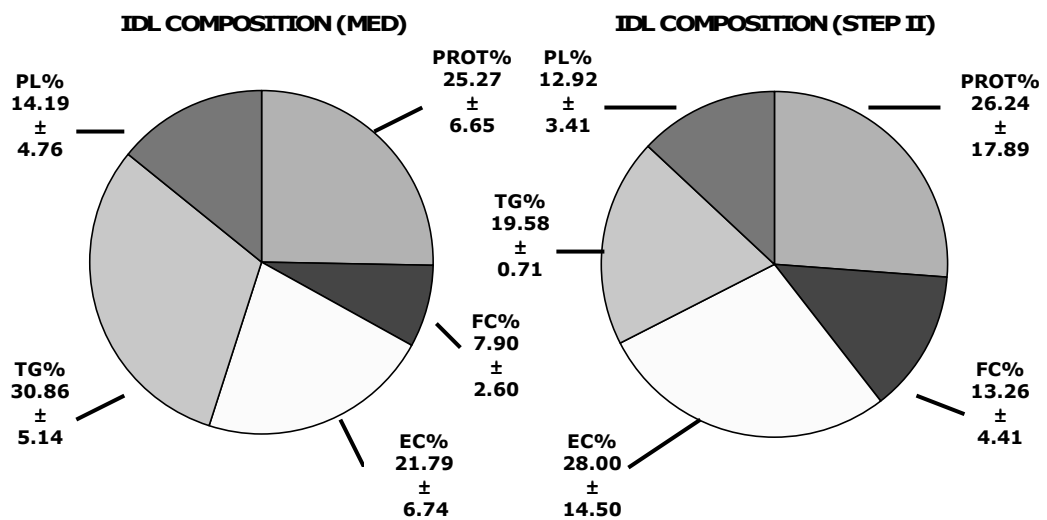
PROT=proteins; FC=free cholesterol; EC=esterified cholesterol;

TG=triglycerides; PL=phospholipids.

(B)



(C)



MED (Mediterranean diet) N=4 / STEP II diet N=3.

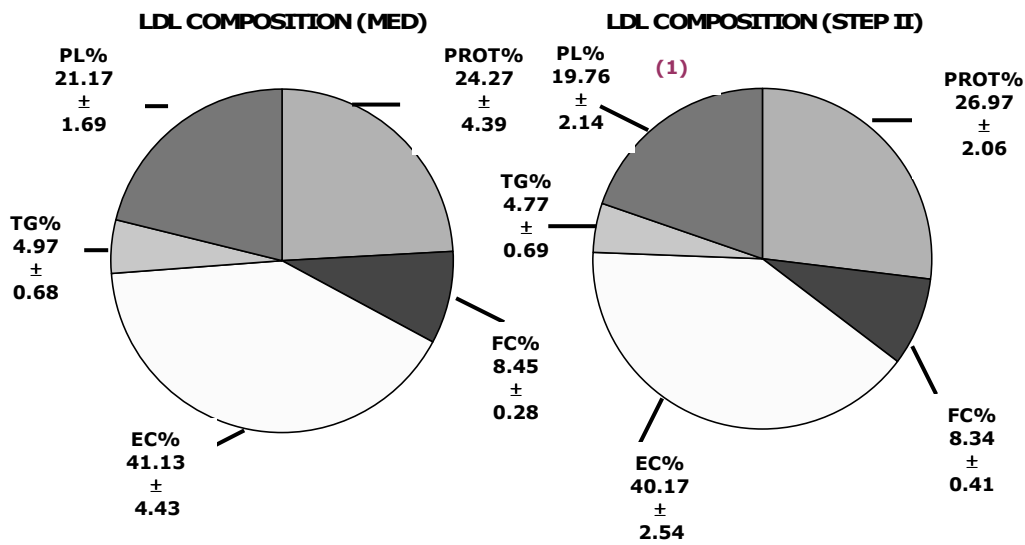
Results expressed as mean ± standard deviation (SD).

(1) $P=0.03$.

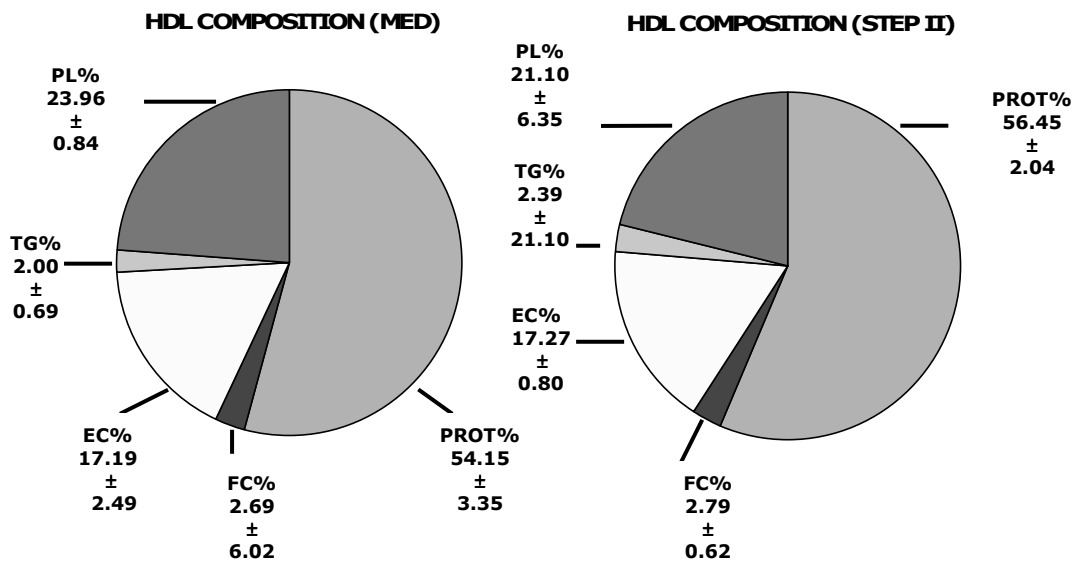
PROT=proteins; FC=free cholesterol; EC=esterified cholesterol;

TG=triglycerides; PL=phospholipids.

(D)



(E)



MED (Mediterranean diet) N=4 / STEP II diet N=3.

Results expressed as mean ± standard deviation (SD).

(1) P=0.05.

PROT=proteins; FC=free cholesterol; EC=esterified cholesterol;

TG=triglycerides; PL=phospholipids.

Table 46. Composition of lipoprotein particles (core, CE:TG, and surface, FC:PL) in the kinetic subset of volunteers (day of the kinetic study).

		MEDITERRANEAN DIET (KINETICS) (N=4)	STEP II DIET (KINETICS) (N=3)	MED vs. STEP II P
VLDL1	EC:TG	0.18 ± 0.05	0.37 ± 0.30	0.33
	FC:PL	0.54 ± 0.27	1.50 ± 0.58	0.04
VLDL2	EC:TG	0.34 ± 0.06	0.87 ± 0.65	0.30
	FC:PL	0.55 ± 0.35	1.16 ± 1.00	0.32
IDL	EC:TG	0.72 ± 0.27	1.42 ± 0.70	0.36
	FC:PL	0.62 ± 0.33	1.04 ± 0.34	0.10
LDL	EC:TG	8.37 ± 1.25	8.55 ± 1.38	0.30
	FC:PL	0.40 ± 0.05	0.43 ± 0.06	0.60
HDL	EC:TG	9.60 ± 4.38	8.17 ± 3.62	0.60
	FC:PL	0.11 ± 0.04	0.14 ± 0.04	0.11

MED=Mediterranean; vs.=versus.

Results expressed as mean ± standard deviation (SD).

Significance $P < 0.05$.

EC=esterified cholesterol; TG=triglycerides; PL=phospholipids.

Table 47. Plasma concentrations of lipoprotein fractions (derived from compositional analysis) in the kinetic subset of volunteers (day of the kinetic study).

		MEDITERRANEAN DIET (KINETICS) (N=4)	STEP II DIET (KINETICS) (N=3)	MED vs. STEP II P
VLDL1	(g/l)	0.32 ± 0.19	0.27 ± 0.06	0.78
VLDL2	(g/l)	0.14 ± 0.08	0.15 ± 0.06	0.95
IDL	(g/l)	0.31 ± 0.06	0.40 ± 0.17	0.57
LDL	(g/l)	3.69 ± 0.32	3.46 ± 1.30	0.63
HDL	(g/l)	2.72 ± 0.39	2.60 ± 1.01	0.86

MED=Mediterranean; vs.=versus.

Results expressed as mean ± standard deviation (SD).

Significance $P < 0.05$.

2.1.4.3.2 Lipoprotein kinetic studies

The preliminary kinetic parameters were obtained from 7 kinetic studies conducted in 4 volunteers. The kinetic parameters obtained were for apo B-100 VLDL₁, VLDL₂, IDL and LDL and apoA-I and A-II HDL for each dietary intervention period.

Lipoprotein kinetic parameters were calculated with the SAAM II program using several biochemical measurements and isotopic enrichment data from GC-MS, which are summarised in Figures 44 and 45.

2.1.4.3.2.1 Fitting observed data to SAAM II calculated parameters

Observed (also termed experimental) masses were fitted to the SAAM II program based on the multi-compartmental models used to calculate masses. The weighted least squares approach was used to minimise differences between model predicted and observed data. Fitting was necessary for subsequent accurate kinetic parameter calculation. A summary of the observed parameters and how they were calculated is shown in Figure 44.

Calculated versus observed masses were expressed as percentage observed/calculated (Table 48, 49). If best fit was performed, there were less differences between calculated and observed parameters values and ratio approximated to 100%:

- * regarding apo B-100-rich lipoproteins parameters. Calculated SAAM II parameters approached observed values for apo B-100-rich lipoproteins in both dietary interventions; except for IDL (198.29% ± 179.15). In the STEP II diet, albeit only from one kinetic study, we obtained low observed apo B-100 IDL pool size values (due to a low total protein concentration) and high observed apo B-100 LDL pool size values (due to a high total protein concentration). Thus, observed total protein concentration differed from expected protein content in these lipoprotein fractions. This explained the wide SD values for observed IDL and LDL parameters.

- * regarding apoA-I and A-II HDL parameters. Calculated SAAM II parameters approached observed values for apoA-I and A-II HDL in both dietary interventions.

Figure 44. Flow diagram of biochemical measurements required for final SAAM II data calculation.

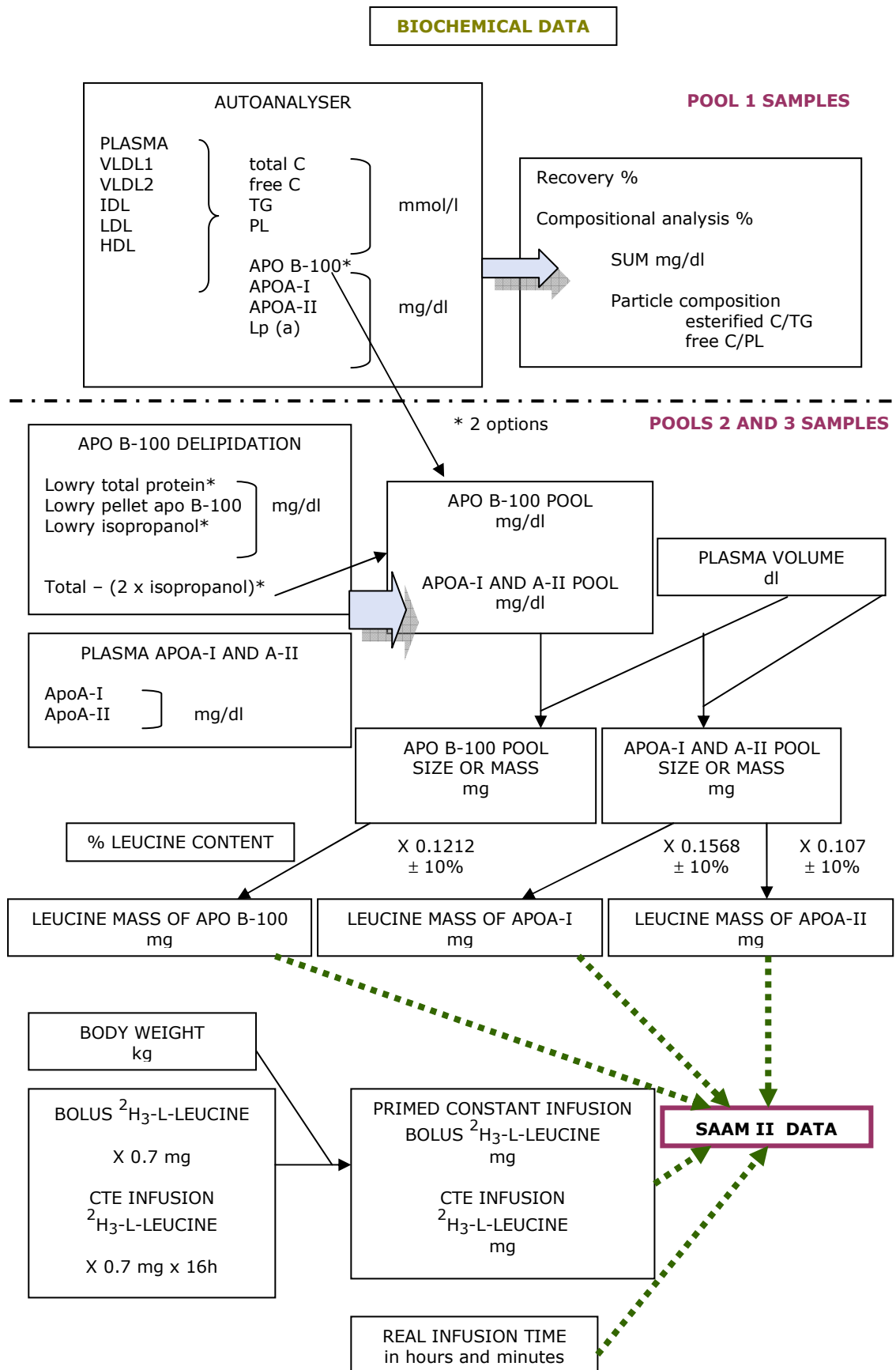


Table 48. Observed and SAAM II calculated apo B-100, and leucine masses for VLDL₁, VLDL₂, IDL and LDL.

	MEDITERRANEAN DIET (N=4)	STEP II DIET (N=3)
SAAM calculated apo B-100 pool size (mg)		
V1	111.94 ± 70.96	96.34 ± 78.77
V2	61.10 ± 42.98	65.07 ± 48.07
IDL	102.95 ± 30.61	93.04 ± 98.22
LDL	2124.59 ± 270.08	1962.32 ± 940.34
Observed apo B-100 pool size (mg)		
V1	112.27 ± 70.80	98.41 ± 77.47
V2	59.48 ± 43.46	63.95 ± 48.96
IDL	102.55 ± 32.14	78.64 ± 107.66 *
LDL	2163.09 ± 226.36	2121.47 ± 1171.69 *
SAAM calculated leucine mass (mg)		
V1	13.57 ± 8.60	11.68 ± 9.55
V2	7.41 ± 5.21	7.89 ± 5.83
IDL	12.48 ± 3.71	11.28 ± 11.90
LDL	257.50 ± 32.73	237.83 ± 113.97
Observed leucine mass (mg)		
V1	13.61 ± 8.58	11.93 ± 9.39
V2	7.21 ± 5.27	7.75 ± 5.93
IDL	12.43 ± 3.90	9.53 ± 13.05 *
LDL	262.17 ± 27.44	257.12 ± 142.01 *
Percentage Calculated/Observed		
V1	99.61 ± 0.50	96.60 ± 6.47
V2	103.81 ± 4.62	103.28 ± 4.34
IDL	100.81 ± 2.14	198.29 ± 179.15 *
LDL	98.10 ± 4.33	95.16 ± 7.81

Results expressed as mean ± standard deviation (SD).

* observed total protein concentration differing from expected protein content known to be present in these lipoprotein fractions.

Table 49. Observed and SAAM II calculated apoA-I, A-II and leucine masses for HDL.

	MEDITERRANEAN DIET (N=4)	STEP II DIET (N=3)
SAAM calculated pool size (mg)		
ApoA-I	4118.75 ± 923.70	3439.58 ± 655.04
Observed pool size (mg)		
ApoA-I	4022.05 ± 834.55	3452.51 ± 674.90
SAAM calculated leucine mass (mg)		
ApoA-I	673.23 ± 152.96	544.82 ± 105.32
Observed leucine mass (mg)		
ApoA-I	659.00 ± 147.79	550.33 ± 104.81
Percentage Calculated/Observed		
ApoA-I	102.16 ± 3.50	99.69 ± 0.49
SAAM calculated pool size (mg)		
ApoA-II	939.25 ± 156.85	831.78 ± 145.99
Observed pool size (mg)		
ApoA-II	940.75 ± 157.65	829.79 ± 146.27
SAAM calculated leucine mass (mg)		
ApoA-II	100.35 ± 16.82	89.22 ± 15.64
Observed leucine mass (mg)		
ApoA-II	100.50 ± 16.78	89.00 ± 15.62
Percentage Calculated/Observed		
ApoA-II	99.85 ± 0.29	100.25 ± 0.13

Results expressed as mean ± standard deviation (SD).

2.1.4.3.2.2 Enrichment curves obtained using SAAM II

Observed (also termed experimental) enrichment curves were used for subsequent kinetic parameter calculation (Figure 45). Also, best fitting enabled observed tracer/tracee ratios to predict calculated enrichment curves using SAAM II multi-compartmental models. The weighted least squares approach also minimised differences between model predicted and observed data.

The following figures (segregated with respect to each diet for one volunteer) are examples of the enrichment curves (the change in tracer/tracee ratios with time) describing apo B-100-rich lipoproteins, apoA-I and A-II HDL kinetics.

Apo B-100-rich lipoproteins enrichment curves following (Figures 46, 47):

* the Mediterranean and STEP II diets showed:

- plasma constant enrichment over the 16h tracer primed constant infusion.
- different lipoprotein fractions kinetics over the 16h primed constant infusion:
 - the sequential appearance over time of VLDL₁ → VLDL₂ → IDL → LDL.
 - VLDL₁, VLDL₂ and IDL reaching a plateau in the first hours after the infusion due to their rapid turnover rates, whereas LDL occurred hours later due to its slow turnover rate.
- once the primed constant infusion was concluded and up to 48h:
 - plasma, VLDL₁, VLDL₂ and IDL enrichment began to decrease (also due their rapid turnover rates), whereas LDL enrichment was constant (also due to its slow turnover rate).

ApoA-I and A-II HDL enrichment curves following (Figures 48, 49):

* the Mediterranean and STEP II diets showed:

- plasma constant enrichment over the 16h tracer primed constant infusion.
- HDL kinetic over the 16h of primed constant infusion:
 - a simultaneous appearance over time of both major HDL apos.
 - HDL reached a plateau several hours after the infusion due to its slow turnover rate.
- once the primed constant infusion was concluded and up to 48h:
 - plasma enrichment began to decrease, whereas HDL enrichment was constant (also due to its slow turnover rate).

Figure 45. Flow diagram showing isotopic enrichment parameters required for final SAAM II data calculation.

