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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ANNEXES

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Annexes

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ANNEXE I

Facultat de Medicina i Ciències de la Salut	Revisió: 1
Unitat de Recerca de Lípids (URL)	Data: 22/02/06
PROCEDIMENT PER A REALITZAR ESTUDIS CINÈTICS AMB ISÒTOPS ESTABLES DE LIPOPROTEÏNES	Pàg. 1 de 12

1. Fonament i objectiu del procediment

El coneixement dels processos metabòlics es basa en el coneixement de la termodinàmica (la cinètica de les reaccions i les concentracions *in vivo* de metabòlits rellevants) de les diferents rutes metabòliques. Aquest conjunt de dades analitzades poden donar-nos una idea detallada de la via metabòlica a estudi. Tradicionalment, per caracteritzar els desordres en el metabolisme de les lipoproteïnes, s´han fet servir les concentracions de lípids i lipoproteïnes plasmàtics. Les mesures puntuals en el temps no donen informació sobre les concentracions anormals de lipoproteïnes en plasma (que poden ser degudes a alteracions en les taxes de producció i/o de degradació). Els estudis metabòlics que utilitzen molècules traçadores (com ara isòtops estables) aplicant models mecanístics permeten obtenir un major coneixement de la dinàmica dels processos metabòlics, els seus desordres i els efectes dels seus tractaments.

2. Espècimen

Plasma fresc i/o prèviament congelat (millor si és amb algun conservant tipus BHT, sacarosa, etc).

3.Reactius i materials

Veure PNTs per a les tècniques.

- D_3- L- Leucina
  L- Leucina-5,5,5 d3 min 99 ATOM % ref. 486825
  (ISOTEC, Sigma Aldrich)
  Campro Scientific BV (The Netherlands)
  Tf. +31-318 529437
  Fax +31-318 542181
  Persona contacte: Chris van Wakeren (wakeren@campro.nl)

- tubs EDTA K3E (3ml) (BD Vacutainer) ref. 368857
- tubs EDTA K3E (10ml) (BD Vacutainer) ref. 366643
- tubs EDTA K3E (10ml) (Vacutainer ref. VT-100STK
- filtres Posidyne NEO (Pall) ref. NEO96E
- kits per determinacions al COBAS MIRA (Roche)
  Free cholesterol (Wako) ref. 279-47106
  ApoA2-HA (Wako) ref. 416-27301
  PhospholipidsB (Wako) ref. 999-54006

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Apolipoprotein calibrator	(Wako)	ref. 411-27591
TG 2x300 for Synchron®Systems	(Beckman Coulter)	ref. 445850
Apo B-100-100100 for Synchron LX®Systems	(Beckman Coulter)	ref. 467905
ApoA100 for Synchron®Systems	(Beckman Coulter)	ref. 467900
Cholesterol 300 for Synchron®Systems	(Beckman Coulter)	ref. 467825

- test de LAL (*Limulus amebocyte lysate* (LAL) test)

Fontlab2000 SL
Tf. 93 8446495
fontlab2000@teleline.es
Persona de contacte: Marta Pérez

- anàlisi de les mostres per GC-MS i obtenció de resultats amb el programa SAAM II

Dr. Muriel Caslake

Vascular Biochemistry Section

Division of Cardiovascular & Medical Sciences

4th floor, University Block, QEB,

Glasgow Royal Infirmary

G31 2ER

Glasgow

4. Instrumentació

Veure PNTs per a les tècniques i aparells.

5. Procediment

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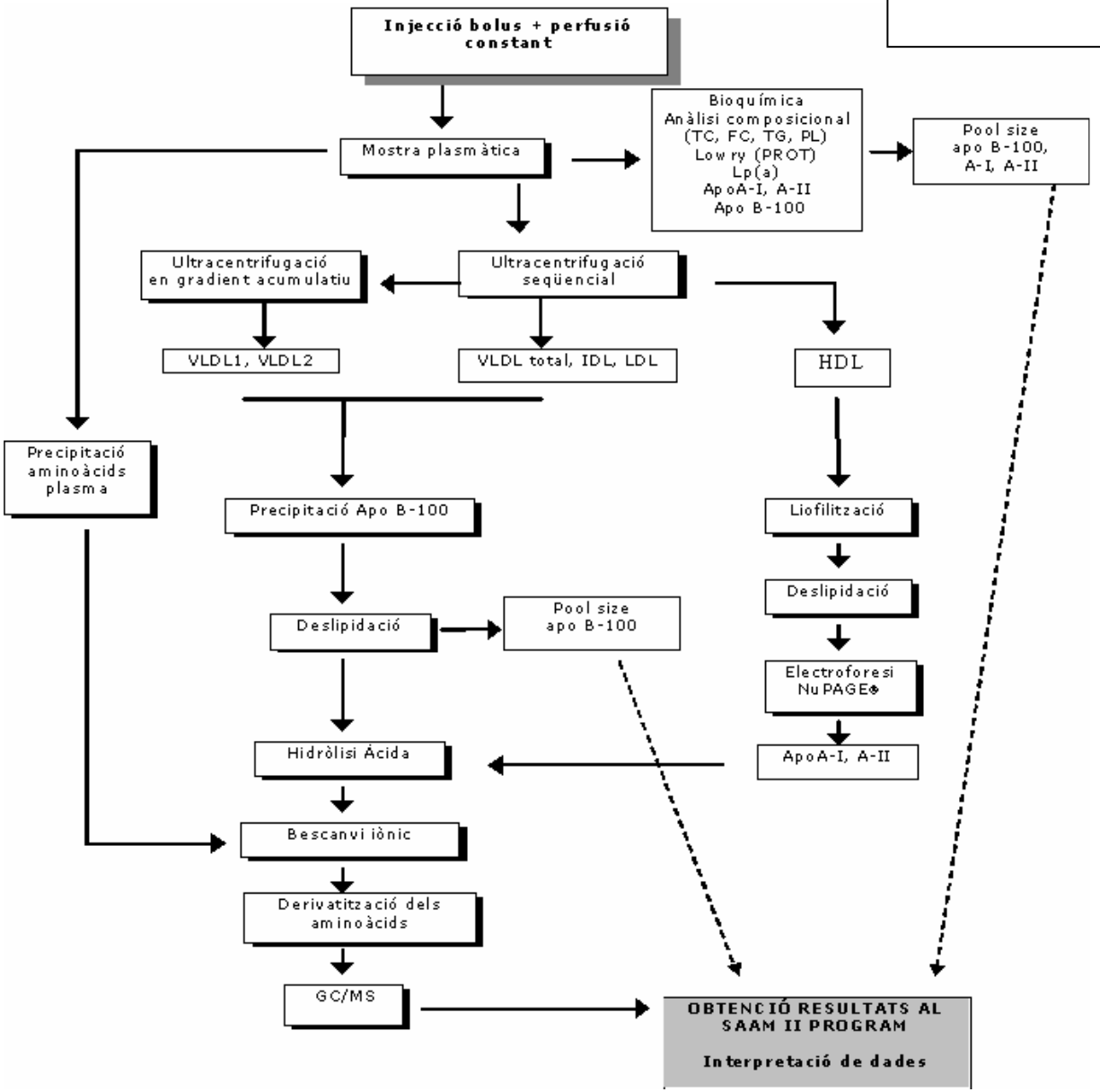
Esquema del procediment metodològic per a dur a terme un estudi de cinètiques de lipoproteïnes



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5.1 Protocol d´intervenció clínica

5.1.1 Preparació de la solució de D_3-L-Leucina per a l´estudi cinètic

5.1.1.1 Preparar una solució de treball de D_3-L-leucina a una concentració de 10 mg/ml

(100 ml totals x 10 mg/ ml) x 1 g/ 1000 mg= 1g D_3-L-leucina

* preparació de la solució de treball (treballar sempre en una campana de cultius!!)