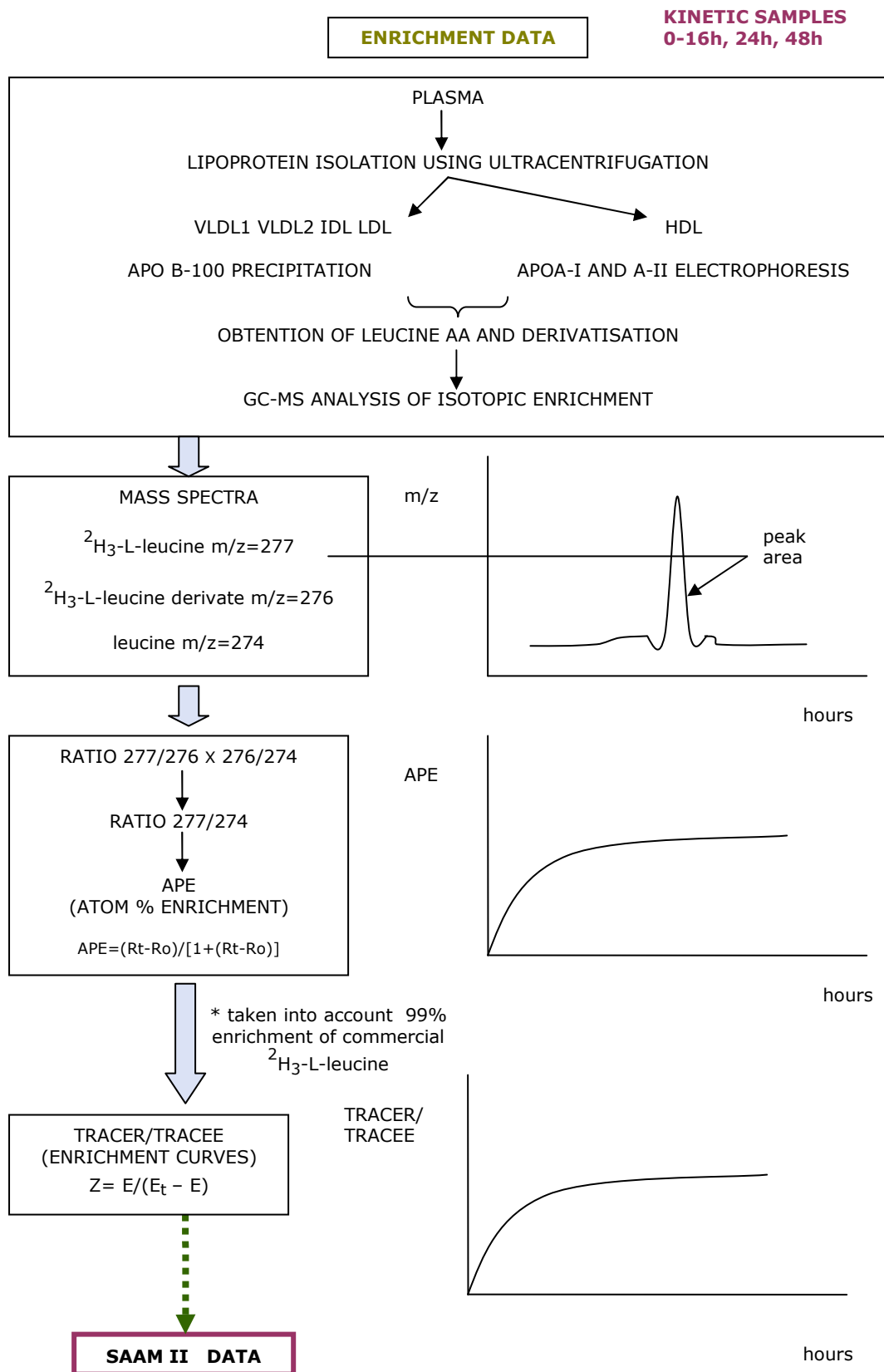
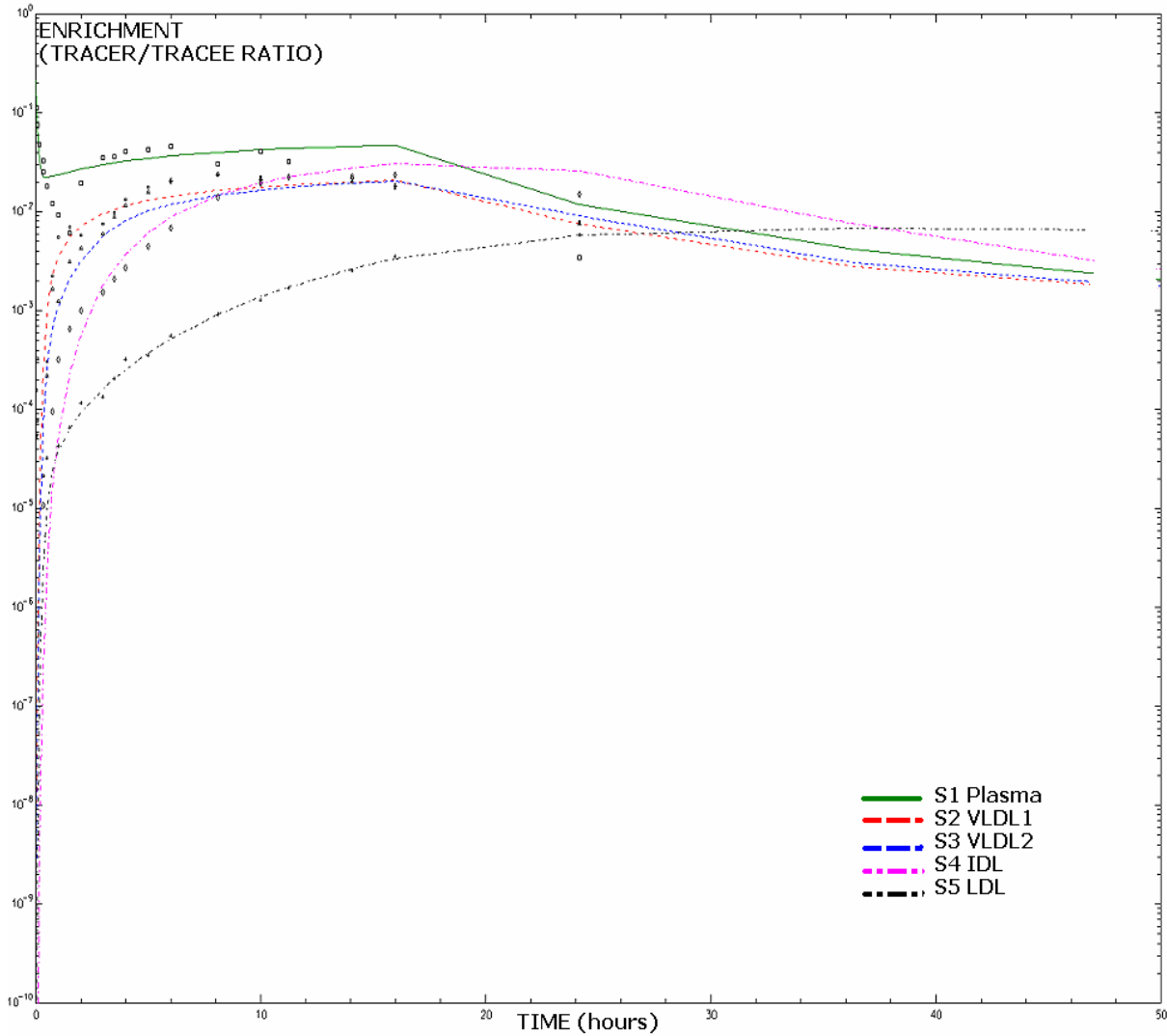


Figure 46. Apo B-100-rich lipoproteins enrichment curves for Mediterranean diet study.



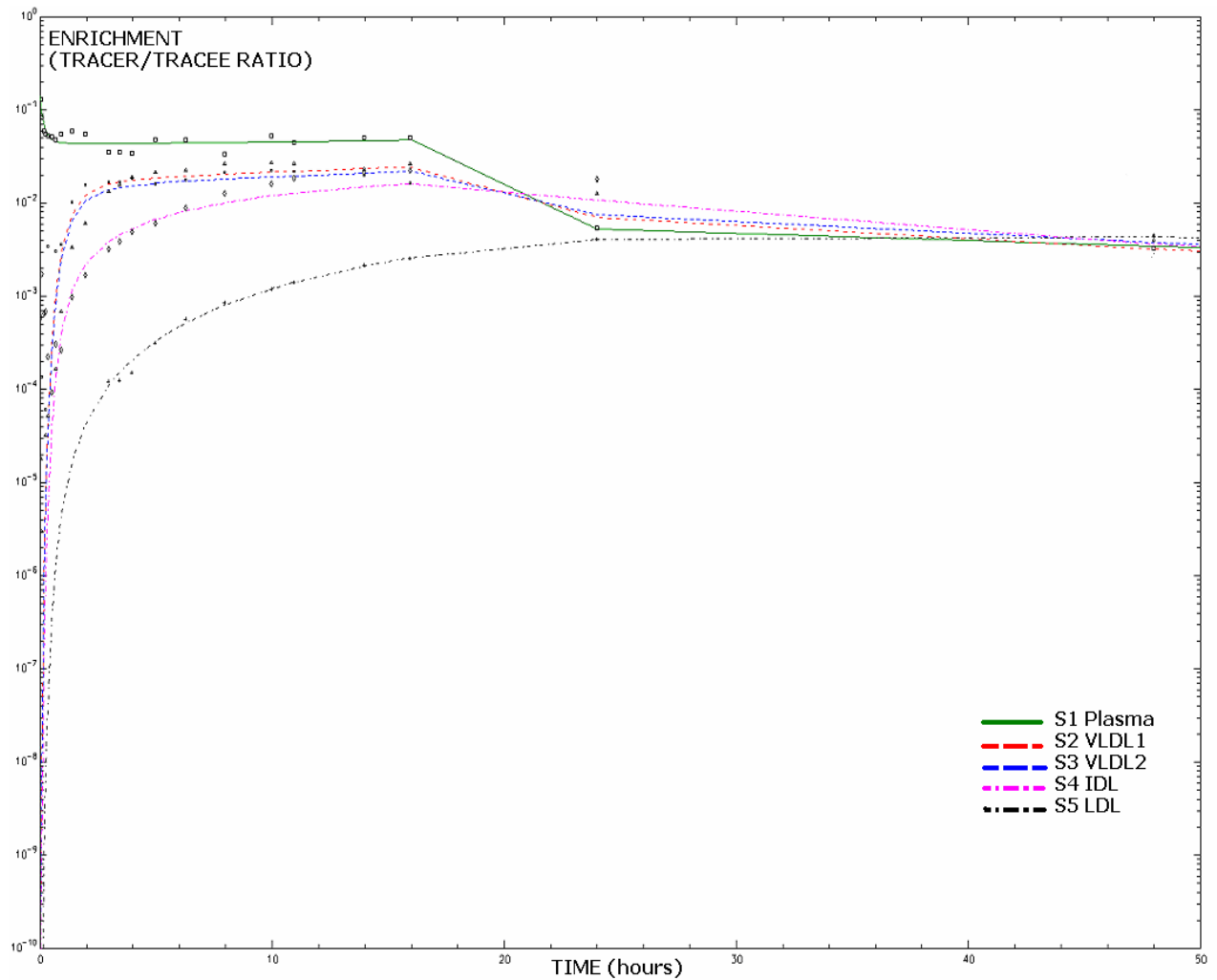
N=1 volunteer.

° (curve points) observed tracer/tracee ratios.

— (curve line) SAAM II calculated enrichment curves from multi-compartmental model.

s=site of determination.

Figure 47. Apo B-100-rich lipoproteins enrichment curves for STEP II diet study.



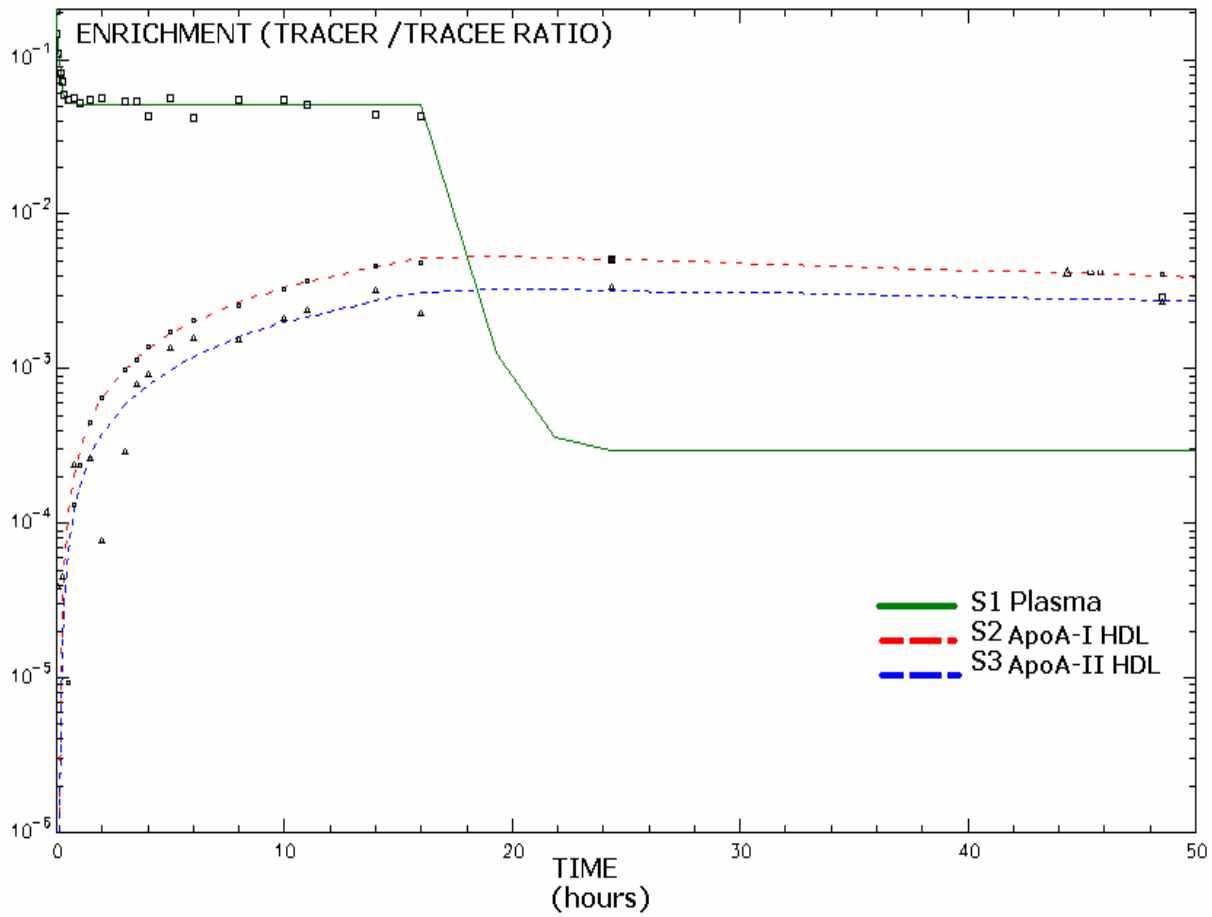
N=1 volunteer.

° (curve points) observed tracer/tracee ratios.

— (curve line) SAAM II calculated enrichment curves from multi-compartmental model.

s=site of determination.

Figure 48. Apo A-I and A-II HDL enrichment curves for Mediterranean diet study.



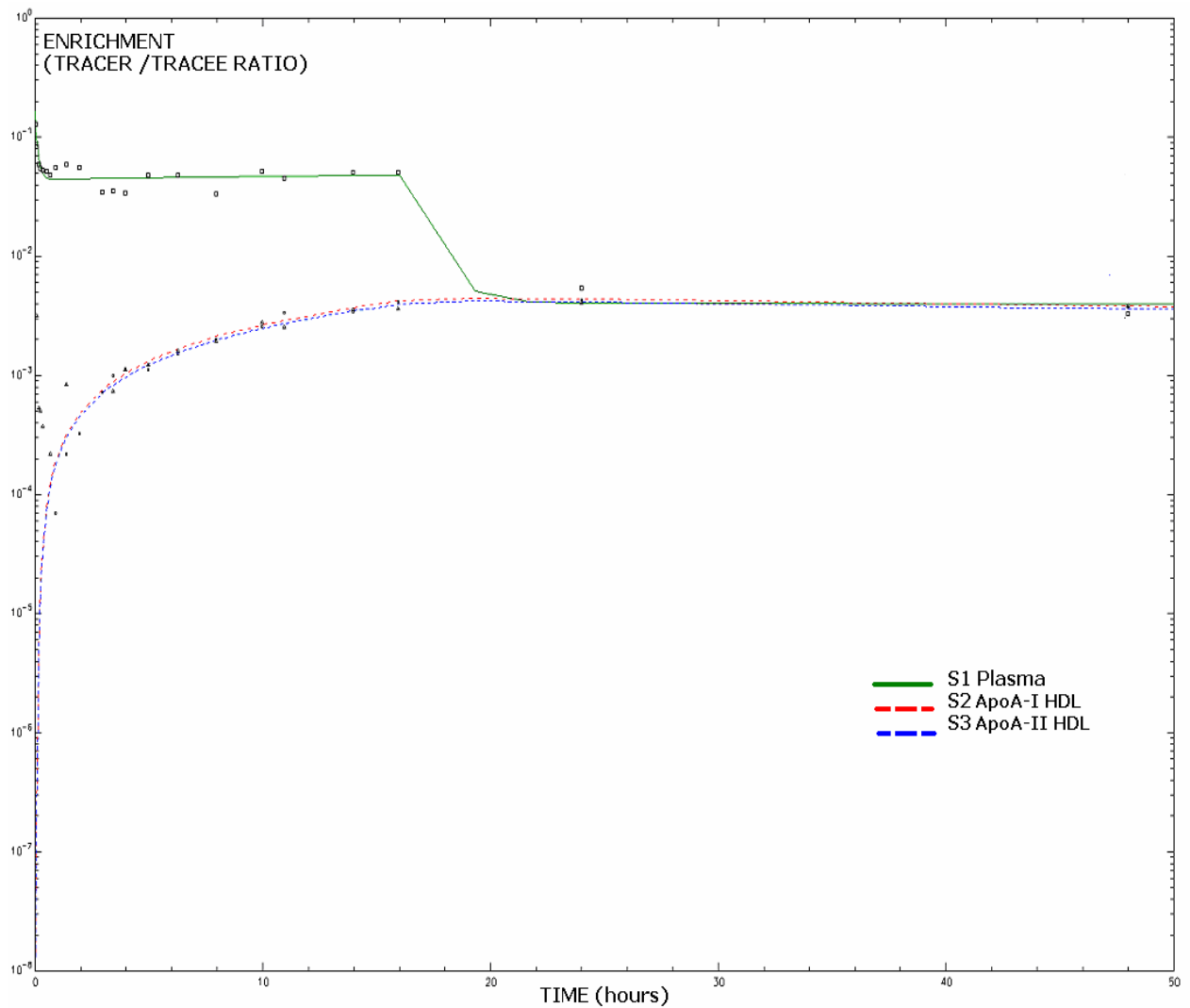
N=1 volunteer.

° (curve points) observed tracer/tracee ratios.

— (curve line) SAAM II calculated enrichment curves from multi-compartmental model.

s=site of determination.

Figure 49. Apo A-I and A-II HDL enrichment curves for STEP II diet study.



N=1 volunteer.

° (curve points) observed tracer/tracee ratios.

— (curve line) SAAM II calculated enrichment curves from multi-compartmental model.

s=site of determination.

2.1.4.3.2.3 Lipoprotein kinetic parameters

The apo B-100-rich lipoproteins kinetic parameters calculated were: VLDL₁, VLDL₂, IDL and LDL apo B-100 pool size (mg), direct fractional catabolic rate (dirFCR; pools/day), fractional transfer rate (FTR; pools/day), overall fractional catabolic rate (FCR; pools/day) and production rate (PR; mg/day or mg/kg/day).

The HDL kinetic parameters calculated were: apoA-I and A-II pool size (mg), production rate (PR; mg/day or mg/kg/day) and fractional catabolic rate (FCR; pools/day).

The results are presented in Tables 50 and 51 and, in simplified multi-compartmental model structures, in Figures 50 and 51.

2.1.4.3.2.3.1 Apo B-100-rich lipoproteins kinetic parameters

Regarding apo B-100-rich lipoproteins and comparing the Mediterranean versus the STEP II diet (Table 50, Figure 50):

* apo B-100 VLDL₁, VLDL₂ and IDL kinetic parameters were very variable between the 4 volunteers. This high variability, together with the limited number of performed kinetic studies, could explain why results obtained regarding these parameters did not reach statistical significance. When comparing the two diets:

- VLDL₁ pool size (111.94 versus 96.34 mg; $P=0.91$), PR (827.75 versus 842.67 mg/day; $P=0.31$), PR corrected for body weight (10.95 versus 12.22 mg/kg/day; $P=0.32$), FTR from VLDL₁ to VLDL₂ (3.77 versus 3.42 pools/day; $P=0.97$) and dirFCR (5.57 versus 6.05 pools/day; $P=0.52$) were similar in both diets.

- VLDL₂ pool size (61.1 versus 65.07 mg; $P=0.96$), PR (262.00 versus 248.67 mg/day; $P=0.86$) and PR corrected for body weight (3.56 versus 3.71 mg/kg/day; $P=0.77$) were similar in both diets. The Mediterranean diet increased, albeit not significantly, FTR from VLDL₂ to IDL (12.06 versus 6.62 pools/day; $P=0.63$) and decreased, albeit not significantly, VLDL₂ dirFCR (1.49 versus 2.81 pools/day; $P=0.68$).

- IDL pool size (102.95 versus 93.04 mg; $P=0.83$) was similar in both diets. The Mediterranean diet increased, albeit not significantly, IDL PR (354.78 versus 148.00 mg/day; $P=0.49$), PR corrected for body weight (4.75 versus 2.17 mg/kg/day; $P=0.52$) and dirFCR (2.45 versus 0.05 pools/day; $P=0.20$). Further, the Mediterranean diet decreased, albeit not significantly, FTR from IDL to LDL (6.64 versus 9.37 pools/day; $P=0.65$).

* LDL pool size was similar in both diets (2124.59 versus 1962.32 mg; $P=0.65$). The Mediterranean diet significantly increased LDL PR (364.25

versus 114.67 mg/day; $P=0.05$). Further, this significance was preserved after correcting LDL PR for body weight (4.95 vs 1.70 mg/kg/day), albeit just not quite reaching statistical significance ($P=0.06$). The Mediterranean diet increased, albeit not significantly, LDL dirFCR (0.45 versus 0.30 pools/day; $P=0.32$).

Table 50. Kinetic parameters describing apo B-100-rich lipoproteins metabolism.

	MEDITERRANEAN DIET (N=4)	STEP II DIET (N=3)	MED vs. STEP II P
Apo pool size (mg)			
Pool V1	111.94 ± 70.96	96.34 ± 78.77	0.91
Pool V2	61.1 ± 42.98	65.07 ± 48.07	0.96
Pool IDL	102.95 ± 30.61	93.04 ± 98.22	0.83
Pool LDL	2124.59 ± 270.08	1962.32 ± 940.34	0.65
Production rate (mg/day)			
PR V1	827.75 ± 672.29	842.67 ± 617.56	0.31
PR V2	262 ± 69.51	248.67 ± 133.81	0.86
PR IDL	354.78 ± 329.94	148 ± 172.57	0.49
PR LDL	364.25 ± 245.02	114.67 ± 128.52	0.05
Production rate corrected for body weight (mg/kg/day)			
PR V1	10.95 ± 7.95	12.22 ± 9.54	0.32
PR V2	3.56 ± 0.64	3.71 ± 2.17	0.77
PR IDL	4.75 ± 4.33	2.17 ± 2.66	0.52
PR LDL	4.95 ± 3.11	1.70 ± 2.01	0.06
Overall fractional catabolic rate			
Fractional transfer rate (pools/day)			
TR V1 to V2	3.77 ± 4.23	3.42 ± 3.01	0.97
TR V2 to IDL	12.06 ± 8.30	6.62 ± 5.59	0.63
TR IDL to LDL	6.64 ± 5.12	9.37 ± 7.66	0.65
Direct Fractional catabolic rate (pools/day)			
dirFCR V1	5.57 ± 6.18	6.05 ± 2.58	0.52
dirFCR V2	1.49 ± 1.75	2.81 ± 4.86	0.68
dirFCR IDL	2.45 ± 1.65	0.05 ± 0.08	0.20
dirFCR LDL	0.45 ± 0.22	0.3 ± 0.12	0.32

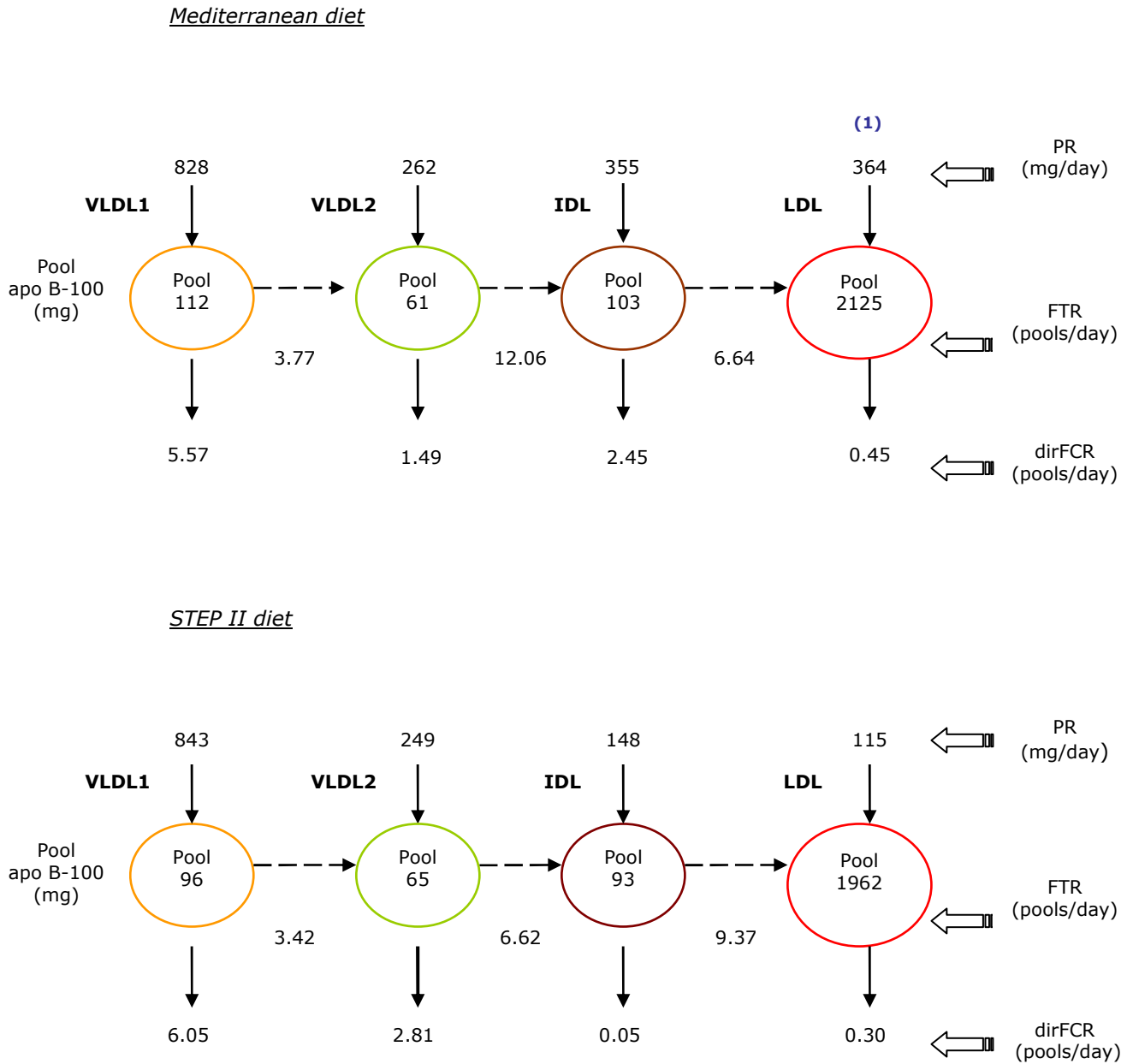
Results expressed as mean ± standard deviation (SD).

Significance $P<0.05$.

MED=Mediterranean; vs.=versus; PR=production rate;

dirFCR=direct fractional catabolic rate; FTR=fractional transfer rate.

Figure 50. Flow diagrams of the kinetic parameters describing apo B-100-rich lipoproteins metabolism.



Mediterranean diet N=4 / STEP II diet N=3

Results expressed as mean.

(1) $P < 0.05$.

PR=production rate; dirFCR=direct fractional catabolic rate;

FTR=fractional transfer rate.

2.1.4.3.2.3.2 ApoA-I and A-II HDL kinetic parameters

Comparing the Mediterranean versus the STEP II diet, no significant differences were observed in kinetic parameters between diets, but there were some trends (Table 51, Figure 51) regarding:

* apoA-I HDL kinetics. The Mediterranean diet tended to increase apoA-I pool size (4118.75 versus 3439.58 mg; $P=0.14$), PR (1260.75 versus 994.00 mg/day; $P=0.10$) and PR corrected for body weight (17.29 versus 14.17 mg/kg/day; $P=0.13$). ApoA-I FCR (0.31 versus 0.29 pools/day; $P=0.52$) was similar in both diets.

* apoA-II HDL kinetics. The Mediterranean diet tended to increase apoA-II pool size (939.25 versus 831.78 mg; $P=0.12$) and to decrease apoA-II PR (263.25 versus 287.00 mg/day; $P=0.13$), apoA-II PR corrected for body weight (3.60 versus 4.10 mg/kg/day; $P=0.17$) and FCR (0.28 versus 0.34 pools/day; $P=0.16$).

Table 51. Kinetic parameters describing apoA-I and A-II HDL metabolism.

	MEDITERRANEAN DIET (N=4)	STEP II DIET (N=3)	MED vs. STEP II P
Apo pool size (mg)			
Pool AI	4118.75 ± 923.70	3439.58 ± 655.04	0.14
Pool AII	939.25 ± 156.85	831.78 ± 145.99	0.12
Production rate (mg/day)			
PR AI	1260.75 ± 148.98	994.00 ± 232.75	0.10
PR AII	263.25 ± 62.24	287.00 ± 90.32	0.13
Production rate corrected for body weight (mg/kg/day)			
PR AI	17.29 ± 0.35	14.17 ± 1.91	0.13
PR AII	3.60 ± 0.74	4.10 ± 1.1	0.17
Overall fractional catabolic rate			
Fractional catabolic rate (pools/day)			
FCR AI	0.31 ± 0.04	0.29 ± 0.01	0.52
FCR AII	0.28 ± 0.05	0.34 ± 0.09	0.16

Results expressed as mean ± standard deviation (SD).

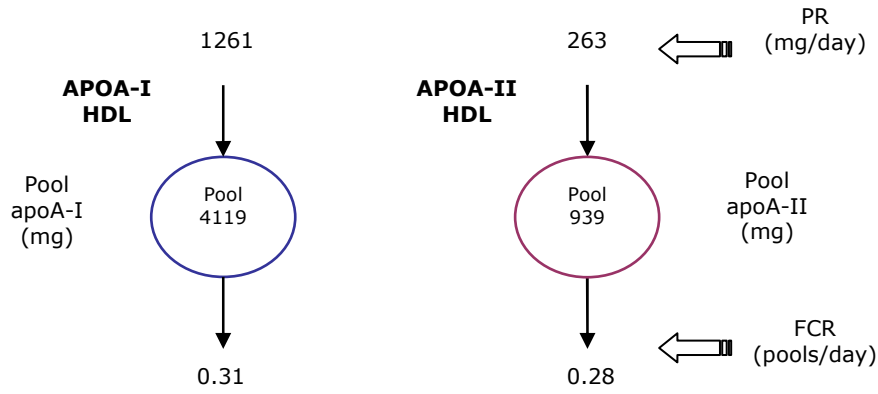
Significance $P<0.05$.

MED=Mediterranean; vs.=versus; PR=production rate;

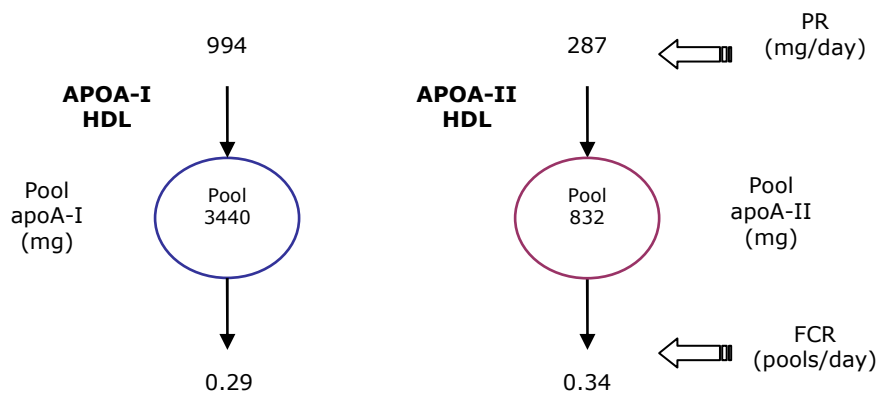
FCR=fractional catabolic rate; FTR=fractional transfer rate.

Figure 51. Flow diagrams of the kinetic parameters describing apoA-I and A-II HDL metabolism.

Mediterranean diet



STEP II diet



Mediterranean diet N=4 / STEP II diet N=3.

Results expressed as mean.

PR= production rate; FCR=fractional catabolic rate;

FTR=fractional transfer rate.

2.1.4.3.2.4 Parameter correlations

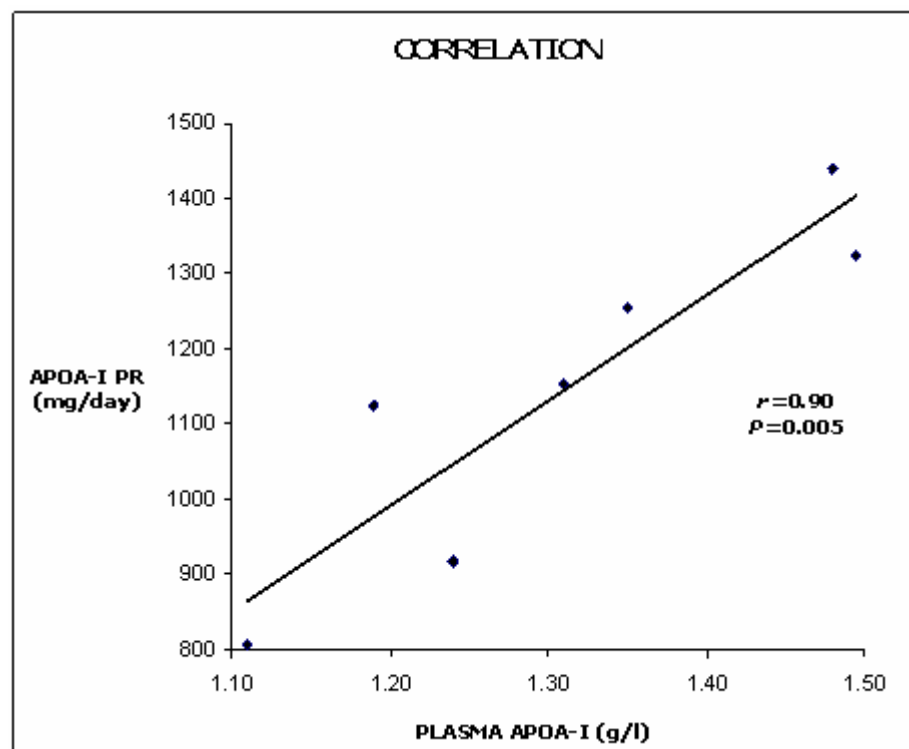
Several correlation coefficients were evaluated to assess linear relationships between measured parameters in the kinetic subset of 4 volunteers.

There was a significant positive correlation between apoA-I plasma concentration (g/l) and apoA-I PR (mg/day) ($r=0.90$; $P=0.005$). Correcting apoA-I PR for body weight (mg/kg/day), the correlation maintained the same, albeit not significant, trend ($r=0.70$; $P=0.07$) (Figure 52).

No other significant correlations were found (Table 52).

Figure 52. Correlation between apoA-I plasma concentrations (g/l) and apoA-I PR (mg/day) (A); and between apoA-I plasma concentrations (g/l) and apoA-I PR (mg/kg/day) (B).

(A)



Mediterranean diet N=4 / STEP II diet N=3.

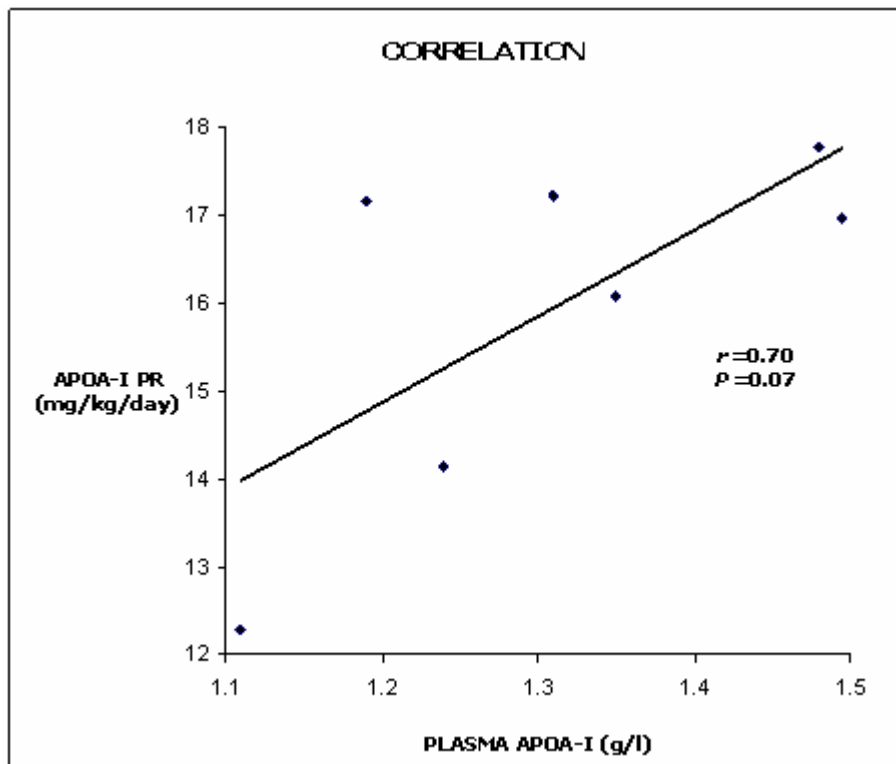
Results expressed as mean.

r =Pearson r .

Significance $P<0.05$.

PR=production rate.

(B)



Mediterranean diet N=4 / STEP II diet N=3.

Results expressed as mean.

r =Pearson r .

Significance $P<0.05$.

PR=production rate.

Table 52. Other correlations between measured parameters.

	<i>r</i>	<i>P</i>
VLDL1 pool (mg) vs. apo B-100 VLDL1 PR (mg/day)	0.31	0.49
VLDL1 pool (mg) vs. apo B-100 LDL FCR (pools/day)	-0.16	0.75
VLDL1 pool (mg) vs. plasma TG (mg/dl)	0.55	0.20
apo B-100 LDL (g/l) vs. apo B-100 LDL PR (mg/day)	-0.06	0.89
apo B-100 LDL (g/l) vs. apo B-100 LDL PR (mg/kg/day)	-0.04	0.94
apo B-100 LDL (g/l) vs. apo B-100 FCR (pools/day)	-0.54	0.20
plasma LDLC (mg/dl) vs. apo B-100 LDL PR (mg/day)	-0.05	0.92
plasma LDLC (mg/dl) vs. apo B-100 LDL PR (mg/kg/day)	-0.06	0.91
plasma LDLC (mg/dl) vs. apo B-100 FCR (pools day)	-0.51	0.30
apoA-I FCR (pools/day) vs. apoA-I plasma (g/l)	-0.33	0.47
apoA-I FCR (pools/day) vs. TG HDL (mg/dl)	0.42	0.35
plasma apoA-II vs. apoA-II PR (mg/day)	0.62	0.14
plasma apoA-II vs. apoA-II PR (mg/kg/day)	0.35	0.45
plasma apoA-II vs. apoA-II FCR (pools/day)	0.03	0.95

Mediterranean diet N=4 / STEP II diet N=3.

r=Pearson *r*.