- tindre preparats 3 flascons de sèrum fisiològic de 100 ml
- obrir un flascó amb pinces i tisores (autoclavades!) i buidar-lo
- obrir un segon flascó, agafar 50 ml amb una pipeta de plàstic (estèril i lliure de pirògens) i posar-los en el flascó buit
- pesar el flascó amb els 50 ml de sèrum en una balança analítica (dins la campana, equilibrada), tarar i pesar, fent servir una espàtula (autoclavada), 1g de D_3-L-leucina (guardar a la nevera fins el moment de fer servir)
- sacsejar lleugerament el flascó per disoldre la D_3-L-leucina
- acabar d´omplir el flascó fins 100 ml de la solució de treball
- tapar el flascó amb parafilm i guardar a la nevera fins el seu ús

5.1.1.2 A partir de la solució de treball, realitzar els càlculs per obtenir les solucions administrades tenint en compte el pes corporal de cada subjecte:

5.1.1.2.1 La injecció puntual o *bolus*, a una concentració de 0,7 mg/kg

(pes (kg) x 0,7 mg D_3-L-leucina) x 1ml/10mg= ml solució de *bolus*

5.1.1.2.2 La solució per la perfusió o infusió constant, a una concetració de 0,7 mg/kg/h

pes (kg) x 0,7 mg D_3-L-leucina x 16h infusió= mg leucina

mg leucina x 1ml/10 mg= ml solució de perfusió

volum final de la perfusió= 12 ml/h x 16h= 192 ml totals

192 ml totals - ml solució de perfusió = ml sèrum fisiològic

* preparació de la solució de perfusió (treballar sempre a una campana de cultius!!)

- obrir el tercer flascó de sèrum fisiològic de 100 ml
- buidar els ml de sèrum fisiològic amb una xeringa de 10 ml (estèril i lliure de pirògens) fins tindre el volum necessari per a preparar la solució de perfusió
- agafar la solució de treball i extreu-re el volum necessari per a preparar la solució de perfusió amb una segona xeringa de 10 ml (estèril i lliure de pirògens)

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- introduir el volum de solució de treball al tercer flascó
- barrejar lleugerament la solució de perfusió, tapar amb parafilm i guardar a la nevera fins que es faci servir

5.1.1.3 Pel control d´endotoxines en el producte final, enviar a un laboratori extern mostres de la solució de treball per que es realitzi la quantificació d´endotoxines bacterianes (piròGens) o test de LAL.

* preparació de les mostres pel test de LAL (treballar sempre a una campana de cultius!!)

- obrir flascons de mostreig
- agafar la solució de treball i extreure amb una xeringa de 5 ml (estèril i lliure de piròGens) de 2 a 4,5 ml
- omplir els flascons de mostreig i guardar a la nevera fins el seu enviament
- al enviar les mostres, adjuntar una fulla complimentada amb nom del producte i número de lot, a més dels càlculs realitzats per preparar la solució de treball i de perfusió
- enviar vist-i-plau dels resultats del test un cop confirmats

5.1.2 Obtenció de mostres el dia de la cinètica

- els subjectes d´estudi han de restar en dejú 10h abans d´iniciar l´estudi cinètic. Són citats a l´Hospital de San Joan a primera hora del matí. Se´ls hi col·loca una via d´entrada amb la solucó de perfusió i el *bolu*s en un braç i una via d´extracció de sang en l´altre braç.

- se´ls realitzen dues extraccions de sang a temps basal (tubs d´ EDTA).

- se´ls injecta el *bolus* de solució D_3-L-leucina a una concentración de 0,7 mg/kg.

- seguidament es realitza la perfusió de forma constant de la mateixa solució de D_3-L-leucina a una concetració de 0,7 mg/kg/h durante 16 h, restant ingressats a l´Hospital. Per la perfusió es fan servir, per major seguretat, filtres especials estèrils (*Posidyne NEOFilter*).

5.1.2.1 Obtenció de mostres per determinacions bioquímiques

A cada subjecte se li realitzen una sèrie de determinacions :

- paràmetres clínics i lipídics generals a les 0h del dia de l´estudi cinètic (colesterol, triglicèrids, etc).

- paràmetres lipídics (colesterol total i lliure, triglicèrids, fosfolípids, apo B-100-100, apoA-I, apoA-II) en mostres de plasma per cada subjecte (**Veure PNT Cobas**)

- anàlisi composicional (colesterol total i lliure, triglicèrids, fosfolípids, apo B-100-100, apoA-I, apoA-II) en mostres de *pools* per cadascuna de les fraccions lipoproteiques (VLDL1, VLDL2, IDL, LDL i HDL) aïllades per cada subjecte (**Veure PNT Cobas**)

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- determinació de proteïnes totals mitjançant mètode de Lowry per l´anàlisi composicional en mostres de *pools* per cadascuna de les fraccions lipoproteiques (VLDL1, VLDL2, IDL, LDL i HDL) aïllades per cada subjecte (**Veure PNT Lowry**)

5.1.2.2 Obtenció de mostres per a la determinació de l´enriquiment isotòpic

Una vegada iniciada la perfusió constant de la solució de D_3-L-leucina, es realitzen una sèrie d´extraccions sanguínies (tubs amb EDTA) a diferents temps i fins un total de 48h (veure Taula). Volum total de les extracions aprox 375 ml de sang total.

El procediment per a l´obtenció de mostres és:

- centrifugar les mostres de sang a 25.000, 10 minutos a 4ºC

- preparar les alíquotes de plasma corresponents a cada temps d´ extracció de la cinètica.