Significance *P*<0.05.

vs.=versus; PR=production rate; FCR=fractional catabolic rate.

UNIVERSITAT ROVIRA I VIRGILI
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DISCUSSION

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1. General objective: implementation of the methodology of lipoprotein kinetic studies using stable isotopes

This thesis describes the setting-up of the necessary methodology to perform apoA-I, apoA-II and apo B-100 kinetic studies in volunteers *in vivo* using stable isotopes to study lipoprotein metabolism.

Lipoprotein metabolism is a complex network of interrelated metabolic pathways. Plasma measurements of lipids, lipoproteins and apos provide static information on their concentrations but do not provide dynamic information on their rates of synthesis and catabolism. Lipoprotein kinetic studies and multi-compartmental modelling have been used for many years to provide an insight into human lipoprotein metabolism *in vivo*. Kinetic data enable an understanding of plasma lipoprotein concentrations which are the net result of *de novo* synthesis, catabolism, and flow of lipoprotein components^{267, 269, 275, 434, 435}.

The use of stable isotopes as metabolic tracers *in vivo* predates the use of radioisotopes by almost 20 years. In 1935, for example, deuterium isotopes were used to study metabolism of fats in mice. With improvements in radioisotope technology, scintillation counting, and the availability of a wide variety of tracers, most kinetic studies between the 1950's and the 1970's used radioisotopes as tracers. In the late 1980's, there was a resurgence of the use of stable isotopes. The complex methodology improved and the cost of GC-MS equipment decreased and became affordable to small research groups. Further, a wide variety of stable isotopes began to become commercially available. All these, together with the questionable use of radioisotopes in human research, made stable isotopes alternative approach more attractive²⁸⁶⁻²⁹².

In the 1980's, our Research Unit in Reus was the first to introduce into Spain the use of radioisotopes for the conduct of human lipoprotein kinetic studies³³⁹⁻³⁴². Our Research Unit then wished to proceed to implement the methodology for the study of lipoprotein kinetics using stable isotopes since there does not appear to be any physiological risk to the patient when a tracer dose is administered⁴³⁶. We already had almost all the required infrastructure in Reus. The methodology is complex, arduous and requires high level of technical expertise when processing the post-injection samples as well as when interpreting the output data. The apoA-I and A-II kinetic studies were performed in our Unit in Reus while the apo B-100 kinetic studies and apos kinetic parameter modelling of data were performed in collaboration with Dr. Caslake and Professor Packard of the Vascular Biochemistry Section, Division of Cardiovascular & Medical Sciences, Royal Infirmary, University of Glasgow, Glasgow, Scotland. The aim of setting-up this methodology was to assess the effects of dietary interventions.

1.1 General methodological aspects

Specific considerations needed to be taken into account in the design and execution of lipoprotein kinetic studies using stable isotopes, especially with respect to labelling, tracer administration, sampling protocols, choice of laboratory methodology and kinetic parameters determination.

1.1.1 Labelling method and the use of leucine as tracer

Since the 1990's, endogenous labelling with stable isotopes has seen a safe option and the primary method in human *in vivo* studies for the characterisation of lipoprotein metabolism; albeit sophisticated analytical methods being required. Endogenous labelling with a labelled tracer precursor ensures that the tracer does not alter lipoprotein metabolism and that all particles are labelled proportionately such that the kinetics of nascent as well as remnant particles can be monitored ^{263, 280, 281, 297, 310, 311, 317, 334, 335, 409, 437-451}.

Leucine, in its deuterated form $^2\text{H}_3\text{-L-leucine}$, is the amino acid most-widely chosen to act as protein tracer. We used it to determine the kinetics of apoA-I, apoA-II and apo B-100 ⁴⁴⁵. Multiple-label leucine ensures additional mass and sufficient isotopic enrichment for accurate GC-MS detection.

$^2\text{H}_3\text{-L-leucine}$ can be considered an ideal tracer due to the following characteristics: its physical and chemical properties are identical to those of the studied tracee from which it is metabolically indistinguishable, it does not perturb the system under study (the human body) and it is physically distinguishable using specific analytical techniques.

The use of leucine as tracer has an advantage in that, since apo B-100, apoA-I and apoA-II contain more leucine than other amino acids, measurable incorporation of $^2\text{H}_3\text{-L-leucine}$ is optimised. Further, leucine is an essential amino acid that is metabolised in muscle with a unidirectional pathway of catabolism to its keto acid ³¹¹. We used a dose of 0.7 mg $^2\text{H}_3\text{-L-leucine/kg}$ body weight so as not to perturb the system ^{264, 382-384}.

In general, the disadvantages of endogenous labelling include the difficulty of deriving the kinetics of the precursor amino acid pool. Thus, plasma amino acids can be used as the tracer source when using the precursor pool as a forcing function. In this manner, there is a reliable relationship between the isotopic enrichment of plasma leucine and the enrichment of hepatic precursor pools used for apo synthesis. It rests on the observation that direct tracer input into the system under study cannot be determined and needs to be assumed from indirect measures such as the final synthesised lipoproteins. Further, for particles produced via multiple pathways, it is difficult to identify from which particular pathway the labelled particle

is derived. For example, apoA-I is synthesised and secreted by the intestine as well as the liver ^{259, 274}.

1.1.2 Tracer administration

In the present study, ²H₃-L-leucine was administered as a primed constant infusion (a bolus injection followed by a constant infusion).

The primed constant infusion creates a situation in which the bolus injection enables high levels of isotopic enrichment to be attained at the beginning of the kinetic study while the subsequent constant infusion maintains these levels constant over the time once the effects of the bolus dose have begun to disappear. The advantage of a bolus injection combined with a constant infusion is that lipoproteins with different turnover rates can be studied. A bolus injection provides dynamic information on interconversion and as well as on newly-synthesised particles. As such, it is more suitable for slowly turning over proteins (e.g. apoA-I, A-II HDL and apo B-100 LDL) or proteins with heterogeneous kinetics (e.g. apo B-100 LDL). A constant infusion, on the other hand, provides information on the degree to which equilibrium, or metabolic steady state, has been achieved. As such, it is more suitable for rapidly turning over proteins (e.g. apo B-100 VLDL) ^{267, 275, 297, 312, 325, 332, 392-394, 434, 450, 452, 453}.

The primed constant infusion minimises the problem of recycling of the precursor over the period of study. There is recycling of the tracer via protein breakdown from the moment the infusion begins, and which contributes to the isotopic enrichment. This complicates the requirement of distinguishing between the kinetics of newly-synthesised product from that of a recycled precursor, especially for slowly turning over proteins. In general, the faster the turnover rate, the less the kinetic parameters will be affected by recycling ^{264, 393, 453}.

It is assumed that plasma leucine is the extracellular source of leucine incorporated into apos synthesised in the liver. However, the isotopic enrichment of plasma leucine at its plateau is usually higher than that for lipoprotein apos. This implies an intracellular precursor source of leucine for apos synthesis or for their metabolism since plasma leucine might not always reflect this intracellular pool; especially for slow turning over proteins. The primed constant infusion takes into account these intracellular precursor pools ^{312, 317}.

1.1.3 Sampling protocol

The isotopic enrichment of ²H₃-L-leucine is well detected in slowly turning over apos using shorter infusion (12-16h) and sample collection (24-48h) times. This avoids the use of longer sampling periods (up to 14 days, or more in some cases). A shorter approach may be considered more physiological in the study of lipoprotein kinetics,

and which enables subjects to be studied over shorter time periods and under well-controlled conditions; either fasting or in the fed state ^{312, 331, 335, 394, 454, 455}. For example, time periods ranging between 12 and 16h of constant infusion have been widely used to determine the parameters of apoA-I and apoA-II in HDL kinetic studies; the slow apoA-I and A-II pools appearing in plasma following a delay of <12h ^{297, 332, 456-465}.

Blood sampling protocols have to be adjusted to the lipoproteins under study. The blood volumes collected need to be minimised during time of study while maximising tracer information. The residence time of lipoproteins depend on rapidly or slowly turning over characteristics. For example, frequent samples are required for the first hours following tracer administration in order to determine apo B-100 VLDL delipidation cascade. Further, sufficient samples need to be drawn towards the end of the study in order to characterise the "tail end" of the apo B-100 LDL, apoA-I and A-II HDL enrichment curves ^{331, 454}.

Fasted conditions (overnight pre-administration and during the course of the kinetic study) are needed to ensure a steady state, the lowest possible interference of apos from the liver and from the intestine (e.g. of apo B-100 with apo B-48 or of hepatic apoA-I with intestinal apoA-I), and no dilution of precursor plasma leucine pool with dietary leucine. Further, fasted conditions facilitate a simplification of the mathematics required when using the SAAM II program to derive the kinetic parameters ^{297, 466-468}.

In our study, ²H₃-L-leucine was administered using a primed constant infusion of 16h with the subjects under fasting conditions. We collected a total blood volume of 375 ml, which is similar in volume to that of a simple blood donation (<10% of total blood volume) over the initial 48h of the study.

1.1.4 Laboratory methodology

Laboratory methodology associated with lipoprotein kinetic studies is complex, difficult to perform, and time consuming. All steps are critical and have to be performed with considerable expertise.

We used classical lipoprotein kinetic study techniques followed by SAAM II modelling of the data ^{455, 469}.

VLDL₁ and VLDL₂ were separated from plasma using cumulative ultracentrifugation. IDL, LDL and HDL were separated using sequential preparative ultracentrifugation. Apo B-100 from VLDL₁, VLDL₂, IDL and LDL was isolated using isopropanol precipitation. Apo A-I and A-II from HDL were isolated using NuPAGE®Novex polyacrylamide gel electrophoresis. Commercial NuPAGE®Novex gels are a rapid and efficient electrophoretic variant technique for isolating apoA-I and A-II and, compared to handmade gels, are more easily optimised and improve the reproducibility in

separating proteins^{261, 264, 312, 335, 382, 384, 459, 460, 470-480}. ApoA-II is a dimer and when reducing agents (β -mercaptoethanol) are excluded, the 2 monomers of apoA-II do not split and can be distinguished from apo-C's which have a similar molecular weight as the apoA-II monomers^{325, 480}. ApoA-I is a monomer and can be easily separated under non-reducing conditions. NuPAGE®Novex gels facilitate the separation of proteins, either in reducing or non-reducing conditions.

Isolated apos are, then, hydrolysed, purified if necessary using ion exchange chromatography, derivatised and separated using GC-MS to determine the isotopic enrichments. Similarly, plasma amino acids are also isolated, subsequent to protein precipitation with TCA, purified using ion exchange chromatography, derivatised and separated on GC-MS to determine the isotopic enrichments^{312, 335, 481}. The quadrupole MS in SIR mode is a very sensitive method for the measurement of the isotopic enrichment of apos²⁶⁹.

1.1.5 Kinetic parameters determined using the modelling approach

In the present set of studies, we used multi-compartmental modelling since this is widely considered the best approach in obtaining information on the kinetics of apo B-100-rich lipoproteins and of apoA-I and apoA-II HDL and, as such, on the metabolism of their parent molecules^{310, 312, 317, 449, 482}. The SAAM II program was used to calculate the kinetic parameters.

Multi-compartmental modelling tracer data enables the determination of multi-exponential equations and coefficients characterising the model as well as production and catabolic rates together with pool size transfer rates between particles. Further, when using a modelling approach the derived kinetic parameters are not influenced by the tracer administration protocol^{261, 331, 332, 388-394, 454}.

1.1.5.1 SAAM II program

The SAAM II program was the modelling software used in the design and determination of lipoprotein kinetic parameters. It has several advantages^{337, 338, 483}:

- * it develops models structures (schema) and generates their defining differential equations. Additional user-defined equations or constraints can also be incorporated.
- * it specifies sites and protocols of tracer administration and compartments related to the experimental data.

* it enables curve fitting to, and parameter estimation from, observed data using the weighted least squares approach. This provides the best estimates or fits of parameters, together with a measure of their precision. Of note is that incorrect weighting of data would lead to poor model fit and incorrect parameter estimates.

1.2 Application of kinetic studies to apo B-100-rich lipoproteins metabolism

Apo B-100 is the major structural apo of VLDL, IDL and LDL and is the only apo that remains within the particle throughout its metabolic fate. Hence, apo B-100 is used to study apo B-100-rich lipoprotein metabolism.

Human *in vivo* apo B-100 kinetic parameters were initially studied in the 1970's using exogenous radiolabelling techniques. Cryer et al (1986) were the first to use an endogenous labelling approach with stable isotopes, specifically ^{15}N -glycine, and to describe a primed constant infusion in humans *in vivo* to study apo B-100 metabolism. Since then, this approach has been the most commonly used, together with the enrichment analysis performed with multi-compartmental modelling ^{269, 312, 335, 483-487}.

We used the apo B-100 multi-compartmental model developed by Demant and Packard et al (1996) ^{264, 295, 397, 488, 489}. Apo B-100-rich lipoproteins kinetic data were determined by fitting the enrichment data to multi-compartmental model using the SAAM II program.

The model was designed as a 15 multi-compartmental system to explain apo B-100-rich lipoproteins kinetics ²⁶⁴. The model includes a site for $^2\text{H}_3$ -L-leucine entry into the system via plasma and, from which, tracer is distributed to body protein pools as well as an intracellular compartment. This intracellular compartment acts as the precursor pool for liver apo B-100 synthesis. After a delay of about 0.5h, which is a function of apo B-100 synthesis, tracer appears through the delipidation cascade of the VLDL₁, VLDL₂, IDL and LDL compartments. The model also considers direct liver synthesis of apo B-100 appearing in these lipoprotein fractions, and remnants formation. As such, the model takes into account the kinetically heterogeneous characteristics of these particles, represented by several compartments, and the direct liver secretion of a range of particles of varying sizes as a more physiologically plausible option ^{264, 269, 273, 295, 317, 330, 337, 483-487, 490-492}. We adapted the blood-sampling times as applied to this apo B-100 model i.e. instead of the original method requiring the collection of blood samples over 12-14 days (at 8 a.m. every morning), our system required the collection of samples over 48h.

1.3 Application of kinetic studies to apoA-I and A-II HDL metabolism

ApoA-I and A-II are the major apos of HDL. These, and other apos of HDL, are readily exchanged between plasma lipoproteins but can exist free from attachment to HDL, they can enter cells and be degraded separately from HDL particles. These variables make the study of apoA-I and A-II HDL metabolism somewhat different from apo B-100-rich lipoproteins. HDL kinetic studies require the assumption that at least a certain amount of apos remain associated with the parent HDL particles during the period of the kinetic study^{277, 297, 448, 493-496}.

Human *in vivo* apoA-I kinetics were initially investigated in the 1970's using exogenous radiolabelling techniques. Cohn et al (1990) were the first to use a primed constant infusion and assumed a single plasma apoA-I pool to study apoA-I metabolism in humans *in vivo* using endogenous label with stable isotopes^{293, 312, 457, 461, 497-500}.

Human *in vivo* apoA-II kinetics were initially studied in the 1980's using exogenous radiolabelling techniques. Rader et al (1993) were the first to use a primed constant infusion to study apoA-II metabolism in humans *in vivo* using endogenous label with stable isotopes⁴⁵⁷.

Since then, most studies evaluating aspects of human *in vivo* apoA-I and A-II metabolism have used endogenous labelling with stable isotopes and have combined this with multi-compartmental modelling for the enrichment data analysis^{294, 323, 436, 461, 498, 501, 502}.

We used the apoA-I and A-II HDL multi-compartmental model developed by Packard and Caslake (2006). The apoA-I and A-II multi-compartmental model was developed using 17 published models as reference, particularly the model developed by Barrett et al (Table 53)^{272, 297, 410, 436, 442}. Our current model has not been previously applied nor published. Further, the 17 apoA-I and A-II kinetic studies published to-date (1978-2005) either as dietary interventions or metabolic perturbation studies, have been used in the present study for comparison purposes i.e. a total of 390 subjects had been studied, with a mean apoA-I FCR of 0.24 ± 0.05 pools/day and a mean apoA-I PR of 13.15 ± 2.04 mg/kg/day. These results were in accordance with our current findings (Tables 53, 55).

The model was designed as an 7 multi-compartmental system to explain apoA-I and A-II HDL kinetic data²⁶⁴. The model is simpler than the apo B-100 model and includes a site for ²H₃-L-leucine entry into the system via plasma and, from which, tracer is distributed to body protein pools as well as an intracellular compartment. This intracellular compartment acts as the precursor pool for apoA-I and A-II synthesis. After a delay of about 0.5h, which is a function of apoA-I and A-II synthesis, tracer appears in the HDL compartment for direct synthesis. Without specifying to which study design the model is applied, apoA-I production can be distinguished as being that of liver or of intestinal origin.

Table 53. ApoA-I and A-II HDL multi-compartmental models in published articles and used as reference in developing the model for our current study ^{272, 297, 410, 436, 442}.

Author	Year	Journal	Label
Caslake MJ et al	1978	Metabolism. 27: 437-447	¹²⁵ I or ¹³¹ I-apoA-I/HDL and ¹²⁵ I -HDL
Schaefer EJ, Zech LA et al	1982	J Lipid Res. 23: 850 - 862	¹²⁵ I or ¹³¹ I -HDL
Herbert PN, Thompson PD et al	1984	JAMA. 252: 1034-1037	¹²⁵ I -HDL
Brinton EA, Eisenberg S, Breslow JL	1989	J Clin Invest. 84: 262-269	¹²⁵ I-apoA-I, ¹³¹ I-apoA-II or vv
Chen YD, Reaven GM et al	1991	Hypertension. 17: 386-393	¹²⁵ I -apoA-I/HDL
Brinton EA, Eisenberg S, Breslow JL	1991	J Clin Invest. 87: 536-544	¹²⁵ I apoA-I, ¹³¹ I apoA-II or vv
Terry RB, Thompson PD et al	1992	Metabolism. 41: 1386 - 1392	¹²⁵ I -HDL
Taskinen MR, Shepherd J, Packard CJ et al	1992	Diabetologia. 35:347-356	¹²⁵ I -apoA-I/HDL and ¹³¹ I -apoA-II/HDL
Ikewaki K / Zech LA, Rader DJ et al	1993	J Lipid Res. 34: 2207 - 2215	¹²⁵ I -apoA-I / ¹³ C ₆ - phenylalanine
Brinton EA, Eisenberg S, Breslow JL	1994	Arterio Thromb. 14: 707-720	¹²⁵ I-apoAI, ¹³¹ I-apoAI-I or vv
Recalde D, Ordovas JM et al	2000	Atherosclerosis. 154: 613-623	² H ₃ -leucine 15h primed constant infusion
Velez-Carrasco W, Lichtenstein AH, Schaefer EJ et al	2000	Arterioscler Thromb Vasc Biol. 20: 801-806	² H ₃ -leucine 15h primed constant infusion Fed state
Pont F, Duvillard L et al	2002	Int J Obesity. 26: 1151-1158	¹³ C- leucine 16h primed constant infusion Fed state
Watts GF, Barrett PH, Johnson AG et al	2003	Diabetes. 52: 803-811	² H ₃ -leucine 6h primed constant infusion
Mauger JF, Lamarche B et al	2005	Atherosclerosis. 178: 157-163	² H ₃ -leucine 12h primed constant infusion Fed state
Brousseau ME, Millar JS, Rader DJ et al	2005	Arterioscler Thromb Vasc Biol. 25: 1057-1064	² H ₃ -leucine 15h primed constant infusion Fed state
Ooi EM, Watts GF, Barrett PH et al (pooled data from 13 kinetic studies)	2005	Obes Res. 13: 1008 -1016	¹³ C- phenylalanine, ² H ₃ -leucine, ¹³ C -leucine primed constant infusion (in one case, using bolus injection)

vv=vice versa; ¹²⁵I and ¹³¹I=radioisotopes (iodine)

2. Specific objectives: applying lipoprotein kinetic studies using stable isotopes to compare the effects of two diets on lipoprotein metabolism

We applied lipoprotein kinetic studies to evaluate our original hypothesis i.e. that a Mediterranean diet rich in MUFAs from virgin olive oil, compared to a STEP II diet, increases, or preserves, HDLC concentrations as a result of an increase in apoA-I production and not due to a decrease in its catabolism. Due to the high level of complexity required for the conduct of these studies, we also applied lipoprotein kinetic studies to apo B-100-rich lipoproteins to evaluate the precision of our kinetic data obtained and, as such, to verify the quality of kinetic methodology.

One limitation of the present work is the small number of study subjects (n=10). This was due to the complexity of the methodology, the time investment, the large number of samples needing storage and processing, and the very high costs involved. This small number of subjects studied and the wide variation in the data obtained could explain the lack of statistically significant differences in some measured parameters ^{435, 503}.

Each volunteer followed a Mediterranean and a STEP II diet in a crossover design. At the end of each dietary intervention period, the corresponding kinetic study was performed to measure the underlying metabolic mechanisms that could explain the plasma concentrations of the lipoproteins. This thesis presents the plasma lipoprotein results of the dietary intervention periods from all 10 volunteers and the lipoprotein kinetic results obtained from a 4 volunteers subset. Although our kinetic data have the limitation of the low number of kinetic studies analysed to-date (7 kinetic studies in 4 volunteers following the Mediterranean diet and 3 volunteers following the STEP II diet), the data obtained do provide some insights into the underlying mechanisms of the effects of the two diets.

The dietary intervention periods were designed as a controlled, crossover study where volunteers were randomly assigned to either diet using a computer generated random number sequence and, as such, providing strong, non-biased evidence for our data ⁴³⁵. The dietary protocol consisted of a 2-week stabilisation period and a 4-week dietary intervention period, either a Mediterranean or a STEP II diet. A 4-week washout period was included between dietary periods in order to minimise any possible interactions between diets and sequence order i.e. the carry-over effect. Plasma lipid concentrations are known to stabilise within 4 weeks of FAs dietary modification ⁵⁰⁴.

In the present study, both diets differed, mainly, in their total lipid and their type of FAs content. The Mediterranean diet, compared to the STEP II diet, had a significantly higher total lipid (33.95% of total energy) and MUFAs (19.09% of total energy) content while the STEP II diet had a lower total lipid (25.01% of total energy) and MUFAs (11.89% of total energy) content. Both diets had a low SFAs content, albeit the Mediterranean diet had a significantly higher SFAs content than the STEP II diet (7.29% versus 5.60% of total energy, respectively). This

corresponded to a difference in the percentage of CH intake in both diets. The Mediterranean diet, compared to the STEP II diet, had a lower CH content (44.96% and 52.23% of total energy, respectively).

We need to highlight that virgin olive oil was exclusively used in both diets and corresponded to about 30-35 g/day in the STEP II and about 50-60 g/day in the Mediterranean diet. Consumed virgin olive oil was of the Arbequina olive variety. This variety has an α -tocopherol content between 130-270 mg/kg, a total phenol content between 170-270 mg/kg and a mean FFA content, or percentage of oleic acid, of $0.12\% \pm 0.04$. Its composition was: oleic acid of $73.01\% \pm 1.03$, palmitic acid of $12.43\% \pm 1.07$, linoleic acid of $9.76\% \pm 0.56$, stearic acid of $2.10\% \pm 0.20$, palmitoleic acid of $1.22\% \pm 0.20$, arachidic acid of $0.56\% \pm 0.26$ and linolenic acid of $0.46\% \pm 0.18$ ⁵⁰⁵. The Mediterranean diet provided about twice the amount of virgin olive oil than the STEP II diet and thus, twice the supposed amount of MUFAs and polyphenols. However, without a proper analysis, it is difficult to know exactly their contents and the effects if doubled. This aspect needs to be studied in depth.

Other foods consumed (vegetables, fruits, legumes, fish) in both diets were similar, although their relative amounts differed slightly in order to maintain an isocaloric intake. Of note is that a change of >2% weight would have an impact on lipoproteins profiles⁵⁰⁶. In the present study, the volunteers' body weight variation in the course of the study was 1.01%.

Our results confirm that a Mediterranean diet rich in MUFAs from virgin olive oil induces a more favourable lipoprotein profile in relation to CVD risk factors, than does a STEP II diet. To-date, published studies have shown that a Mediterranean diet intake preserves plasma apoA-I and HDLC concentrations. Conversely, a STEP II diet significantly decreases plasma apoA-I and HDLC concentrations. Our results confirm these findings, and which are of considerable clinical relevance in the management of CVD. The differences observed, especially in plasma apoA-I concentrations, may be due to virgin olive oil content as part of the composition of an optimal diet such as the Mediterranean diet, rather than the STEP II diet. Hence, a Mediterranean diet rich in MUFAs from virgin olive oil could be used as a dietary tool to induce an increase in apoA-I and HDLC concentrations, or to maintain them^{134, 213, 242, 507-525}.

Large amounts of MUFAs (approx. 80% as oleic acid) consumed, mainly, in the form of olive oil in the Mediterranean diet, are considered to be responsible for the protective effect of the diet against CVD. Olive oil, especially virgin olive oil, is the natural juice from the first pressing of the olive. The process results in other minor non-lipid constituents of olive oil such as vitamin E, sitosterols, carotenoids and polyphenols, all of which have been shown to exhibit protective activity against CVD^{143, 243, 526-528}.

In 2004, the Food and Drug Administration (FDA) provided a qualified health claim for monounsaturated fat from olive oil and CHD risk reduction; limited but not conclusive scientific evidence suggesting that a daily intake of about 2 tablespoons

(23 g) of olive oil may reduce CHD risk, due to MUFA content of olive oil. To achieve this possible benefit, olive oil would need to replace a similar amount of SFAs, without increasing the total calories ingested per day ⁵²⁸.

2.1 Our study of the effects on lipoprotein metabolism of a Mediterranean-type diet rich in MUFAs from virgin olive oil

2.1.1 Dietary interventions and kinetics to study lipoprotein metabolism

2.1.1.1 Anthropometric and biochemical parameters

From the results of the 10 volunteers studied, we observed that the Mediterranean diet, compared to the STEP II diet, significantly increased plasma apoA-I concentrations (+9.96%). Further, the Mediterranean diet showed a trend, albeit not significant, towards an increase in plasma HDLC concentrations (+10.87%). We confirmed this effect on plasma apoA-I concentrations using repeated measurements in the 10 volunteers and in the 4 volunteers subset. Of note is that in the 4 volunteers subset as well, the Mediterranean diet, compared to the STEP II diet, significantly increased plasma apoA-I concentrations (+9.30%). Further, the measurements made on the day of the kinetic studies reflected this same trend in which the Mediterranean diet, compared to the STEP II diet, significantly increased plasma apoA-I concentrations (+10.22%).

Our results are in concordance with data of several published studies showing that whereas a STEP II diet decreases HDLC concentrations, a Mediterranean diet maintains, or even increases, HDLC ^{60-62, 183, 507-525}.

In the group of 10 volunteers we observed that the Mediterranean diet, compared to the STEP II diet, significantly reduced the apo B-100/apoA-I ratio (+14.97%). This was due, mainly, to a reduction in plasma apo B-100, albeit not significantly, together with a maintenance of plasma apoA-I concentrations. The opposite effect was observed in the STEP II diet; plasma apo B-100 concentrations were maintained together with a significant decrease in plasma apoA-I concentrations.

Our results are in agreement with data from recent studies which reflect this clinically relevant ratio (q.v. the INTERHEART study) and in which the apo B-100/apoA-I ratio was shown to be the best risk marker reflecting the relationship between atherogenic and anti-atherogenic particles in plasma. The INTERHEART results showed that the apo B-100/apoA-I ratio differences between CVD cases and control subjects were approximately 6% (with a mean ratio of 0.85 in CVD cases and of 0.80 in controls). It showed, as well, this ratio to be a marker of myocardial infarction, irrespective of the geographic distributions of the populations studied. Recently, the apo B-100/apoA-I ratio has been linked to the risk of fatal stroke in a manner that is similar to that for myocardial infarction and other ischemic events ^{156, 158, 171, 529, 530}.

ApoA-II data were available only from the blood samples taken on the day of the kinetic study. From the results of the 4 volunteers subset, the Mediterranean diet, compared to the STEP II diet, showed a trend, albeit not significant, towards an increase in plasma apoA-II concentrations and in the apoA-II of HDL (+7.65%).

The role of plasma apoA-II concentrations in relation to CVD has not, as yet, been well defined, although apoA-II is known to modulate several steps in HDL metabolism and would be, therefore, a modulator of RCT ^{183, 189-199, 293}.

From the results of the 10 volunteers, we observed that Mediterranean and the STEP II diets *per se* caused a decrease of plasma total C concentrations (-3.13% and -5.59%, respectively), although significance was reached only after the STEP II diet. The Mediterranean and STEP II diets decreased, albeit not significant, plasma LDLC concentrations to a similar extent (-5.57% and -4.51%, respectively). In relation to plasma apo B-100 concentrations, the Mediterranean diet induced a decrease (-5.55%), albeit not significant, and the STEP II diet maintained the values (-0.28%). There were no statistically significant differences between the diets. From the results of the 4 volunteers subset, comparing both diets, we observed no significant differences between diets. However, the result from the day of the kinetic study showed a difference in which the Mediterranean diet, compared to the STEP II diet, significantly increased total C (+3.37%).

Our results agree with data of several published studies which show that both, the Mediterranean and STEP II diets, have a lowering capacity with respect to total C and LDLC ^{60-62, 172-175, 507-525, 531}.

From the results from the day of the kinetic study in the 4 volunteers subset, we observed that the Mediterranean diet, compared to the STEP II diet, induced several compositional changes in the lipoprotein fractions. Percentage PL in VLDL₁, VLDL₂, IDL and LDL increased in the Mediterranean diet, although only significantly in VLDL₂ (+52.82%) and LDL (+6.66%) fractions. Further, the Mediterranean diet induced core-surface lipoprotein fractions changes. The Mediterranean diet decreased the FC:PL ratio in VLDL₁, VLDL₂, IDL, LDL and HDL and the EC:TG ratio in VLDL₁, VLDL₂, IDL and LDL; albeit the decrease was only significant for VLDL₁ FC:PL ratio (-6.90%).

The interest of analysing the composition of lipoprotein fractions is that, once we have concluded the kinetic studies in all 10 volunteers, we would have a better understanding of the relationships between plasma lipid concentrations and the kinetic parameters of lipoprotein metabolism ⁴³⁵.

From the results of the 10 volunteers, we observed that the Mediterranean diet *per se* significantly decreased the diastolic blood pressure (-3.71 mmHg) in our normal blood pressure volunteers. There were no statistically significant differences between the diets.

Our result agree with published data showing that the traditional Mediterranean diet has an impact on blood pressure. More recent studies show that replacement of some CH with plant-source protein or with MUFAs could lower blood pressure further (e.g.

Dietary Approaches to Stop Hypertension, DASH, diet). Our result showing diastolic blood pressure reduction is greater than the -2.6 mmHg observed when sodium intake is reduced by 75 mg/day (DASH diet) or than the -3.4 mmHg observed when dietary potassium supplements of 50 to 120 mg/day are administered (reducing diastolic blood pressure similar to that of salt restriction). Thus, virgin olive oil could be included in the recognised dietary modifications that have the effect of lowering blood pressure (e.g. salt intake reduction, weight loss induced by an energy deficit, moderated alcohol consumption among imbibers, increased potassium intake and consumption of an overall healthy DASH-based diet). What may be the cause of this blood pressure improvement is unclear, although several studies have shown that olive oil intake *per se* and olive oil compounds improve blood pressure since olive oil antioxidant compounds might inactivate effects of free radicals and lipid peroxides and which could affect arterial stiffness ^{208, 213, 242, 246, 251, 520, 523, 532-534}.

We designed the diets to be isocaloric ⁵³⁵. Despite this, volunteers' body weight decreased significantly (-1.05 kg) following the STEP II diet, while body weight did not change significantly (-0.31 kg) following the Mediterranean diet. The same trend was observed in BMI (-0.37 and -0.12 kg/m², respectively). The Mediterranean diet, compared to the STEP II diet, induced a not significant change in body weight by a mean of $\leq 1\%$. However, in the 4 volunteers subset, we observed that the Mediterranean diet significantly decreased the body weight (-0.58 kg) and showed a trend, albeit not significant, towards a decreased BMI (-0.21 kg/m²). The Mediterranean diet, compared to the STEP II diet, did not show statistically significant differences in any of these measured anthropometric parameters.

From the results of the 10 volunteers and the 4 volunteers subset, we observed that after each dietary intervention period and comparing both diets, no significant differences were found in relation to plasma TG concentrations with either diet.

The Mediterranean and STEP II diets have shown, in several published studies, to have a TG-concentration lowering capacity ^{143, 242, 496, 516, 518, 535-542}.

From the results of the 10 volunteers and the 4 volunteers subset after each dietary intervention period and comparing both diets, we observed no significant differences in plasma markers of inflammation (hs CRP, IL-6, sICAM-1 and sVCAM-1) and oxidation (ox LDL and ox LDL-Ab).

Our results are not in accord with several published studies that have focussed on the effects of the traditional Mediterranean diet; reductions in these markers being observed ^{144, 228, 238, 241, 243, 526-528, 543-558}.

2.1.1.2 Preliminary lipoprotein kinetic parameters

Results are available for only the 4 volunteers in whom the 7 kinetic studies have been analysed to-date (4 studies following the Mediterranean diet and 3 following the

STEP II diet). Although the number of kinetic studies performed is low, the results of the 7 kinetic studies do provide some insight into the effects of the diets.

To our knowledge, the present study is the first to compare the impact of a Mediterranean diet rich in MUFAs from virgin olive oil on apoA-I HDL kinetic parameters. Further, the current work is more complete because it addresses the effects of the diets on apo B-100 VLDL₁, VLDL₂, IDL and LDL as well (Tables 54, 55, 56, 57) ^{269, 497, 505, 559-577}.

Few studies have reported the effects of dietary changes on plasma apoA-I and apoA-II kinetic parameters. Uniquely, Desroches et al (2004) performed a very similar study to ours in relation to diets. They evaluated the impact of a low fat-diet and a high-MUFA diet without specifying quality (from virgin olive oil or other MUFA sources) or quantity. Specifically, they performed kinetic studies of apo B-100 VLDL, apoA-I and apoA-II HDL pre- and post-dietary intervention periods of between 6-7 weeks. Some of their dietary intervention and kinetic study design differed from ours:

- * they studied two parallel groups of young men (mean age 37.5 years) who were normolipidaemic and moderately obese. The diets were *ad libitum* (with unrestricted intake). All meals were prepared and consumed in their Metabolic Unit and 3-day food records were completed at baseline.
- * they performed their kinetic study before and after each dietary intervention period under constant feeding conditions. Blood samples were collected over a shorter period of time (12h) and kinetic data were obtained using the mono-exponential approach.

We need to highlight that our kinetic data are within the same range of values as that of published studies. Kinetic parameters for apo B-100 LDL, apoA-I and A-II HDL did not differ as much as those for apo B-100 VLDL and IDL. VLDL and IDL kinetic parameter variability between studies has been considerable and may be related to differences in the type of subjects selected, differences in methodology (modelling) and, as well, the accuracy in the measurements of plasma pool masses ³³¹.

The present work confirms our hypothesis with respect to plasma apoA-I and HDLC concentrations and apoA-I HDL kinetics. However, it is unable to confirm that both diets significantly decrease plasma LDLC concentrations, although differences in apo B-100 LDL kinetic parameters would seem to suggest such a decrease.

2.1.1.2.1 ApoA-I and A-II HDL kinetic parameters

All plasma measurements showed that the Mediterranean diet, compared to the STEP II diet, significantly increased plasma apoA-I concentrations. This was reflected in apoA-I kinetic parameters of the 4 volunteers subset. The Mediterranean diet, compared to the STEP II diet, showed a trend, albeit not significant, towards an

increase in apoA-I HDL PR (+26.84%). When corrected for body weight, apoA-I HDL PR showed the same tendency (+22.02%). ApoA-I HDL FCR was similar in both diets.

Due to methodological limitations, we measured plasma apoA-II concentrations only at the end of each dietary intervention, on the day of the kinetic study. The Mediterranean diet, compared to the STEP II diet, showed a trend, albeit not significant, towards an increase in plasma apoA-II concentrations. Kinetic parameters from the 4 volunteers subset indicated that the Mediterranean diet, compared to the STEP II diet, induced a trend, albeit not significant, towards a decrease in apoA-II HDL PR (-9.02%). This tendency did not change following a correction for body weight (-13.89%). Also, the Mediterranean diet showed a trend, albeit not significant, towards a decrease in apoA-II HDL FCR (-21.43%) and towards an increase in apoA-II pool size (+12.92%).

Our results are in accordance with data of several published studies which show that a STEP II diet low in SFAs and C significantly decreases plasma HDLC concentrations in humans. The evidence supports the proposition that a low-fat and, perhaps, low-MUFA ($\leq 12\%$) diets are associated with a decrease in plasma HDLC concentrations due to a reduction in apoA-I PR. The majority of published kinetic studies suggest that FCR is the main determinant of HDLC concentrations, except in the case of dietary studies. Variation in fat content and different types of FAs affect, mainly, HDL PR or transport rates ²⁷⁴.

Few kinetic studies have reported the effects of dietary changes on apoA-I and apoA-II plasma kinetic parameters. Kinetic studies performed using radioisotopes and stable isotopes have provided similar data (Tables 54, 55) ⁵⁵⁹⁻⁵⁶⁶.

Brinton et al (1990) found, following a very-low-fat low-C diet compared to a high-fat diet, a significantly decrease in HDL PR together with a significant decrease in plasma HDLC concentrations. They did not observe any changes either in plasma apoA-II concentrations or in the kinetic parameters ⁵⁶⁰.

Vélez-Carrasco et al (1999) found, following an isocaloric STEP II diet compared to baseline, a significant decrease in plasma HDLC and apoA-I concentrations, and the decrease was due to a significant reduction in apoA-I PR. These results could have been as a result of the reduction in total fat content; the baseline diet having 35% of total energy and STEP II diet having 25%. They did not find any changes in plasma apoA-II concentrations or in the kinetic parameters ⁵⁶¹.

Desroches et al (2004) found, in relation to the percentage changes between pre- and post-low-fat dietary intervention periods, a significant decrease in plasma apoA-I concentrations due to a significant reduction in apoA-I PR and in FCR. Further, they observed a significant decrease in plasma apoA-II concentrations due to an increase, albeit not significant, of apoA-II PR and FCR. In relation to the percentage change between pre- and post-high-MUFA dietary intervention period, they observed a decrease, albeit not significant, in plasma apoA-I concentrations due to a significant increase in FCR (+9.60). Of note is that the high-MUFA diet increased, albeit not

significantly, apoA-I PR. A significant decrease in plasma apoA-II concentrations (-16.00%) was found, and was due to a significant increase of FCR (+40.90%). The possible effects that their subjects' body weight decrease of >2% might have had on apoA-I kinetic parameters need to be taken into account. The significant variations in body weight could have been due to the *ad libitum* nature of the dietary study. The heterogeneity in the subjects' grades of obesity make it impossible to compare the impact of the experimental diets on kinetic parameters in subgroups of lean and obese individuals ⁵⁶⁵.

From the results of the 4 volunteers subset with 7 analysed kinetic studies conducted with the two diets, we observed a significant correlation ($r=0.90$; $P=0.005$) between plasma apoA-I concentrations and apoA-I PR. This same correlation was observed even after correcting for body weight ($r=0.70$; $P=0.07$). Hence, our results suggest that a high apoA-I PR is the determinant of a high apoA-I plasma concentrations, and not variations in its catabolism.