Els volums d´alíquotes de les mostres de plasma per cada temps d´extracció de la cinètica segons el seu posterior processament són:

* 2 x 1ml (eppendorf de 1,5 ml) per la precipitació d´aminoàcids del plasma o *plasma method* i per reserva

* 2 x 2 ml per la ultracentrifugació seqüencial preparativa (criotubs de 3,5 ml) i per reserva

* 2 ml de reserva (en cas que no es puguin fer els volums anteriors de reserva, guardar un mínim de 2 ml de plasma)

A partir de 0.2-0.5 ml de plasma de cada mostra pels diferents temps de la cinètica es preparen *pools* o barreges dels plasmes (per triplicat: *pool* 1, *pool* 2, *pool* 3). Els volums d´alíquotes de les mostres de *pools* són:

* 14 ml totals de la barreja de plasmes repartits en
    * P1, P2, P3 cadascun de 2 ml per la ultracentrifugació seqüencial preparativa
    * P1, P2, P3 cadascun de 2 ml per reserva

No s´ha de pipetejar exacte, sinó s´ha d´agafar una mica més de volum per evitar errors de pipeteig (ex. 1,2 ml o 2,2 ml)!!

- les alíquotes de plasma es poden emmagatzemar a – 70ºC fins el seu posterior processament seguint el procediment metodològic.

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DIA (HORA)	VOLUM DE SANG	PLASMA METHOD	ULTRACENTRÍFUGA	ANÂLISI VLDL1, VLDL2,IDL,LDL,HDL
0 h (8:00)	30 ml	1 ml	2 X 2 ml	Lípids / bioquímica / enriquiment isotòpic
1 min (8:01)	15 ml	1 ml	2 ml	enriquiment isotòpic
2 min (8:02)	15 ml	1 ml	2 ml	enriquiment isotòpic
5 min (8:05)	15 ml	1 ml	2 ml	enriquiment isotòpic
10 min (8:10)	15 ml	1 ml	2 ml	enriquiment isotòpic
15 min (8:15)	15 ml	1 ml	2 ml	enriquiment isotòpic
20 min (8:20)	15 ml	1 ml	2 ml	enriquiment isotòpic
30 min (8:30)	15 ml	1 ml	2 ml	enriquiment isotòpic
45 min (8:45)	15 ml	1 ml	2 ml	enriquiment isotòpic
1 h (9:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
1.5 h (9:30)	15 ml	1 ml	2 ml	enriquiment isotòpic
2 h (10:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
3 h (11:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
3.5 h (11:30)	15 ml	1 ml	2 ml	enriquiment isotòpic
4 h (12:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
5 h (13:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
6 h (14:00)	15 ml	1 ml	2 ml	enriquiment isotòpic

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8 h (16:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
10 h (18:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
11 h (19:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
14 h (22:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
16 h (24:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
24 h (8:00)	15 ml	1 ml	2 ml	enriquiment isotòpic
48 h (8:00)	15 ml	1 ml	2 ml	enriquiment isotòpic

POOLS or PLASMA MIX (x3)	
ULTRACENTRÍFUGA	**ANÂLISI VLDL1, VLDL2, IDL, LDL**
2 ml	bioquímica

5.2 Protocol pel procediment metodològic d´anàlisi de mostres

5.2.1 Obtenció de les diferents fraccions lipoproteiques

5.2.1.1 Ultracentrifugació seqüencial preparativa

De cadascuna de les extraccions sanguínies realitzades (0h fins 48h a més de P1, P2 i P3), s´aïllen les diferents fraccions lipoproteiques (VLDL, IDL, LDL, HDL) per ultracentrifugació seqüencial preparativa (**Veure PNT Ultracentrifugació Seqüencial Preparativa i PNT Ultracentrífuges**).

5.2.1.2 Ultacentrifugació en gradient acumulatiu o swing out

De cadascuna de les extraccions realizadas (0h fins 48h a més de P1, P2 i P3) s´aïllen, a partir de las VLDL totals les subfraccions VLDL1 i VLDL2 per ultracentrifugació en gradient acumulatiu (**Veure PNT Ultracentrifugació en gradient acumulatiu o Swingout i PNT Ultracentrífuges**).

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5.2.2 Obtenció de l´apo B-100-100

5.2.2.1 Precipitació amb isopropanol

Per obtindre l´apo B-100-100 aïllada de les diferents fraccions lipoproteiques (VLDL1, VLDL2, IDL i LDL) per les diferents mostres (0h fins 48h a més de P2 i P3) es fa servir el mètode de precipitació amb isopropanol descrit per Egusa et al. (**Veure PNT Precipitació i deslipidació de l´apo B-100-100**).

5.2.2.2 Deslipidació de l´apo B-100-100 amb etanol: èter

Per deslipidar l´apo B-100-100 obtinguda de les diferents fraccions lipoproteiques (VLDL1, VLDL2, IDL i LDL) per les diferents mostres (0h fins 48h a més de P2 i P3) es fa servir el mètode clàssic amb disolvents orgànics (**Veure PNT Precipitació i deslipidació de l´apo B-100-100**).

5.2.3 Obtenció de l´apoA-I i A-II

5.2.3.1 Desalació de les HDL per cromatografia de filtració en gel

Les HDL per les diferents mostres (0h fins 48h) són desalades per cromatografia de filtració en gel fent servir col·lumnes comercials (**Veure PNT Desalació per cromatografia de filtració en gel**).

5.2.3.2 Liofilització de les HDL

Una vegada desalades les HDL, es liofilitzen (**Veure PNT Liofilitzador i Deslipidació d´HDL**).

5.2.3.3 Deslipidació de les HDL

El procés de deslipidació de les HDL per les diferents mostres (0h fins 48h) es basa en el mateix principi que la deslipidació de les fraccions lipoproteiques riques en apo B-100-100 però amb una barreja de disolvents específica (**Veure PNT Deslipidació d´HDL**).

5.2.3.4 Aïllament de l´apoA-I i A-II

Per separar específicament l´apoA-I i A-II d´altres apoproteïnes característiques de les HDL per les diferents mostres (0h fins 48h) es fa servir una variant del mètode d´electroforesi en gels de poliacrilamida en condicions desnaturalizants o SDS-PAGE (**Veure PNT Aïllament de l´apoproteïna AI i A-II per electroforesi desnaturalitzant SDS-PAGE**) .

5.2.4 Hidròlisi àcida de les mostres

5.2.4.1 Hidròlisi àcida de l´apo B-100-100

Per obtindre els aminoàcids que formen part de les apolipoproteïnes i que, finalment, seran analizats en un GC-MS, es duu a terme la hidròlisi amb àcid clorhídric de l´apo B-100-100 obtinguda de les diferents fraccions lipoproteiques (VLDL1, VLDL2, IDL i LDL) per les diferents mostres (0h fins 48h a més de P2 i P3) (**Veure PNT Hidròlisi àcida de proteïnes**).

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5.2.4.2 Hidròlisi àcida de l´apoA-I i A-II

Per purificar els aminoàcids d´una proteïna separada per electroforesi en gels de poliacrialmida és necesari tallar les bandes corresponents a l´apoA-I i A-II obtingudes de HDL per les diferents mostres (0h fins 48h) i dur a terme la seva hidròlisi àcida (**Veure PNT Hidròlisi àcida de proteïnes**).