2.1.1.2.2 Apo B-100-rich lipoproteins kinetic parameters

Results from the 4 volunteers subset showed that apo B-100 VLDL₁, VLDL₂ and IDL kinetic parameters were very variable for the Mediterranean diet as well as for the STEP II diet. This, together with the limited number of kinetic studies analysed, might explain the lack of statistical significance in the kinetic parameters when comparing the two diets.

Results from the 4 volunteers subset showed that the Mediterranean diet, compared to the STEP II diet caused an increase, albeit not significant, in plasma LDLC concentrations. This was observed in apo B-100 LDL kinetic parameters from the 4 volunteers. The Mediterranean diet, compared to the STEP II diet, significantly increased apo B-100 LDL PR (+217.65%). This difference was similar, albeit not significant, when corrected for body weight (+191.18%). However, the Mediterranean diet, compared to STEP II diet, showed a trend, albeit not significant, towards an increase in apo B-100 LDL dirFCR (+33.33%).

Our results agree with data of several published studies showing that the STEP II diet might decrease LDL synthesis by the liver ⁵⁷⁸. It needs to be taken into account that different diets may act through different metabolic ways, although the effects in plasma may be similar ^{273, 497}.

Few kinetic studies have reported the effects of low-fat diets and/or high-MUFA diets on apo B-100-rich lipoproteins plasma kinetic parameters. Kinetic studies performed using radioisotopes and stable isotopes have provided similar data (Tables 56, 57) ⁵⁶³⁻⁵⁷⁷.

Cortese et al (1983) found, following a low-fat diet compared to a high-fat diet, a significant decrease in plasma LDLC concentrations due to a significant decrease in

apo B-100 LDL PR and, possibly, an increased FCR as well. Apo B-100 VLDL PR and FCR did not change ⁵⁶⁹.

Abbott et al (1990) found, following a low-fat high-CH diet compared to a high-fat diet, a significant decrease in the plasma concentrations of total C, LDLC and HDLC while TG increased, but not significantly. Apo B-100 VLDL and LDL PR and FCR did not change, whereas a significant decrease in VLDL pool size was due to a decrease in transfer from VLDL to IDL and from IDL to LDL ⁵⁷¹.

Brinton et al (1990) found, following a very-low-fat high-CH diet compared to a high-fat diet, a significant decrease in the plasma concentrations of total C and LDLC but they performed no apo B-100 VLDL₁, VLDL₂, IDL and/or LDL kinetic studies ⁵⁶⁰.

Gill et al (2003) found, following increasing high-MUFA diets, a significant decrease in plasma C, LDLC, atherogenic plasma subclass LDL₃ and apo B-100. Plasma TG did not change significantly. VLDL₁ and VLDL₂ PR and FCR did not change significantly. Increased LDL clearance was due, probably, to an up-regulation of LDLR. However, they performed no apo B-100 IDL and/or LDL kinetic studies ⁵⁶³.

Desroches et al (2004) found that neither within nor between a low-fat and a high-MUFA diets were there differences in apoB-100 VLDL PR or FCR ⁵⁶⁵.

2.1.1.2.3 Kinetic parameters as a key to understanding plasma lipoproteins

Apos have a distinctive metabolic behaviour that reflects differences in their interactions with enzymes, lipid transfer proteins and receptors. Thus, changes in apos production and catabolism in kinetic studies may reflect changes in gene transcription, protein maturation, lipoprotein synthesis and degradation. Studies in cell and animal models have provided some information on molecular mechanisms by which dietary compounds might have an effect on lipid metabolism (a regulation through different mechanisms). Even so, there is much to be determined with respect to interactions of dietary compounds, and between genes and proteins of the lipoprotein metabolism. For example ^{230, 578-584}:

- * MUFAs and CH have been shown to decrease CETP activity.
- * MUFAs increase LDLR mRNA and protein abundance and activity.
- * low-fat high-CH diets decrease LPL expression.
- * olive oil increases apoA-I mRNA expression and plasma apoA-I concentrations.
- * promoters of apo B-100, apoA-I and apoA-II genes have binding sites for HNF-4 transcription factor (glucose regulates apoA-II gene through HNF-4).
- * promoters of apoA-I, apoA-II and LPL genes have binding sites for PPAR α which interacts with unsaturated FAs.

2.1.2 The rationale for therapeutic interventions targeting HDLC

Currently, the existing therapies have only modest effects on HDLC levels. HDL metabolism is complex and related to C flux through the RCT pathway. The protective capacity of HDL against CVD may be related to this and other aspects of HDL functionality. The clinical benefits of HDL-targeting therapies indicate that plasma HDLC concentrations alone are almost certainly not an adequate predictor of CVD risk⁵²⁴. As such, there is a considerable need to identify novel markers and kinetic methods to assess the effects of novel interventions on HDL metabolism. In the past decade, there have been major improvements in therapies for reducing LDLC and, as a consequence, cardiovascular events. The next two decades are likely to witness major advances in the novel therapies targeting HDLC metabolism. The goal would be towards the prevention or regression of atherosclerosis, and a further substantial reduction in cardiovascular events⁵⁸⁵. These include several life-style recommendations (e.g. soluble fibre intake, moderate alcohol or MUFAs consumption, avoiding cigarette smoke and increase in physical activity) to increase plasma HDLC concentrations⁵⁸⁶. Recommendations for MUFAs intake, specifically that derived from virgin olive oil, are within CVD prevention guidelines because it is a dietary factor that maintains, or preserves, plasma HDLC and increases apoA-I concentrations.

Table 54. Nutritional interventions in human subjects applying kinetics to study apoA-I and A-II HDL metabolism ⁵⁵⁹⁻⁵⁶⁶.

Reference	Subjects	Conclusions
Blum et al JCI. 1977. 60: 795-807 (radioisotope)	3 man / 5 women (healthy subjects)	<u>A high-CH, very low-fat diet</u> enhanced HDL protein FCR compared to a normal diet.
Shepherd et al JCI. 1978. 61: 1582-1592 (radioisotope)	4 men (healthy subjects)	A PUFA diet decreased plasma C and TG levels, changed FA composition of all plasma lipoprotein fractions. It also decreased plasma apoA-I.
Brinton et al JCI. 1990. 85: 144-151 (radioisotope)	5 men / 8 women (healthy subjects)	<u>A very low-fat, low-CH diet</u> decreased HDL apoA-I synthesis and enhanced apoA-I FCR compared to a high-fat diet. Diet induced decreases in HDLC, correlated with decreases in apo A-I synthesis.
Vélez-Carrasco et al Arterioscler Thromb Vasc Biol. 1999. 19: 918-924	14 men/ 7 women (healthy subjects)	<u>A STEP II diet</u> decreased HDL apoA-I synthesis compared to a normal diet.
Frenais et al Atheros. 2001. 157: 131-135	1 men / 4 postmenopausal women (type II diabetes mellitus)	Effect of maxEPA® capsules on kinetics of apoA-I (decreased FCR and production) in type II diabetes mellitus, probably linked to changes in plasma TG levels (plasma TG decreased).
Gill et al Am J Clin Nutr. 2003. 78: 47-56	17 men/ 18 women (moderately hypercholesterolemic, middle-aged subjects)	Effect of <u>replacing SFAs with MUFAs</u> had no changes in HDL and apoA-I.
Matthan et al Arterioscler Thromb Vasc Biol. 2004. 24: 1092-1097	8 women (postmenopausal)	The mechanism for the adverse lipoprotein profile observed with hydrogenated fat intake was determined in part by increased apoA-I.
Desroches et al J Lipid Res 2004. 45: 2331-2338	65 men (18 men for kinetic study) (moderate obese, normolipidemic subjects)	<u>A low-fat diet</u> reduction in apoA-I production explained decrease of HDLC. A decrease in apoA-I FCR possibly attributable to body weight reduction) <u>A high-MUFA diet</u> reduction in HDL3-C mediated by an increase in apoA-II FCR.
Chan et al Am J Clin Nutr. 2006. 84: 37-43	48 men (insulin resistant, obese)	Fish oils supplementation (Omacor® capsules) decreased HDL apoA-I and A-II FCR and production.
Our study	10 men (moderate hypercholesterolemic)	<u>A Mediterranean-type diet</u> , compared with a <u>STEP II diet</u> , increased plasma apoA-I levels, showed a trend towards an increase in plasma HDL C levels and decreased apo B-100/apoA-I ratio. <u>A Mediterranean-type diet</u> , compared with a <u>STEP II diet</u> , showed a high apoA-I HDL production rate. A higher apoA-I production rate is the determinant of high plasma apoA-I levels.

underlined=similar to our study;
 FCR=fractional catabolic rate; PR=production rate;
 CH=carbohydrates; SFAs=saturated fatty acids;
 MUFAs=monounsaturated fatty acids;
 PUFAs=polyunsaturated fatty acids.

Table 55. Nutritional interventions in human subjects applying stable isotope kinetics to study apoA-I and A-II HDL metabolism: study design and kinetic parameters ⁵⁵⁹⁻
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Reference	Design																																				
Vélez-Carrasco et al Arterioscler Thromb Vasc Biol. 1999. 19: 918-924	<p>2 separate metabolic studies (similar data from both, thus added): 1st a 6-wk baseline average American diet + 24-wk STEP II diet 2nd a 6-wk baseline average American diet + 6-wk STEP II diet Max. 4-wk of non regulated diet between phases Composition of diets determined by chemical analysis of food</p> <table border="1"> <thead> <tr> <th></th> <th>Baseline diet</th> <th>STEP II diet</th> </tr> </thead> <tbody> <tr> <td>CH %</td> <td>49.4 ± 2.2</td> <td>58.8 ± 1.8</td> </tr> <tr> <td>Prot %</td> <td>15 ± 1.2</td> <td>16.3 ± 0.7</td> </tr> <tr> <td>Total fat %</td> <td>35.4 ± 2.3</td> <td>25.5 ± 1.8</td> </tr> <tr> <td> SFA %</td> <td>14.1 ± 2.2</td> <td>4 ± 0.4</td> </tr> <tr> <td> MUFA %</td> <td>14.5 ± 1</td> <td>10.8 ± 0.9</td> </tr> <tr> <td> PUFA %</td> <td>6.9 ± 1.2</td> <td>10.5 ± 0.2</td> </tr> <tr> <td>C (mg/1000 kcal)</td> <td>147 ± 27</td> <td>45 ± 15</td> </tr> </tbody> </table> <p>All meals and drinks prepared and provided (metabolic unit) Body weight maintained constant</p>		Baseline diet	STEP II diet	CH %	49.4 ± 2.2	58.8 ± 1.8	Prot %	15 ± 1.2	16.3 ± 0.7	Total fat %	35.4 ± 2.3	25.5 ± 1.8	SFA %	14.1 ± 2.2	4 ± 0.4	MUFA %	14.5 ± 1	10.8 ± 0.9	PUFA %	6.9 ± 1.2	10.5 ± 0.2	C (mg/1000 kcal)	147 ± 27	45 ± 15												
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MUFA %	14.5 ± 1	10.8 ± 0.9																																			
PUFA %	6.9 ± 1.2	10.5 ± 0.2																																			
C (mg/1000 kcal)	147 ± 27	45 ± 15																																			
Frenais et al Atheros. 2001. 157: 131-135	<p>Weight maintenance diet (during 1 month) before kinetic study CH % 50 Fat % 35 Prot % 15 Kinetic study before and 2 months after 8 wk treatment with MaxEPA® capsules (fish oil supplementation) 1000 mg EPA 120 mg DHA 1.75 mg α-tocopherol acetate</p>																																				
Matthan et al Arterioscler Thromb Vasc Biol. 2004. 24: 1092-1097	<p>Randomised, blinded, crossover study 3 diets for 5-wk periods with 2/3 of fats from soybean oil for unsaturated fat stick margarine for hydrogenated fat butter for saturated fat Composition of diets determined by chemical analysis of food</p> <table border="1"> <thead> <tr> <th></th> <th>Unsaturated fat</th> <th>Hydrogenated fat</th> <th>Saturated fat</th> </tr> </thead> <tbody> <tr> <td>CH %</td> <td>55.8</td> <td>53.5</td> <td>54</td> </tr> <tr> <td>Prot %</td> <td>15.7</td> <td>16.7</td> <td>16.9</td> </tr> <tr> <td>Total fat %</td> <td>28.5</td> <td>29.7</td> <td>29.1</td> </tr> <tr> <td> SFA %</td> <td>7.3</td> <td>8.5</td> <td>16.7</td> </tr> <tr> <td> MUFA %</td> <td>8.1</td> <td>8.5</td> <td>8.1</td> </tr> <tr> <td> PUFA %</td> <td>12.5</td> <td>6.3</td> <td>2.4</td> </tr> <tr> <td> Trans %</td> <td>0.6</td> <td>6.7</td> <td>1.3</td> </tr> <tr> <td>C (mg/1000 kcal)</td> <td>66</td> <td>67</td> <td>121</td> </tr> </tbody> </table> <p>Body weight maintained constant Analysis of the macronutrient of diets</p>		Unsaturated fat	Hydrogenated fat	Saturated fat	CH %	55.8	53.5	54	Prot %	15.7	16.7	16.9	Total fat %	28.5	29.7	29.1	SFA %	7.3	8.5	16.7	MUFA %	8.1	8.5	8.1	PUFA %	12.5	6.3	2.4	Trans %	0.6	6.7	1.3	C (mg/1000 kcal)	66	67	121
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wk=week; CH=carbohydrates; SFAs=saturated fatty acids;
 MUFAs=monounsaturated fatty acid;
 PUFAs=polyunsaturated fatty acids; EPA=eicosapentanoic acid,
 DHA=docosahexanoic acid.

Method	Kinetic parameters			
² H ₃ -L-leucine Primed constant infusion for 15 hours Blood samples collected until 15 hours 12 hours O/N fasted + fed hourly for 20 hours Mono-exponential function	<u>Baseline diet</u> <u>STEP II diet</u>			
	ApoA-I			
	Plasma (mg/dl)	125 ± 15	110 ± 13	(1)
	Pool size (mg)	4308 ± 759	3781 ± 734	(1)
	FCR (pools/day)	0.219 ± 0.052	0.220 ± 0.043	
	PR (mg/kg/day)	12.26 ± 3.07	10.84 ± 2.11	(2)
	ApoA-II			
	Plasma (mg/dl)	27 ± 3	27 ± 4	
	Pool size (mg)	937 ± 173	916 ± 215	
	FCR (pools/day)	0.175 ± 0.042	0.171 ± 0.038	
PR (mg/kg/day)	2.15 ± 0.61	2.04 ± 0.53		
	(1) P < 0.001			
	(2) P < 0.05			
² H ₃ -L-leucine Primed constant infusion for 14 hours Blood samples collected until 14 hours Fasted condition Mono-exponential function	<u>Before supplementation</u> <u>After maxEPA® capsules</u>			
	ApoA-I			
	Plasma (mg/dl)	111.8 ± 25.4	116 ± 25.6	
	FCR (pools/day)	0.37 ± 0.08	0.27 ± 0.09	(1)
	PR (mg/kg/day)	16.1 ± 3.3	12.1 ± 2.8	(1)
	(1) P < 0.05			
² H ₃ -L-leucine Primed constant infusion for 15 hours Blood samples collected until 15 hours 12 hours O/N fasted + fed hourly for 20 hours Multi-compartmental model	After experimental diets			
	<u>Unsaturated fat</u> <u>Hydrogenated fat</u> <u>Saturated fat</u>			
	ApoA-I			
	Plasma (mg/dl)	117 ± 13	114 ± 14	118 ± 12 (1)
	Pool size (mg)	3326 ± 287	3211 ± 358	3353 ± 198 (1)
	FCR (pool/day)	0.19 ± 0.04	0.20 ± 0.04	0.18 ± 0.03 (1)
	PR mg/kg/day)	9.79 ± 0.89	10.14 ± 1.94	9.53 ± 1.12
		(1) P < 0.05		

O/N=over night; FCR=fractional catabolic rate; PR=production rate.

Reference	Design																																								
Desroches et al J Lipid Res 2004. 45. 2331-2338	<p>Randomised, parallel, two-group study 2 diets: 6-7-wk of a low-fat/high-CH diet or a high-fat diet rich in MUFAs Both diets with the same meals but different amount of fat and CH Unsaturated fat, mainly olive oil CH, mainly whole grain and vegetables Composition of diets determined using nutrition data bases and software</p> <table border="1"> <thead> <tr> <th></th> <th>Low-fat/high-CH diet</th> <th>High-MUFA diet</th> <th></th> </tr> </thead> <tbody> <tr> <td>CH %</td> <td>58.3</td> <td>44.7</td> <td>(1)</td> </tr> <tr> <td>Prot %</td> <td>15.9</td> <td>15.2</td> <td></td> </tr> <tr> <td>Total fat %</td> <td>25.8</td> <td>40.1</td> <td>(1)</td> </tr> <tr> <td>SFA %</td> <td>6</td> <td>8.2</td> <td>(1)</td> </tr> <tr> <td>MUFA %</td> <td>13.3</td> <td>22.5</td> <td>(1)</td> </tr> <tr> <td>PUFA %</td> <td>5.1</td> <td>7.6</td> <td>(1)</td> </tr> <tr> <td>PUFA/SFA ratio</td> <td>0.87</td> <td>0.93</td> <td>(1)</td> </tr> <tr> <td>C (mg/1000 kcal)</td> <td>105.8</td> <td>110.1</td> <td></td> </tr> <tr> <td>Total fibers (g/1000 kcal)</td> <td>14.2</td> <td>10.1</td> <td></td> </tr> </tbody> </table> <p>(1) $P < 0.01$</p> <p>All meals prepared and provided (metabolic unit) Diets consumed <i>ad libitum</i> 3-day food records at baseline</p>		Low-fat/high-CH diet	High-MUFA diet		CH %	58.3	44.7	(1)	Prot %	15.9	15.2		Total fat %	25.8	40.1	(1)	SFA %	6	8.2	(1)	MUFA %	13.3	22.5	(1)	PUFA %	5.1	7.6	(1)	PUFA/SFA ratio	0.87	0.93	(1)	C (mg/1000 kcal)	105.8	110.1		Total fibers (g/1000 kcal)	14.2	10.1	
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Chan et al Am J Clin Nutr. 2006. 84: 37-43	<p>Randomized, doubled blind, placebo controlled intervention study 3-wk run in period 6-wk treatment period (atorvastatin, fish oils capsules Omacor®, atorv + fish, atorv + placebo capsules) Atorvastatin 40 mg/day Omacor® capsules 4 g/day fish oils (taken at night) 45 % EPA 39 % DHA Placebo capsules 4 g/day (corn oil)</p> <p>Checked capsules intake Composition of diets determined using nutrition software 24h dietary diaries at the beginning and end of the study Physical activity assessed by a 7-day recall questionnaire Body weight maintained constant (variations < 2%)</p>																																								

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 DHA=docosahexanoic acid.