5.2.4.2.1 Cromatografía de bescanvi iònic

En aquest mètode s´han de purificacar els aminoàcids obtinguts de la hidròlisi àcida per cromatografia bescanvi iònic per eliminar posibles restes de poliacrilamida, que podrien interferir en les posteriors determinacions (**Veure PNT Purificació d´amoniàcids per cromatografia de bescanvi iònic, *VacElut* o sistema d´elució de col·lumnes per buit**).

5.2.5 Obtenció dels aminoàcids lliures del plasma o *plasma method*

L´obtenció dels aminoàcids lliures en plasma per les diferents mostres (0h fins 48h) es fa per precipitació amb disolvents orgànics o altres compostos com ara l´àcid tricloracètic (TCA) (**Veure PNT Obtenció dels aminoàcids del plasma o *Plasma method*, Purificació d´amoniàcids per cromatografia de bescanvi iònic, VacElut o sistema d´elució de col·lumnes per buit**).

5.2.6 Evaporació per centrifugació al buit

Una vegada finalitzada la hidròlisi àcida de les mostres per totes les fraccions lipoproteiques s´ha d´evaporar el disolvent àcid per continuar el processament dels aminoàcids.
Igualment, finalitzada l´obtenció d´aminoàcids lliures en plasma un cop obtinguts per cromatografia de bescanvi iònic en hidròxid d´amoni és necessària la seva evaporació (**Veure PNT Evaporació al buit de mostres en solució, Evaporador de buit**).

5.2.7 Derivatització d´aminoàcids pel seu anàlisi en un GC-MS

El procés de derivatització de les mostres d´apo B-100-100, apoA-I i apoA-II té lloc un cop finalitzada l´evaporació dels aminoàcids obtinguts per hidròlisi àcida.
Aquest pas és realitzat al laboratori de la Dr. Muriel Caslake, Vascular Biochemistry Section, Division of Cardiovascular & Medical Sciences, Royal Infirmary, Glasgow.

5.2.8 Anàlisi d´aminoàcids derivatizats en un GC-MS i obtenció de resultats amb el programa SAAM II
El procés d´obtenció de dades de les mostres processades a partir del seu anàlisi en un GC-MS també es realitza al laboratori de la Dr. Muriel Caslake, Vascular Biochemistry Section, Division of Cardiovascular & Medical Sciences, Royal Infirmary, Glasgow.

5.2.8.1 Anàlisi de resultats cinètics mitjançant l´aplicació de models compartimentals

Aquest pas és realitzat al laboratori de la Dr. Muriel Caslake, Dep. Pathological Biochemistry, University of Glasgow (**Veure full de dades Excel de la carpeta Cinètiques**).

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

Per introduir les dades cinètiques al SAAM II program s´han de calcular a part una sèrie de dades (**Veure full de dades Excel de la carpeta Cinètiques**):

- càlcul de la massa o concentració total o *pool size* de apo B-100-100, apoA-I i A-II en les diferents fraccions en mostres de *pools* per cadasquna de les fraccions lipoproteiques (VLDL1, VLDL2, IDL, LDL i HDL) aïllades per cada subjecte

- càlcul de la massa o concentració de leucina o *leucine mass* injectada en cada subjecte

5.2.9 Tècniques complementàries en l´estudi de cinètiques de lipoproteïnes

5.2.9.1 Determinació de proteïnes pel mètode de *Lowry*

Per calcular la concentració total o *pool size* d´apo B-100-100 en les fraccions lipoproteiques VLDL1, VLDL2, IDL i LDL es fa servir el mètode de determinació de proteïnes de *Lowry*. Aquesta determinació es realitza a dues mostres de *pools* de plasma (P2, P3) a partir de les quals s´aïllen per ultracentrifugació les diferents fraccions lipoproteiques. Després de la seva deslipidació, es determina per *Lowry* (**Veure PNT *Lowry***):

- el precipitat d´apo B-100-100

- les proteïnes que puguin quedar en el sobrenadant d´isopropanol. Compte de guardar el volum d´isopropanol després de la precipitació de l´apo B-100-100.

5.2.9.1.1 Determinació de mostres aquoses

Després de la precipitació de l´apo B-100-100 amb isopropanol de les dues mostres de *pools* per VLDL1, VLDL2, IDL i LDL, es determina per *Lowry* el sobrenadant d´isopropanol (**Veure PNT *Lowry***).

5.2.9.1.2 Determinació de mostres precipitades

Després de la deslipidació de l´apo B-100-100 amb etanol:èter de les dues mostres de *pools* per VLDL1, VLDL2, IDL i LDL, es determina per *Lowry* el precipitat d´apo B-100-100 (**Veure PNT *Lowry***).

5.2.9.2 Determinació de proteïnes pel mètode de *Bradford*

Per calcular el contingut de proteïna total de les HDL després de desalar-les es fa servir el mètode de *Bradford* (**Veure PNT *Bradford***).

5.2.9.3 Anàlisi composicional de lipoproteïnes

Es realitza l´anàlisi composicional:
- en VLDL1, VLDL2, IDL, LDL i HDL obtingudes a partir d´una mostra de *pool* (P1)
- una mostra de plasma a temps inicial (0h)

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Es calcula la concentració de colesterol total, colesterol esterificat i lliure, triglicèrids, fosfolípids, apo B-100-100, apoA-I i A-II fent servir un autoanalitzador Cobas Mira (**Veure PNT Cobas Mira**).
Per calcular la concentració total de proteïnes es fa servir el mètode de *Lowry* (modificació amb SDS) (**Veure PNT *Lowry***). Aquests valors de proteïnes totals dels P1 també es fan servir pel càlcul del *pool size* d´apo B-100-100.
També es determina la concentració de Lp(a) en les mostres de plasma inicial (pel posterior càlcul del *pool size* d´apo B-100 s´han de corregir els valors de colesterol total per les concentracions de Lp(a) dels voluntaris).

6. Bibliografia

Veure referències bibliogràfies de cada metodologia en el seu corresponent PNT.

Demant T, Packard CJ. et al. Sensitive methods to study human apo B-100-100 metabolism using stable isotope-labeled amino acids. Am. J. Physiol. Endocrinol. Metab. 1996. 270: 1022-36.

Hugh P, Barrett R, Foster DM. Design and analysis of lipid tracer kinetic studies. Curr Opin Lipidol. 1996. 7; 3: 143-8.

Cobelli C, Foster DM. Compartmental models: theory and practice using the SAAM II software system. Adv Exp Med Biol. 1998. 445: 79-101.

Packard CJ, Demant T et al. Apolipoprotein B metabolism and the distribution of VLDL and LDL subfractions. J Lipid Res. 2000. 41; 2: 305-18.