Method	Kinetic parameters					
² H ₃ -L-leucine Primed constant infusion for 12 hours Blood samples collected until 12 hours 12 hours O/N fasted + fed cookies every 30 min for 15 hours Monoexponen tial function	<u>Low-fat/High-CH diet</u>					
		Before	After	ApoA-I % change	After versus Before	
	Plasma (mg/dl)	112 ± 18	99 ± 12	-11.60	(1)	
	Pool size (mg)	4439 ± 914	3828 ± 527	-13.80	(2)	
	FCR (pools/day)	0.226 ± 0.052	0.171 ± 0.040	-24.30	(1)	
	PR (mg/kg/day)	11.10 ± 1.50	7.60 ± 2.20	-31.50	(3)	
	<u>High-MUFA diet</u>					
		Before	After	ApoA-I % change	After versus Before	
	Plasma (mg)	121 ± 18	113 ± 11	-6.60		
	Pool size (mg)	4956 ± 890	4458 ± 771	-10.00	(1)	
	FCR (pools/day)	0.198 ± 0.034	0.217 ± 0.032	+9.60	(1)	
	PR (mg/kg/day)	10.60 ± 1.40	11 ± 2.00	+3.80		
	Between diets FCR (2) / PR (1)					
	<u>Low-fat/High-CH diet</u>					
		Before	After	ApoA-II % change	After versus Before	
	Plasma (mg/dl)	31.1 ± 5.4	28.1 ± 4.8	-9.60	(1)	
	Pool size (mg)	1241 ± 302	1098 ± 245	-11.50	(1)	
	FCR (pools/day)	0.210 ± 0.070	0.230 ± 0.073	+9.50		
	PR (mg/kg/day)	2.83 ± 0.68	2.85 ± 0.81	+0.70		
	<u>High-MUFA diet</u>					
	Before	After	ApoA-II % change	After versus Before		
Plasma (mg/dl)	35 ± 5.00	29.40 ± 1.1	-16.00	(2)		
Pool size (mg)	1429 ± 184	1170 ± 259	-18.10	(3)		
FCR (pools/day)	0.186 ± 0.039	0.262 ± 0.075	+40.90	(1)		
PR (mg/kg/day)	2.30 ± 0.76	3.47 ± 1.10	+18.40			
(1) P<0.05; (2) P<0.01; (3) P<0.001						
² H ₃ -L-leucine Single bolus injection Blood samples collected until 96 hours Fasted condition Multicompart mental model	After intervention diets					
		<u>Placebo</u>	<u>Fish oils</u>	<u>Atorv</u>	<u>Fish oil +Atorv</u>	<u>% Main effect fish oils</u>
	ApoA-I					
	<u>Baseline</u>					
	Plasma (mg/dl)	128 ± 5.00	118 ± 4.00	119 ± 5.00	128 ± 6.00	
	Pool size (mg)	5051 ± 158	4491 ± 202	4707 ± 255	5132 ± 237	
	FCR (pools/day)	0.27 ± 0.02	0.31 ± 0.03	0.23 ± 0.02	0.25 ± 0.02	
	PR (mg/kg/day)	14.6 ± 1.4	14 ± 1.3	11.8 ± 1	15.9 ± 2.9	
	<u>Week 6</u>					
	Plasma (mg/dl)	126 ± 4.00	121 ± 4.00	123 ± 4.00	135 ± 9.00	
	Pool size (mg)	4942 ± 130	4597 ± 195	4844 ± 220	5400 ± 312	
	FCR (pools/day)	0.26 ± 0.02	0.24 ± 0.01	0.24 ± 0.01	0.22 ± 0.02	-0.04 (1)
	PR (mg/kg/day)	13.60 ± 1.20	10.80 ± 0.80	11.80 ± 1.00	13.3 ± 2.00	-1.6 (2)
	ApoA-II					
	<u>Baseline</u>					
	Plasma (mg/dl)	35 ± 1.00	30 ± 1.00	30 ± 1.00	33 ± 1.00	
	Pool size (mg)	1389 ± 56	1118 ± 39	1174 ± 63	1307 ± 59	
	FCR (pools/day)	0.24 ± 0.02	0.26 ± 0.02	0.19 ± 0.02	0.21 ± 0.02	
	PR (mg/kg/day)	14.60 ± 1.40	14 ± 1.30	11.80 ± 1.00	15.90 ± 2.90	
	<u>Week 6</u>					
Plasma (mg/dl)	33 ± 1.00	26 ± 1.00	29 ± 1.00	31 ± 2.00		
Pool size (mg)	1282 ± 46	982 ± 34	1141 ± 77	1247 ± 70		
FCR (pools/day)	0.21 ± 0.02	0.20 ± 0.01	0.20 ± 0.02	0.19 ± 0.02	-0.04 (3)	
PR (mg/kg/day)	13.60 ± 1.20	10.80 ± 0.80	11.80 ± 1.00	13.30 ± 2.00	-0.4 (4)	
(1) P=0.012; (2) P=0.034; (3) P=0.014; (4) P=0.013						

O/N=over night; FCR=fractional catabolic rate; PR=production rate.

Reference	Design																																
Our study	<p>Randomised, crossover study a 2-wk stabilisation period + two dietary intervention periods of 4-wk each (Mediterranean / STEP II diet) a 4 wk-washout period between them</p> <p>Stabilisation diet had the compositional characteristics of the Catalan diet Mediterranean diet compositional characteristics adjusted to the Spanish recommendations for prevention and treatment of CVD STEP II diet compositional characteristics adjusted to the AHA recommendations</p> <p>Composition of diets determined using nutrition data bases and software</p> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th></th> <th style="text-align: center;">Stabilisation diet</th> <th style="text-align: center;">Mediterranean diet</th> <th style="text-align: center;">STEP II diet</th> </tr> </thead> <tbody> <tr> <td>CH %</td> <td style="text-align: center;">42.33 ± 1.89</td> <td style="text-align: center;">44.96 ± 5.09</td> <td style="text-align: center;">52.23 ± 5.15</td> </tr> <tr> <td>Prot %</td> <td style="text-align: center;">14.87 ± 0.42</td> <td style="text-align: center;">14.20 ± 2.02</td> <td style="text-align: center;">15.51 ± 2.35</td> </tr> <tr> <td>Total fat %</td> <td style="text-align: center;">36.31 ± 2.27</td> <td style="text-align: center;">33.95 ± 2.82</td> <td style="text-align: center;">25.01 ± 4.84 (1)</td> </tr> <tr> <td> SFA %</td> <td style="text-align: center;">11.17 ± 1.08</td> <td style="text-align: center;">7.29 ± 0.90</td> <td style="text-align: center;">5.60 ± 1.21 (1)</td> </tr> <tr> <td> MUFA %</td> <td style="text-align: center;">17,35 ± 3,78</td> <td style="text-align: center;">19.09 ± 2.09</td> <td style="text-align: center;">11.89 ± 2.39 (2)</td> </tr> <tr> <td> PUFA %</td> <td style="text-align: center;">7.00 ± 0.81</td> <td style="text-align: center;">4.20 ± 0.60</td> <td style="text-align: center;">4.36 ± 2.59</td> </tr> <tr> <td>C (mg/1000 kcal)</td> <td style="text-align: center;">192.00 ± 85.99</td> <td style="text-align: center;">219.90 ± 85.99</td> <td style="text-align: center;">171.89 ± 77.72</td> </tr> </tbody> </table> <p>(1) P=0.02 (2) P=0.01</p> <p>Body weight maintained constant Meals from readily available foods Volunteers followed dietitian recommendations and had lunch together with a dietitian from Monday to Friday. Volunteers had everyday breakfast and suppers, together with weekends meals at home 7 food records during all study (one 5-day food frequency record + six 3-day food records) Physical activity record</p>		Stabilisation diet	Mediterranean diet	STEP II diet	CH %	42.33 ± 1.89	44.96 ± 5.09	52.23 ± 5.15	Prot %	14.87 ± 0.42	14.20 ± 2.02	15.51 ± 2.35	Total fat %	36.31 ± 2.27	33.95 ± 2.82	25.01 ± 4.84 (1)	SFA %	11.17 ± 1.08	7.29 ± 0.90	5.60 ± 1.21 (1)	MUFA %	17,35 ± 3,78	19.09 ± 2.09	11.89 ± 2.39 (2)	PUFA %	7.00 ± 0.81	4.20 ± 0.60	4.36 ± 2.59	C (mg/1000 kcal)	192.00 ± 85.99	219.90 ± 85.99	171.89 ± 77.72
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 MUFAs=monounsaturated fatty acids;
 PUFAs=polyunsaturated fatty acids; EPA=eicosapentanoic acid;
 DHA=docosahexanoic acid.

Method	Kinetic parameters		
² H ₃ -L-leucine Primed constant infusion for 16 hours Blood samples collected until 48 hours Fasted condition Multicompartmental model		ApoA-I	
		<u>After Mediterranean diet</u>	<u>After STEP II diet</u>
	Plasma (mg/dl)	136.88 ± 14.57	123.33 ± 12.01
	Pool size (mg)	4118.75 ± 923.70	3439.58 ± 655.04
	FCR (pools/day)	0.31 ± 0.04	0.29 ± 0.01
	PR (mg/kg/day)	17.29 ± 0.35	14.17 ± 1.91
		ApoA-II	
		<u>After Mediterranean diet</u>	<u>After STEP II diet</u>
	Plasma (mg/dl)	32.13 ± 2.32	29.67 ± 2.08
	Pool size (mg)	939.25 ± 156.85	831.78 ± 145.99
FCR (pools/day)	0.28 ± 0.05	0.34 ± 0.09	
PR (mg/kg/day)	3.60 ± 0.74	4.10 ± 1.11	

O/N=over night; FCR=fractional catabolic rate; PR=production rate.

Table 56. Nutritional interventions in human subjects applying kinetics to study apoB-100-rich lipoproteins metabolism ⁵⁶³⁻⁵⁷⁷.

Reference	Subjects	Conclusions
Shepherd et al Clin Chim Acta. 1978. 87: 395-404 (radioisotope)	1 patient (type IIB hyperlipoproteinaemia)	A SFA fat diet increased production and plasma pool of VLDL apo B-100. It also increased plasma pool and reduced FCR of LDL apo B-100 A PUFA diet decreased plasma C and TG levels, changed FA composition of all plasma lipoprotein fractions. It also decreased apo B-100 reducing apo B-100 production.
Huff MW et al Metabolism. 1982. 31:493-498 (radioisotope)	6 healthy subjects	A high CH diet increased VLDL apo B-100 pool size and production whereas decreased FCR.
Cortese C et al Eur J Clin Invest. 1983. 13:79-85 (radioisotope)	8 hyperlipidemic subjects	<u>A low-fat diet</u> (25% of energy) reduced LDL concentration, LDL apo B-100 production and increased FCR. Exchanging a PUFA diet for a SFA diet (fat providing 45% of energy on both occasions), VLDL and LDL apo B-100 production decreased.
Nestel et al J Clin Invest. 1984. 74: 82-89 (radioisotope)	7 subjects (5 healthy / 2 hypertriglyceridemic)	Fish oils (supplements provided, MaxEPA® capsules) lowered VLDL lipids and apo B-100. VLDL apo B-100 production decreased after fish oil supplementation.
Abbot et al. J Clin Invest. 1990. 86: 642-650 (radioisotope)	14 Pima indians (10 men / 4 women) (healthy / NIDDM)	<u>A low-fat, high-CH diet</u> decreased VLDL to LDL conversion, thus decreasing LDLc. Evidence in some subjects of increased LDL catabolism.
Stacpoole PW et al J Lipid Res. 1991. 32: 1837-1848 (radioisotope)	9 subjects (4 healthy, 4 FH heterozygous / 1 FH homozygous)	In FH patients, Vivonex® (90% CH, 1% fat) suppressed C synthesis, enhanced production of large VLDL particles and increased VLDL intrahepatic assemblage time. It also increased LDL catabolism, thus decreasing plasma LDL levels.
Pacy PJ et al Atherosclerosis. 1993. 103: 231-243	5 patients (1 men / 4 women) (non functional LPL)	Increased rate of production of VLDL apo B-100 in patients with non functional LPL.
Gylling et al J Lipid Res. 1996. 37: 1776-1785 (radioisotope)	8 patients (mild hypercholesterolemic, NIDDM middle-aged)	Pravastatin enhanced FCR and decreased production of LDL apo B-100 combined with sitosterols, lowered C total, LDLc and LDL apo B-100 because of reduced LDL apoB-100 production.
Fisher et al J Lipid Res. 1998. 39: 388-401 (radioisotope)	5 patients (4 men / 1 women) (hypertriglyceridemic, NIDDM)	Fish oils increased LDL apo B-100 (decreasing IDL apo B-100 catabolism and increasing their conversion to LDL apo B-100) and LDLc and decreased VLDL apo B-100 (decreased production) and TG levels.

underlined=similar to our study;
 FCR=fractional catabolic rate; PR=production rate;
 CH=carbohydrates; SFAs=saturated fatty acids;
 MUFAs=monounsaturated fatty acids;
 PUFAs=polyunsaturated fatty acids.

Reference	Subjects	Conclusions
Bordin et al Eur J Clin Nutr. 1998. 58: 104-109	10 men (healthy subjects)	Dietary supplementation with fish oil decreased VLDL TG levels through a decreased VLDL apo B-100 production.
Chan et al Diabetes. 2002. 51: 2377-2386.	48 men (dyslipidemic, insulin resistant, obese)	Fish oils decreased plasma TG levels by decreasing VLDL apo B-100 production but not by altering the catabolism of apo B-100-rich lipoproteins.
Gill et al Am J Clin Nutr. 2003. 78: 47-56	17 men/ 18 women (moderately hypercholesterolemic middle-aged subjects)	Effect of <u>replacing SFAs with MUFAs</u> limited to LDL: reduced LDLc, reduced plasma apo B-100, enhanced LDL catabolism.
Chan et al Am J Clin Nutr. 2003. 77: 300-307	24 men (obese, dyslipidemic)	Fish oils decreased plasma TG levels by decreasing VLDL apo B-100 production but not by altering the catabolism of apo B-100-rich lipoproteins.
Matthan et al Arterioscler Thromb Vasc Biol. 2004. 24: 1092-1097	8 women (postmenopausal)	The mechanism for the adverse lipoprotein profile observed with hydrogenated fat intake is determined in part by a decreased LDL apo B-100 catabolism.
Desroches et al J Lipid Res 2004. 45: 2331-2338	65 men (18 men for kinetic study) (moderately obese, normolipidemic subjects)	No difference in VLDL apoB-100 kinetics between <u>low-fat diet and high-MUFA diets</u> .
Our study	10 men (moderate hypercholesterolemic)	A <u>Mediterranean diet</u> , compared to the <u>STEP II diet</u> , showed a high apo B-100 LDL production rate and direct fractional catabolic rate. Both diets decreased similarly plasma LDLc levels.

underlined=similar to our study;
 NIDDM=non insulin dependent diabetes mellitus ;
 FCR=fractional catabolic rate; PR=production rate;
 CH=carbohydrates; SFAs=saturated fatty acids;
 MUFAs=monounsaturated fatty acids;
 PUFAs=polyunsaturated fatty acids.

Table 57. Nutritional interventions in human subjects applying stable isotope kinetics to study apoB-100-rich lipoproteins metabolism: study design and kinetic parameters
 563-577

Reference	Design
Pacy PJ et al Atherosclerosis. 1993. 103: 231-243	A <u>therapeutic low-fat diet</u> (20-25 g/day) Kinetic study after 3 days on either isocaloric diets a minimal fat diet (< 15 g/day) or a moderate fat diet (45-50 g/day) Compared to 5 control subjects on a normal fat diet (70-90 g/day)
Bordin et al Eur J Clin Nutr. 1998. 58: 104-109	4-wk treatment with 10 g fish oil concentrate (MaxEPA® capsules) 1.8 g EPA 1.2 g DHA No changes in subjects diet and physical activity 5-day food records at the beginning and the end of treatment
Chan et al Diabetes. 2002. 51: 2377-2386.	Randomised, two-arm, parallel group study 3-wk run in diet stabilizing period 6-wk treatment period of supplementation fish oils (Omacor® capsules , 4 g/day) or placebo capsules (corn oil) Isocaloric diets Checked by capsules count

underlined=similar to our study;
 wk=week; CH=carbohydrates; SFAs=saturated fatty acids;
 MUFAs=monounsaturated fatty acids; PUFAs=polyunsaturated fatty acids;
 EPA=eicosapentanoic acid; DHA=docosahexanoic acid.

Method	Kinetic parameters					
Primed constant infusion ¹³ C -leucine during 9 hours Fasted conditions Algebraic functions (linear regression)	<u>Minimal fat diet</u> <u>Moderate fat diet</u> <u>Controls</u>					
	VLDL apo B-100					
	FSR (pools/h)	0.10 ± 0.04	0.09 ± 0.03	0.15 ± 0.02		
	PR (mg/kg)	3.56 ± 1.69	3.52 ± 1.94	1.06 ± 0.35		
Primed constant infusion ¹³ C -leucine during 6 h injection at the end of heparin (to measure LPL and HL activity) Fasted condition Monocompartmental model	Fish oil treatment (MaxEPA® capsules)					
	<u>Before</u> <u>After</u>					
	Apo B-100 pool (mg / dl)	6.50 ± 3.50	3.69 ± 1.00	(1)		
	VLDL					
	FSR (pools/h)	0.37 ± 0.12	0.42 ± 0.09	(2)		
	PR (mg/dl/h)	2.17 ± 0.66	1.54 ± 0.49	(2)		
	(1) P=0.014					
	(2) P=0.012					
Single bolus injection ² H ₃ -L-leucine Blood samples collected until 96 hours Fasted condition Multicompartmental model	After intervention supplementation					
		<u>Placebo</u>	<u>Fish oils</u>	<u>Atorv</u>	<u>Fish+Atorv</u>	<u>% Main effect fish oils</u>
		Apo B-100				
	Baseline					
	Plasma apoB-100 (mg/dl)	129 ± 4.00	122 ± 6.00	128 ± 6.00	134 ± 6.00	
	Pool size (mg)					
	Total	2798 ± 167	27801 ± 163	2641 ± 151	2850 ± 196	
	VLDL	390 ± 46	367 ± 39	338 ± 39	404 ± 53	
	IDL	145 ± 11	161 ± 11	159 ± 14	162 ± 12	
	LDL	2263 ± 140	2253 ± 139	2144 ± 134	2285 ± 186	
	FCR (pools/day)					
	VLDL	4.30 ± 0.40	3.80 ± 0.30	4.50 ± 0.50	3.70 ± 0.30	
	IDL	5.30 ± 0.60	3.70 ± 0.30	3.90 ± 0.50	4.20 ± 0.20	
	LDL	0.29 ± 0.02	0.25 ± 0.03	0.27 ± 0.04	0.28 ± 0.04	
	PR (mg/kg/day)					
	VLDL	16.20 ± 2.0	12.80 ± 1.70	14.80 ± 2.30	14.70 ± 2.10	
	IDL	8.20 ± 1.20	5.50 ± 0.70	5.60 ± 0.70	6.80 ± 0.60	
	LDL	6.60 ± 0.50	4.80 ± 0.50	5.40 ± 0.80	5.80 ± 0.60	
	Week 6					
	Plasma apoB-100 (mg/dl)	123 ± 3.00	69 ± 3.00	118 ± 6.00	73 ± 5.00	-49 ± 4.00 (3)
Pool size (mg)						
Total	2681 ± 145	1606 ± 90	2638 ± 185	1627 ± 13	-1107 ± 86 (3)	
VLDL	346 ± 39	228 ± 28	235 ± 27	148 ± 18	+112 ± 20 (3)	
IDL	135 ± 10	115 ± 11	152 ± 16	106 ± 10	-40.3 ± 7.50 (3)	
LDL	2199 ± 118	1263 ± 77	2252 ± 155	1372 ± 126	-949 ± 76 (3)	
FCR (pools/day)						
VLDL	4.50 ± 0.40	6.30 ± 0.80	4.30 ± 0.30	6.20 ± 0.80	+ 2.20 ± 0.60 (3)	
IDL	5.0 ± 0.70	5.0 ± 0.30	4.30 ± 0.40	6.20 ± 0.70	+ 1.50 ± 0.40 (4)	
LDL	0.27 ± 0.02	0.50 ± 0.30	0.32 ± 0.03	0.50 ± 0.05	+0.22 ± 0.04 (3)	
PR (mg/kg/day)						
VLDL	15.70 ± 2.50	13.80 ± 2.0	10.10 ± 1.20	8.80 ± 1.10		
IDL	7.20 ± 0.90	5.80 ± 0.80	5.80 ± 0.70	5.80 ± 0.50		
LDL	6.10 ± 0.50	5.80 ± 0.70	6.80 ± 0.80	6.40 ± 0.6		
	(3) P=0.001					
	(4) P=0.002					

O/N=over night; FCR=fractional catabolic rate; PR=production rate.