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ANNEXE II

Facultat de Medicina i Ciències de la Salut	Revisió: 1
Unitat de Recerca de Lípids (URL)	Data: 17/07/04
MESURA DE DENSITAT DE LÍQUIDS: REFRACTÒMETRES I PICNÒMETRES	Pàg. 1 de 5

Picnòmetre 5324/25 Afora	Codi inventari URV:
Refractòmetre AO Model 10406/15	Codi inventari URV: 012419
Refractòmetre Comecta Model C-1	Codi inventari URV:

Fabricant:
Afora
American Optical Company, USA
Comecta, SA

Funcions:
Mesurar la densitat o contingut de sal de diverses solucions líquides fent servir:
- picnòmetre, recipient calibrat que permet pesar un volum de líquid amb molta precisió
- refractòmetre, aparell que permet mesurar l´índex de refracció d´una substància

Instal·lació:
El refractòmetre AO s´ha de connectar al corrent elèctric.

Instruccions d'ús:
Existeix un manual d'usuari proporcionat per la casa comercial Comecta que es troba en la carpeta Manuals d' Aparells.

1. Mesurar la densitat de diverses solucions:

1.1 Fent servir el picnòmetre

1.1.1 Pesar en una balança de precisió el picnòmetre, prèviament netejat i assecat, juntament amb el seu tap (veure Annex). Fer servir una balança analítica ± 0.001g si es vol ser molt acurat.

1.1.2 Registrar el pes en sec (Po).
1.1.3 Omplir el picnòmetre amb aigua destil·lada, evitant de fer bombolles.

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1.1.4 Col·locar el tap del picnòmetre i assegurar-se que el líquid puja pel capil·lar intern.

1.1.5 Eixugar la part externa del picnòmetre de les possibles restes de líquid que hagin sobreeixit.

1.1.6 Pesar el picnòmetre. Registrar el pes ple (P_H).

1.1.7 Buidar el picnòmetre i assecar bé.

1.1.8 Omplir el picnòmetre amb la solució problema que volem mesurar i seguir el passos 1.1.3-1.1.5.

1.1.9 Pesar el picnòmetre. Registrar el pes ple (P_P).

1.1.10 Calcular la densitat de la solució problema amb la fórmula:

$$\text{Densitat (g/ml)} = (P_P - Po)) / (P_H - Po)$$

1.1.11 Un cop acabat de fer servir, netejar, assecar bé i guardar.

1.2 Fent servir el refractòmetre Comecta

1.2.1 Enfocar el refractòmetre sota un focus de llum i ajustar les diòptries de l´operari amb l´anell de diòptries.

1.2.2 Obrir la tapa i deixar caure 1 o 2 gotes de la solució problema. en la superfície del prisma (veure Annex).

1.2.3 Tancar la tapa.

1.2.4 Fer la lectura corresponent a la línia de posició que limita la zona de clar/fosc que informa del percentatge de sal (%) o densitat (g/ml) de la solució problema (veure Annex).

1.2.5 Per repetir la lectura, netejar el prisma amb aigua destil·lada i assecar bé.

1.2.6 Un cop acabat de fer servir, netejar, assecar bé i guardar el refractòmetre al seu estoig.

1.3 Fent servir el refractòmetre AO

1.3.1 Connectar el refractòmetre al corrent elèctric i encendre el botó de la llum.

1.3.2 Enfocar el refractòmetre cap el focus de llum i ajustar les diòptries de l´operari amb l´anell de diòptries.

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1.3.2 Deixar caure 1 o 2 gotes de la solució problema. en la superfície del prisma (veure Annex).

1.3.2 Tancar la tapa. Al no tenir tapa pròpia, s´ha de fer servir la tapa del refractòmetre Comecta.

1.3.3 Fer la lectura corresponent a la línia de posició que limita la zona de clar/fosc que informa del percentatge de sal (%) o densitat (g/ml) de la solució problema (veure Annex).

1.3.4 Per calcular la densitat es fa servir la fórmula:

Densitat (g/ml)= (lectura refractòmetre X 5.391) – 6.185

1.3.5 Per repetir la lectura, netejar el prisma amb aigua destil·lada i assecar bé.

1.3.6 Un cop acabat de fer servir, netejar, assecar bé i guardar el refractòmetre.

Manteniment Intern:
Cap en especial.

Manteniment extern:
Cap en especial.

Reparacions:
Avisar al Servei de Manteniment.

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ANNEX

Fig.1. Imatge i esquema d´un picnòmetre.

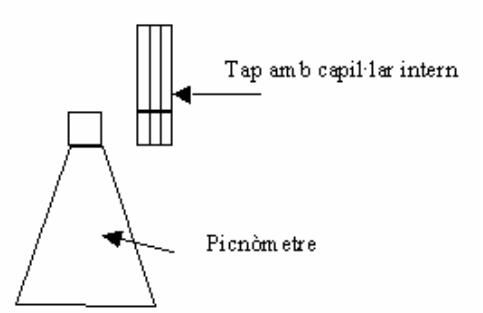

Fig.2. Imatge d´un refractòmetre AO.

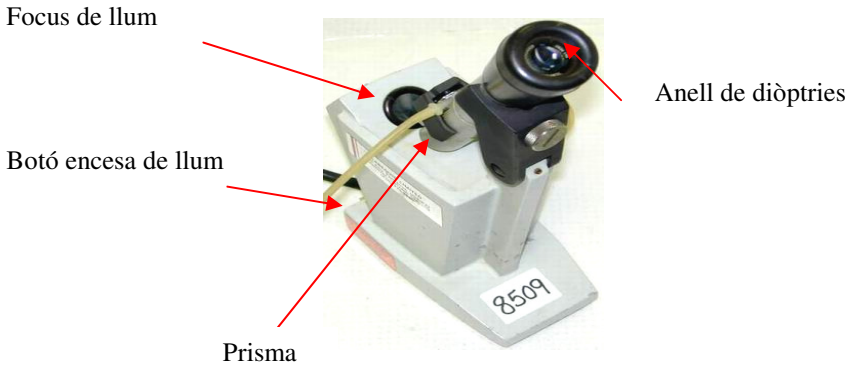

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Fig.3. Imatge d´un refractòmetre Comecta.

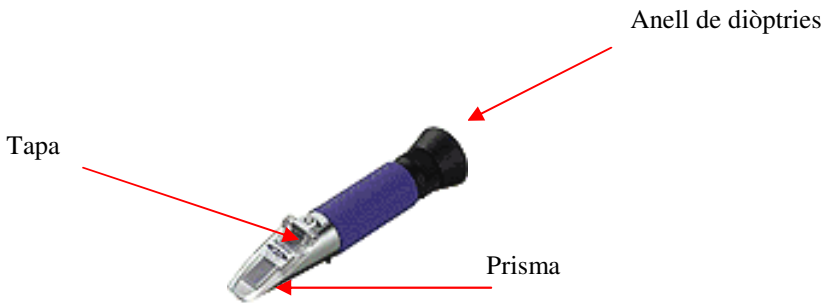
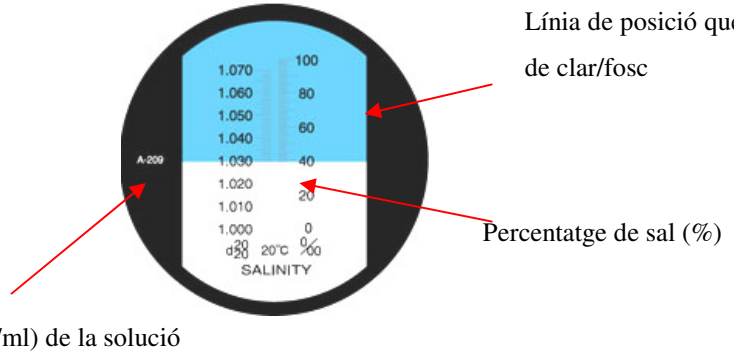


Fig.4. Esquema de lectura de la densitat de diverses solucions



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ANNEXE III

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Unitat de Recerca de Lípids (URL)	Data: 17/07/04
Ultracentrífugues	Pàg. 1 de 5

Centrikon T-1055	Codi inventari URV: 012581
Centrikon T-1075	Codi inventari URV:012580

Fabricant:
Kontron Instruments, Italy.

Funcions:
Centrifugar mostres a elevades revolucions.

Instal·lació:
Connectar al corrent elèctric i mantenir-les en un ambient d´una temperatura poc elevada.

Instruccions d'ús:
Manual d'usuari i d´accessoris proporcionat per la casa comercial que es troba en la carpeta Manuals d' Aparells.

1. Per posar en marxa

1.1 Encendre l'aparell amb els botó ON/OFF situats a la part davantera de l´aparell, en la part inferior dreta.

1.2 Obrir la tapa situada a la part superior esquerra per col·locar el rotor. Anar amb compte degut al considerable pes del rotor. Assegurar-se que queda ben col·locat.

1.3 Tancar la tapa.

1.4 Programar la ultracentrífuga (veure Annex).

1.4.1 Programar la temperatura: prémer el botó Parameter/Temp del panell de control inferior i marcar la temperatura amb els dígits situats a la dreta del panell de control. Després prémer Enter. Per esborrar, prémer Clear i tornar a programar la temperatura. La temperatura programada apareix en el panell superior ROTOR TEMP.

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1.4.2 Programar el temps: prémer el botó Parameter/Time del panell de control inferior i marcar els temps amb els dígits situats a la dreta del panell de control. Després prémer Enter. Per esborrar, prémer Clear i tornar a programar el temps. Per programar hores prémer el dígit i Enter; per programar hores i minuts prémer dígit per hora, prémer • , prémer dígit per minuts i Enter. Per programar minuts, prémer zero, prémer • , prémer dígit per minuts i Enter. La temperatura programada apareix en el panell superior ELAPSED TIME.

1.4.3 Programar les revolucions: prémer el botó Parameter/Speed del panell de control inferior i marcar les rpm amb els dígits situats a la dreta del panell de control. Després prémer Enter. Per esborrar, prémer Clear i tornar a programar el temps. Només permet programar números sencers (ex:39000 rpm, 20000 rpm). La temperatura programada apareix en el panell superior ROTOR SPEED.

1.4.4 Programar delayed: prémer el botó Parameter/Delayed Start del panell de control inferior i marcar el temps de delayed amb els dígits situats a la dreta del panell de control. Després prémer Enter. Per eliminar el delayed, prémer zero i Enter. Comprobar que un cop fet START s´encengui el llum STATUS/DELAYED START del panell de control superior. El copmte enrera del temps de delayed apareix en el panell superior ELAPSED TIME.

1.5 Prémer el botó START del panell de control inferior.

1.6 Apuntar a la llibreta d´incidències les condicions del programa de la ultra i l´operador.

1.7 Esperar fins que la ultracentrífuga estigui rodant a 4000 rpm. Comprovar que no s´encenguin els llums vermells del panell ERROR o STATUS s´encenen per assegurar que tot va bé.

2. Per desconnectar l'aparell

2.1 Quan la ultacentrífuga hagi acabat de rodar, prémer el botó STOP i esperar a què es desfaci el buit. Mirar el panell inferior VACUUM.

2.2 Obrir la tapa i treure el rotor. Col·locar-lo en el seu suport específic perquè no es faci malbé el codi de barres identificat per la ultracentrífuga.

2.3 Netejar el compartiment del rotor de les possibles restes d´oli amb paper i apuntar a la llibreta les revolucions finals corregudes per la ultra. Mirar les rpm totals en el comptador del panell superior.

2.4 Apagar l'aparell prement els botons ON/OFF.

Instruccions d'ús abreujades:

1. Encesa de la ultracentrífuga

1.1 Encendre l'aparell amb el botó ON/OFF.

1.2 Obrir la tapa i col·locar el rotor.

1.3 Tancar la tapa i programar la ultracentrífuga.

1.4 Prémer el botó START.

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2. Desconnexió de l'aparell

2.1 Prémer el botó STOP i esperar a què es desfaci el buit.

2.2 Obrir la tapa i treure el rotor amb les mostres.

2.3 Apagar l'aparell prement el botó ON/OFF.

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ANNEX

Fig.1. Imatge i esquema d´una ultracentrífuga.

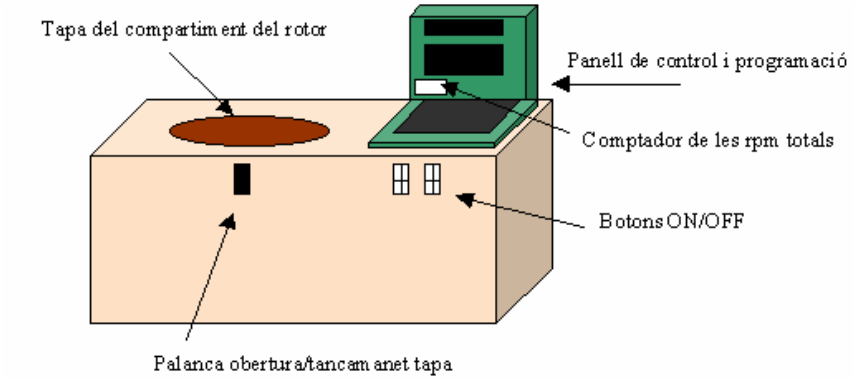

Fig.2. Imatge i esquema dels panells de control.

2a. Panell superior

Rotor temp	Elapsed time	Rotor speed
°C	hrs.min.	rpm

Mode	Error	Status
normal	start fallure	power on
vertical	instrument fallure	program runs
zonal	water cooling	rotor runs
precool	rotor/door	unload
	diffusion pump	open door
Vacuum	drive	delayed start
	rotor unloaded	brake on
low	vacuum	run interrupted
medium	overspeed	
high	rotor temp	

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2b. Panell inferior

Mode	**Parameter**	7	8	9	
		4	5	6	START
normal/ zonal	temp/ rotor code	1	2	3	
vertical/precool	time/ delayed start	•	∞	0	STOP
	speed/ zonal speed	clear		enter	

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ANNEXE IV

Facultat de Medicina i Ciències de la Salut	Revisió: 1
Unitat de Recerca de Lípids (URL)	Data: 17/07/04
ULTRACENTRIFUGACIÓ EN GRADIENT ACUMULATIU O SWING OUT	Pàg. 1 de 6

1. Fonament i objectiu de la prova

La ultracentrifugació en gradient acumulatiu o *swing out* permet la separació de les diferents fraccions lipoproteiques segons la seva densitat i constant de flotació (Sf) en un gradient de solucions de diferent densitat.