Reference	Design																																																				
Gill et al Am J Clin Nutr. 2003. 78: 47-56	<p>Randomised, blinded, crossover study 3 dietary interventions for 6-wk</p> <table border="1"> <thead> <tr> <th></th> <th>Low-MUFA diet</th> <th>Moderate-MUFA diet</th> <th>High-MUFA diet</th> </tr> </thead> <tbody> <tr> <td>MUFAs</td> <td>25 %</td> <td>35 %</td> <td>51 %</td> </tr> <tr> <td>SFAs</td> <td>50 %</td> <td>37 %</td> <td>24 %</td> </tr> <tr> <td>cis PUFAs</td> <td>21 %</td> <td>24 %</td> <td>21 %</td> </tr> <tr> <td>trans PUFAs</td> <td>4 %</td> <td>4 %</td> <td>4 %</td> </tr> </tbody> </table> <p>Washout period between diet ≥ 8-wk</p> <p>Composition of diets determined using nutrition data bases and software</p> <table border="1"> <thead> <tr> <th></th> <th>Low-MUFA</th> <th>Moderate-MUFA</th> <th>High-MUFA</th> </tr> </thead> <tbody> <tr> <td>CH %</td> <td>52.20 ± 0.60</td> <td>52.50 ± 0.70</td> <td>53.20 ± 0.80</td> </tr> <tr> <td>Prot %</td> <td>12.40 ± 0.40</td> <td>12.20 ± 0.30</td> <td>12.30 ± 0.40</td> </tr> <tr> <td>Total fat %</td> <td>69.10 ± 2.40</td> <td>69.50 ± 2.30</td> <td>68.20 ± 2.50</td> </tr> <tr> <td>SFA %</td> <td>14.70 ± 0.30</td> <td>11.20 ± 0.20</td> <td>7.30 ± 0.10</td> </tr> <tr> <td>MUFA %</td> <td>7.80 ± 0.20</td> <td>10.30 ± 0.20</td> <td>13.70 ± 0.20</td> </tr> <tr> <td>cis PUFAs %</td> <td>6.10 ± 0.10</td> <td>7.20 ± 0.10</td> <td>6.80 ± 0.0</td> </tr> <tr> <td>trans PUFAs%</td> <td>1.20 ± 0.03</td> <td>0.90 ± 0.02</td> <td>0.90 ± 0.02</td> </tr> </tbody> </table> <p>(1) P<0.01</p> <p>Manufactured test fats provided as margarine, spread and baked products (Anglia Oil Ltd & Chemtech International Ltd) Body weight maintained constant 7-day food records before study 5-day records during study</p>		Low-MUFA diet	Moderate-MUFA diet	High-MUFA diet	MUFAs	25 %	35 %	51 %	SFAs	50 %	37 %	24 %	cis PUFAs	21 %	24 %	21 %	trans PUFAs	4 %	4 %	4 %		Low-MUFA	Moderate-MUFA	High-MUFA	CH %	52.20 ± 0.60	52.50 ± 0.70	53.20 ± 0.80	Prot %	12.40 ± 0.40	12.20 ± 0.30	12.30 ± 0.40	Total fat %	69.10 ± 2.40	69.50 ± 2.30	68.20 ± 2.50	SFA %	14.70 ± 0.30	11.20 ± 0.20	7.30 ± 0.10	MUFA %	7.80 ± 0.20	10.30 ± 0.20	13.70 ± 0.20	cis PUFAs %	6.10 ± 0.10	7.20 ± 0.10	6.80 ± 0.0	trans PUFAs%	1.20 ± 0.03	0.90 ± 0.02	0.90 ± 0.02
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Chan et al Am J Clin Nutr. 2003. 77: 300-307	<p>Randomized, two-arm, parallel group study 3-wk run in diet stabilizing period 6-wk treatment period of supplementation fish oils (Omacor® capsules, 4 g/day) or placebo capsules (corn oil)</p> <p>Isocaloric diets Checked by capsules count</p>																																																				
Matthan et al Arterioscler Thromb Vasc Biol. 2004. 24: 1092-1097	<p>Randomized, blinded, crossover study 3 diets for 5-wk periods with 2/3 of fats from soybean oil for unsaturated fat stick margarine for hydrogenated fat butter for saturated fat</p> <p>Composition of diets determined by chemical analysis of food</p> <table border="1"> <thead> <tr> <th></th> <th>Unsaturated fat</th> <th>Hydrogenated fat</th> <th>Saturated fat</th> </tr> </thead> <tbody> <tr> <td>CH %</td> <td>55.8</td> <td>53.5</td> <td>54</td> </tr> <tr> <td>Prot %</td> <td>15.7</td> <td>16.7</td> <td>16.9</td> </tr> <tr> <td>Total fat %</td> <td>28.5</td> <td>29.7</td> <td>29.1</td> </tr> <tr> <td>SFA %</td> <td>7.3</td> <td>8.5</td> <td>16.7</td> </tr> <tr> <td>MUFA %</td> <td>8.1</td> <td>8.5</td> <td>8.1</td> </tr> <tr> <td>PUFA %</td> <td>12.5</td> <td>6.3</td> <td>2.4</td> </tr> <tr> <td>Trans %</td> <td>0.6</td> <td>6.7</td> <td>1.3</td> </tr> <tr> <td>C (mg/1000 kcal)</td> <td>66</td> <td>67</td> <td>121</td> </tr> </tbody> </table> <p>Body weight maintained constant Analysis of the macronutrient of diets</p>		Unsaturated fat	Hydrogenated fat	Saturated fat	CH %	55.8	53.5	54	Prot %	15.7	16.7	16.9	Total fat %	28.5	29.7	29.1	SFA %	7.3	8.5	16.7	MUFA %	8.1	8.5	8.1	PUFA %	12.5	6.3	2.4	Trans %	0.6	6.7	1.3	C (mg/1000 kcal)	66	67	121																
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wk=week; CH=carbohydrates; SFAs=saturated fatty acids;
 MUFAs=monounsaturated fatty acids PUFAs=polyunsaturated fatty acids.

Method	Kinetic parameters					
		Low-MUFA diet	High-MUFA diet			
² H ₃ -L-leucine Bolus infusion Blood samples collected until 48 hours Fasted condition Multicompartmental model	Plasma apo B-100 (mg/dl)	120.80 ± 3.10	113.10 ± 3.10	(1)		
	VLDL1					
	Pool size (mg)	119 ± 22	117 ± 18			
	Production (mg/day)	626 ± 112	737 ± 146			
	FCR (pools/day)					
	dir FCR (pools/day)	1.00 ± 0.50	0.80 ± 0.40			
	Transfer to VLDL2 (pools/day)	6.10 ± 1.10	5.90 ± 0.80			
	VLDL2					
	Pool size (mg)	202 ± 17	206 ± 18			
	Production (mg/day)	171 ± 25	190 ± 25			
	FCR (pools/day)					
	dir FCR (pools/day)	0.50 ± 0.20	0.20 ± 0.05			
	Transfer to VLDL2 (pools/day)	3.30 ± 0.40	3.70 ± 0.50			
		(1) P<0.01				
Single bolus injection ² H ₃ -L-leucine Blood samples collected until 96 hours Fasted condition Multicompartmental model		Fish oil		Placebo		
		Before	After	Before	After	
		Apo B-100				
	Plasma apo B-100 (mg/dl)	128 ± 0.06	118 ± 0.0	129 ± 0.04	123 ± 0.03	
	Pool size (mg)					
	VLDL	338 ± 39	234 ± 27	390 ± 46	346 ± 39	(1)
	IDL	159 ± 14	152 ± 16	145 ± 11	135 ± 10	
	LDL	2144 ± 134	2252 ± 155	2263 ± 140	2199 ± 118	
	FCR (pools/day)					
	VLDL	4.51 ± 0.46	4.32 ± 0.31	4.25 ± 0.44	4.53 ± 0.38	
	IDL	3.92 ± 0.49	4.25 ± 0.41	5.33 ± 0.58	5.03 ± 0.65	
	LDL	0.27 ± 0.04	0.32 ± 0.03	0.29 ± 0.02	0.27 ± 0.02	
	PR (mg/kg/day)					
	VLDL	14.80 ± 2.30	10.10 ± 1.20	16.20 ± 2.00	15.70 ± 2.40	(1)
	IDL	5.63 ± 0.73	5.84 ± 0.69	8.20 ± 1.19	7.17 ± 0.89	
	LDL	5.43 ± 0.84	6.80 ± 0.84	6.57 ± 0.53	6.06 ± 0.50	
		(1) P<0.05				
	² H ₃ -L-leucine Primed constant infusion for 15 hours Blood samples collected until 15 hours 12 hours O/N fasted + fed hourly for 20 hours Multicompartmental model		Experimental diets		Saturated fat	
		Unsaturated fat	Hydrogenated fat			
IDL apo B-100						
Plasma IDL apo B-100 (mg/dl)		2.40 ± 1.40	2.90 ± 1.60	2.80 ± 1.20		
Pool size (mg)		69 ± 39	83 ± 45	80 ± 36		
FCR (pools/day)		8.20 ± 2.90	7.90 ± 2.90	7.80 ± 30		
PR (mg/kg/day)		7.90 ± 3.40	8.70 ± 2.10	8.80 ± 2.50		
LDL apo B-100						
Plasma LDL apo B-100 (mg/dl)		80.50 ± 8.90	81.40 ± 11.70	87.70 ± 8.80	(1)	
Pool size (mg)		2267 ± 233	2319 ± 413	2507 ± 305	(1)	
FCR (pools/day)		0.39 ± 0.07	0.33 ± 0.08	0.30 ± 0.05	(1)	
PR (mg/kg/day)		14.30 ± 2.90	11.60 ± 2.80	12.80 ± 16		
		(1) P<0.05				

O/N=over night; FCR=fractional catabolic rate; PR=production rate.

Reference	Design																																								
Desroches et al J Lipid Res 2004. 45: 2331-2338	<p>Randomised, parallel, two-group study 2 diets: 6-7-wk of a low-fat/high-CH diet or a high-fat diet rich in MUFAs Both diets with the same meals but different amount of fat and CH Unsaturated fat, mainly olive oil CH, mainly whole grain and vegetables</p> <p>Composition of diets determined using nutrition data bases and software</p> <table border="1"> <thead> <tr> <th></th> <th>Low-fat/high-CH diet</th> <th>High-MUFA diet</th> <th></th> </tr> </thead> <tbody> <tr> <td>CH %</td> <td>58.3</td> <td>44.7</td> <td>(1)</td> </tr> <tr> <td>Prot %</td> <td>15.9</td> <td>15.2</td> <td></td> </tr> <tr> <td>Total fat %</td> <td>25.8</td> <td>40.1</td> <td>(1)</td> </tr> <tr> <td> SFA %</td> <td>6</td> <td>8.2</td> <td>(1)</td> </tr> <tr> <td> MUFA %</td> <td>13.3</td> <td>22.5</td> <td>(1)</td> </tr> <tr> <td> PUFA %</td> <td>5.1</td> <td>7.6</td> <td>(1)</td> </tr> <tr> <td> PUFA/SFA ratio</td> <td>0.87</td> <td>0.93</td> <td>(1)</td> </tr> <tr> <td>C (mg/1000 kcal)</td> <td>105.8</td> <td>110.1</td> <td></td> </tr> <tr> <td>Total fibers (g/1000 kcal)</td> <td>14.2</td> <td>10.1</td> <td></td> </tr> </tbody> </table> <p>(1) P<0.01</p> <p>All meals prepared and provided (metabolic unit) Diets consumed <i>ad libitum</i> 3-day food records at baseline</p>		Low-fat/high-CH diet	High-MUFA diet		CH %	58.3	44.7	(1)	Prot %	15.9	15.2		Total fat %	25.8	40.1	(1)	SFA %	6	8.2	(1)	MUFA %	13.3	22.5	(1)	PUFA %	5.1	7.6	(1)	PUFA/SFA ratio	0.87	0.93	(1)	C (mg/1000 kcal)	105.8	110.1		Total fibers (g/1000 kcal)	14.2	10.1	
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Our study	<p>Randomized, crossover study a 2-wk stabilisation period + two dietary intervention periods of 4-wk each (Mediterranean / STEP II diet) a 4-wk washout period between them</p> <p>Stabilisation diet had the compositional characteristics of the Catalan diet Mediterranean diet compositional characteristics adjusted to the Spanish recommendations for prevention and treatment of CVD STEP II diet compositional characteristics adjusted to the AHA recommendations</p> <p>Composition of diets determined using nutrition data bases and software</p> <table border="1"> <thead> <tr> <th></th> <th>Stabilisation diet</th> <th>Mediterranean diet</th> <th>STEP II diet</th> <th></th> </tr> </thead> <tbody> <tr> <td>CH %</td> <td>42.33 ± 1.89</td> <td>44.96 ± 5.09</td> <td>52.23 ± 5.15</td> <td></td> </tr> <tr> <td>Prot %</td> <td>14.87 ± 0.42</td> <td>14.20 ± 2.02</td> <td>15.51 ± 2.35</td> <td></td> </tr> <tr> <td>Total fat %</td> <td>36.31 ± 2.27</td> <td>33.95 ± 2.82</td> <td>25.01 ± 4.84</td> <td>(1)</td> </tr> <tr> <td> SFA %</td> <td>11.17 ± 1.08</td> <td>7.29 ± 0.90</td> <td>5.60 ± 1.21</td> <td>(1)</td> </tr> <tr> <td> MUFA %</td> <td>17.35 ± 3.78</td> <td>19.09 ± 2.09</td> <td>11.89 ± 2.39</td> <td>(2)</td> </tr> <tr> <td> PUFA %</td> <td>7.00 ± 0.81</td> <td>4.20 ± 0.60</td> <td>4.36 ± 2.59</td> <td></td> </tr> <tr> <td>C (mg/1000 kcal)</td> <td>192.00 ± 85.99</td> <td>219.90 ± 85.99</td> <td>171.89 ± 77.72</td> <td></td> </tr> </tbody> </table> <p>(1) P=0.02 (2) P=0.01</p> <p>Body weight maintained constant Meals from readily available foods Volunteers followed dietitian recommendations and had lunch together with a dietitian from Monday to Friday. Volunteers had everyday breakfast and suppers, together with weekends meals at home 7 food records during all study (one 5-day food frequency record + six 3-day food records) Physical activity record</p>		Stabilisation diet	Mediterranean diet	STEP II diet		CH %	42.33 ± 1.89	44.96 ± 5.09	52.23 ± 5.15		Prot %	14.87 ± 0.42	14.20 ± 2.02	15.51 ± 2.35		Total fat %	36.31 ± 2.27	33.95 ± 2.82	25.01 ± 4.84	(1)	SFA %	11.17 ± 1.08	7.29 ± 0.90	5.60 ± 1.21	(1)	MUFA %	17.35 ± 3.78	19.09 ± 2.09	11.89 ± 2.39	(2)	PUFA %	7.00 ± 0.81	4.20 ± 0.60	4.36 ± 2.59		C (mg/1000 kcal)	192.00 ± 85.99	219.90 ± 85.99	171.89 ± 77.72	
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 MUFAs=monounsaturated fatty acids; PUFAs=polyunsaturated fatty acids.

Method	Kinetic parameters			
$^2\text{H}_3$ -L-leucine Primed constant infusion for 12 hours Blood samples collected until 12 hours 12 hours O/N fasted + fed cookies every 30 min for 15 hours Mono- exponential function	<u>Low-fat/High-CH diet</u>			
		<u>Before</u>	<u>After</u>	<u>% change</u>
	Plasma apoB-100 (mg/dl)	111 ± 6.50	127 ± 9.30	+14.40
	VLDL Apo B-100 Pool size (mg)	481 ± 354	548 ± 515	+13.9
	FCR (pools/day)	39.71 ± 21.08	33.42 ± 12.74	-15.8
	PR (mg/kg/day)	2.83 ± 0.68	2.85 ± 0.81	+0.7
		<u>High-MUFA diet</u>		
		<u>Before</u>	<u>After</u>	<u>% change</u>
	Plasma apoB-100 (mg/dl)	175 ± 6.20	141 ± 5.60	-19.40
	VLDL Apo B-100 Pool size (mg)	711 ± 225	559 ± 236	-21.40
	FCR (pools/day)	7.86 ± 6.90	7.39 ± 3.70	-3.90
	PR (mg/kg/day)	58.80 ± 50.67	41.44 ± 16.20	-29.50
		Between diets significant differences in Plasma LDL Apo B-100 (1)		
		(1) $P < 0.05$		
$^2\text{H}_3$ -L-leucine Primed constant infusion for 16 hours Blood samples collected until 48 hours Fasted condition Multi-compartmental model	<u>After Mediterranean diet</u>		<u>After STEP II diet</u>	
	Plasma Apo B-100 (mg/dl)	103.50 ± 9.68	106.33 ± 7.51	
	VLDL1 Apo B-100 Pool size (mg)	111.94 ± 70.96	96.34 ± 78.77	
	dir FCR (pools/day)	5.57 ± 6.18	6.05 ± 2.58	
	PR (mg/kg/day)	10.95 ± 7.95	12.22 ± 9.54	
	VLDL2 Apo B-100 Pool size (mg)	61.10 ± 42.98	65.07 ± 48.07	
	dir FCR (pools/day)	1.49 ± 1.75	2.81 ± 4.86	
	PR (mg/kg/day)	3.56 ± 0.64	3.71 ± 2.17	
	IDL Apo B-100 Pool size (mg)	102.95 ± 30.61	93.04 ± 98.22	
	dir FCR (pools/day)	2.45 ± 1.65	0.05 ± 0.08	
	PR (mg/kg/day)	4.75 ± 4.33	2.17 ± 2.66	
	LDL Apo B-100 Pool size (mg)	2124.59 ± 270.08	1962 ± 940.34	
	dir FCR (pools/day)	0.45 ± 0.22	0.3 ± 0.12	
	PR (mg/kg/day)	4.95 ± 3.11	1.70 ± 2.01	

O/N=over night; FCR=fractional catabolic rate; PR=production rate.

UNIVERSITAT ROVIRA I VIRGILI
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CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
IMPLEMENTATION OF STABLE ISOTOPES LIPOPROTEIN KINETIC STUDIES: EFFECTS ON HDL METABOLISM OF A MEDITERRANEAN TYPE DIET RICH
IN MUFAS FROM VIRGIN OLIVE OIL.
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Conclusions

1. We have, for the first time in Spain, implemented the necessary methodology to perform apo B-100, apoA-I and A-II kinetic studies using stable isotopes in human subjects *in vivo*.
2. The present study is the first, using stable isotope kinetics to study lipoproteins, to compare the impact of a Mediterranean diet high in MUFAs from virgin olive oil with that of a STEP II diet.
3. In this study, supervision and evaluation of dietary recommendations have been strict and instructions provided by the dietician have been kept simple so as to be easy-to-follow at home and which reflected real-life conditions with respect to meal preparation.
4. The Mediterranean diet, compared with the STEP II diet, significantly increases the plasma concentrations of apoA-I and shows a trend, albeit not significant, towards an increase in the plasma concentrations of HDLC and a significant decrease in the apo B-100/apoA-I ratio.
5. Both diets induce similar decreases in the plasma concentrations of LDLC, albeit statistically not significant.
6. Because of the labour-intensive nature of the studies and the need for high-technology equipment, our lipoprotein assessments have the limitation of the low number of kinetic study analyses, to-date. Despite these limitations, we are able to document that:
 - 6a. the Mediterranean diet induces a high apoA-I HDL production rate compared to the STEP II diet.
 - 6b. the Mediterranean diet induces a high apo B-100 LDL production rate and direct fractional catabolic rate compared to the STEP II diet.
7. A higher apoA-I production rate is the determinant of the high plasma concentrations of apoA-I and not a result of variations in its catabolism.
8. The Mediterranean diet reduces diastolic blood pressure.
9. Neither of the two diets evaluated have any effect on the inflammation and oxidation markers studied.
10. Lipoprotein kinetic studies enable the monitoring of lipoprotein metabolic parameters and the investigation of nutritional and pharmacologic interventions in

CONCLUSIONS

the primary and secondary prevention of CVD in subjects who may be predisposed to the disease.

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