2. Espècimen

Plasma.

3. Reactius, controls i altres materials

3.1. Solucions de densitat
- Veure Annex 1.

3.2. Material
- Coated Ultra-Clear Tubes (Beckman, ref. 344060)
- pipetes Pasteur de vidre (Normax, ref. 5426015)
- matraus aforats de diferents volums

4. Instrumentació
- centrífuga *Centricon* 75 (Kontron Instruments, Italy)
- rotor pendular Kontron TST 41.14, 41000 rpm (Kontron Instruments, Italy)
- balança electrònica (FX-400, AND Electronic Balance)
- agitador magnètic (HC1202, Bibby)

5. Procediment

5.1 Preparació de les mostres

5.1.1 Preparar vials de plàstic petits, ben identificats i afegir-hi 0,341 g de NaCl.

5.1.2 Afegir als vials 2 ml de plasma o 2 ml de VLDL (veure PNT ultracentrifugació seqüencial preparativa), resuspendre suaument i deixar a temperatura ambient un temps fins que es dissolgui. Si només s´afegeix 1 ml de plasma o de VLDL, corregir amb 1 ml de solució de densitat 1,006 g/ml.

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5.1.3 Preparar els tubs Beckman ben identificats en un rac i afegir-hi 0.5 ml de la solució de densitat 1.182 g/ml.

5.1.4 A continuació, afegir el volum de plasma o VLDL dissolts en NaCl i el volum de les diferents solucions de densitat segons l´ordre d´*over layer* (veure Annex 2).

5.1.5 Posar els tubs dins del caputxons del rotor. Identificar cada mostra amb el número de cada caputxó ja que durant tot el procés els tubs no es podran extreure. Les mostres no s´ equilibren a la balança; cada caputxó i el seu tap ja estan equilibrats entre ells i les mostres i solucions s´ afegeixen fent servir una pipeta automàtica (calibrar anteriorment).

5.1.6 Afegir una mica de greix als taps i tancar els caputxons. Mirar que aquests estiguin equilibrats, que encaixin bé en la seva posició en el rotor i col·locar aquest en la ultracentrífuga de forma correcta. Tornar a repassar que els caputxons estiguin ben col·locats (veure Annex 3).

5.2 Extracció de les diferents fraccions lipoproteiques

5.2.1 Extracció de VLDL1 (Sf 60-400)

5.2.1.1 Centrifugar les mostres preparades a partir de 2 ml de plasma o VLDL a 39000 rpm, 23ºC, 1.38 hores i desacceleració zero.

6.2.1.2 Extreure 1 ml de la fracció VLDL1. Extreure amb una pipeta Pasteur de vidre i enrasant en un matrau aforat de 1 ml la fracció que queda en la part superior del tub, sense extreure dels caputxons.

5.2.2 Extracció de VLDL2 (Sf20-60)

5.2.2.1 Afegir en *over layer* 1 ml de la solució 6 (veure Annex 2).

5.2.2.2 Centrifugar a les condicions adequades (veure Annex 3).

5.2.2.3 Extreure 0.5 ml de la fracció VLDL2. Extreure amb una pipeta Pasteur de vidre i enrasant en un matrau aforat de 0.5 ml la fracció que queda en la part superior del tub, sense extreure dels caputxons.

5.2.3 Extracció de IDL (Sf 12-20) i LDL (Sf 0-12)

5.2.3.1 Centrifugar a les condicions adequades (veure Annex 3).

5.2.3.2 Extreure 0.5 ml de la fracció IDL. Extreure amb una pipeta Pasteur de vidre i enrasant en un matrau aforat de 0.5ml la fracció que queda en la part superior del tub, sense extreure dels caputxons.

5.2.3.3 Extreure 1 ml de la fracció LDL. Extreure amb una pipeta Pasteur de vidre i enrasant en un matrau aforat de 1 ml la fracció que queda en la part superior del tub, sense extreure dels caputxons.

6. Bibliografia

Lingren et al. Blood lipids and lipoproteins:quantitation, composition and metabolism. Wiley-Interscience, New York, 1972, p221-245.

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

ANNEX 1 : SOLUCIONS DE DENSITAT

Material:

- NaCl (JT Baker, ref. 0278)
- EDTA Na2 (Panreac, ref. 131669)
- NaOH (Fluka, ref. 71690)
- NaBr (Panreac, ref. 121646)

Solucions de densitat:

- Densitat 1.006 g/ml

  22.8 g NaCl, 0.2 g EDTA Na2, 2 ml NaOH 1 N (1M), 2 l H20 destil·lada (veure PNT ultracentrifugació seqüencial preparativa). Dissoldre el NaCl i l´EDTA en 2 l H20 destil·lada i afegir el NaOH. Mirar la densitat en el densitòmetre (veure PNT Densitòmetre) i ajustar-la si és necessari amb NaCl.

- Densitat 1.182 g/ml

  249.80 g NaBr, 1 l sol. 1.006 g/ml. Dissoldre el NaBr a poc a poc per què no precipiti. Mirar la densitat en el densitòmetre (veure PNT Densitòmetre) i ajustar-la si és necessari amb NaBr.

- Altres solucions de densitat es preparen a partir d´ una barreja de les dues solucions anteriors (veure Taula 1). Mirar la densitat en el densitòmetre (veure PNT Densitòmetre) i ajustar-la si és necessari amb una o altra solució.

Taula 1. Altres solucions de densitat.

Sol.	Densitat g/ml	ml sol. 1.006	ml sol. 1.182
1	1.0988	25/50	27.89/55.78
2	1.0860	25/50	20.83/41.66
3	1.0790	25/75	17.72/53.16
4	1.0722	25/75	15.05/46.50
5	1.0641	25/75	12.31/36.93
6	1.0588	25/100	10.73/42.92

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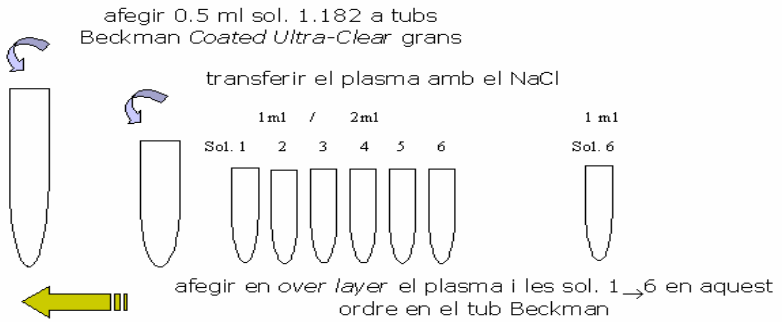
ANNEX 2 : PREPARACIÓ DE LA MOSTRA

Taula 2. Preparació de la mostra: *over layer* de les diferents solucions de densitat.

Ordre	Solució	Densitat g/ml	Volum ml
1º		1.182	0.5
2º		plasma	2
3º	1	1.0988	1
4º	2	1.0860	1
5º	3	1.0790	2
6º	4	1.0722	2
7º	5	1.0641	2
8º	6	1.0588	2

Figure 2. Preparació de la mostra: *over layer* de les diferents solucions de densitat.



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ANNEX 3 : CENTRIFUGACIÓ DE LES MOSTRES

Taula 3. Condicions de centrifugació.

VLDL1 (9.81 ω^2t)*	VLDL2 (2.12 ω^2t)	IDL (1.55 ω^2t)	LDL (7.52 ω^2t)
1.38 h 39.000 rpm	15.41h 18.500 rpm	2.35h 39.000 rpm	21.10h 30.000 rpm
	12.03h 21.100 rpm	3.12h 35.000 rpm	17.30h 33.000 rpm
	17.31h 17.500 rpm	3.24h 34.000 rpm	16.29h 34.000 rpm
	18.08h 17.200 rpm	3.50h 32.000 rpm	18.36h 32.000 rpm
	14.52h 19.000 rpm		18.02h 32.500 rpm
	16.34h 18.000 rpm		19.12h 31.500 rpm
	20.58h 16.000 rpm		

***ω^2t=[(2 X 3.1416 X rpm)/60 s]^2 X temps (s)**

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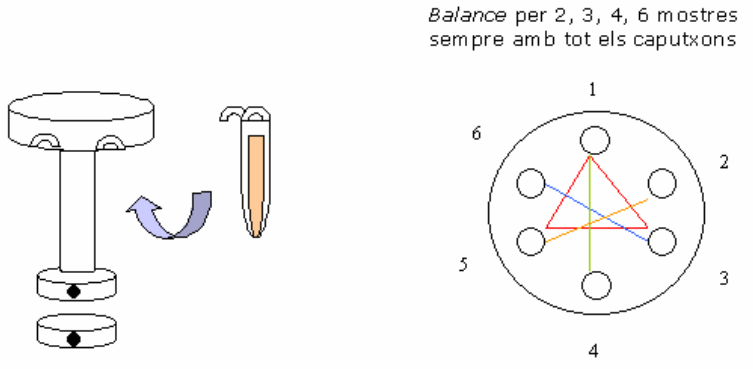
Figure 3. Condicions de centrifugació.



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ANNEXE V

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 10/07/04
PRECIPITACIÓ I DESLIPIDACIÓ DE L´ APOPROTEÏNA B	Pàg. 1 de 3

1. Fonament i objectiu de la prova

El procés de deslipidació permet eliminar els lípids de les partícules de lipoproteïnes i obtindre les apoproteïnes per posteriors determinacions, evitant- se així possibles interferències. Per deslipidar lipoproteïnes es fan servir dissolvents polars com ara barreges d´alcohols i/o cloroform.

La precipitació amb isopropanol de l´apo B-100 i posterior deslipidació amb dissolvents orgànics permet obtindre la major part d´apo B-100 present en les fraccions lipoproteiques VLDL, IDL i/o LDL.

2. Espècimen

Fraccions lipoproteiques riques en apo B-100 aïllades per ultracentrifugació seqüencial o en gradient.

3. Reactius i materials

3.1. Solucions de precipitació i deslipidació
- isopropanol (Merk, ref. 1.00993.1000)
- etanol (Merk, ref.1.11727.1000)
- èter (Merk, ref.1.11727.1000)
- àcid nítric HNO3 60% (Panreac, ref. 131036)

3.2. Material
- tubs de vidre (Schott Duran®, nº ref. 26 135 11 55)
- pipetes Pasteur de vidre (Normax, ref. 5426015)

4. Instrumentació
- campana d´ extraccions (F+, Vallés)
- font de llum directa
- vòrtex (D-051, Dinko)
- pipetus automàtic (Bibbyjet, Bibby)
- dispensador de volums (Dispensette, ref. 4700140; Brand, ref.4700130)
- centrífuga (H-103 R S, Kokusan)

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5. Procediment

5.1 Preparació de les mostres

5.1.1 Afegir un volum de mostra determinat als tubs de vidre correctament rotulats i identificats. En cas de mostres a analitzar posteriorment en un GC/MS, rentar prèviament els tubs amb àcid nítric -aigua, aigua, aigua destil·lada i deixar-los assecar.

5.2 Precipitació de l´apo B-100 amb isopropanol

5.2.1 Afegir isopropanol en una relació 1:1 respecte al volum de mostra, agitar amb vòrtex i deixar a 4ºC O/N (overnight). Guardar l´isopropanol a temperatura ambient. En cas de mostres analitzades posteriorment en un GC/MS, apuntar en cada rac el procés que s´ ha realitzat per cada fracció lipoproteica.

5.2.1.1 Volums a afegir segons el tipus de mostra:

1 ml	VLDL1	+	1 ml	isopropanol
0.5 ml	VLDL2	+	0.5 ml	isopropanol
2 ml	IDL	+	2 ml	isopropanol
1 ml	LDL	+	1 ml	isopropanol

5.2.2 Centrifugar les mostres amb *brake off* a 3.000 rpm, 30 minuts, 4ºC. Anar amb compte perquè en alguns casos el precipitat por ser molt inestable.

5.2.3 Eliminar el sobrenadant d´isopropanol, amb compte de no agafar restes de precipitat flotant, amb una pipeta Pasteur de vidre. Mirar a contrallum d´una font de llum directa. Treure el màxim d´isopropanol possible sense arrossegar gaire precipitat (no cal treure´l tot). Tenir en compte que en alguns casos el precipitat pot ser molt inestable. En cas de mostres analitzades posteriorment en un GC/MS, com en estudis cinètics d´apoproteïnes, aquest procés es realitza també per les mostres de pools (per duplicat). En aquest cas, no es tira el sobrenadant d´isopropanol, sinó que es guarda per mesurar la proteïna pel mètode de *Lowry* (veure protocol *Lowry*).

5.3 Deslipidació de l´apo B-100 amb etanol:èter

5.3.1 Un cop extret el sobrenadant d´isopropanol dels tubs, afegir 3 ml d´etanol:èter (3:1), agitar amb vòrtex i deixar a 4ºC O/N. Mirar que el precipitat es desfaci bé i que no es quedi per les parets del tub. L´etanol:èter (3:1) es guarda a 4ºC. A les fraccions IDL i LDL s´hi poden afegir 6 ml al tenir més component lípidic. També es poden deixar les mostres a 4ºC durant 2 dies seguits si cal.

5.3.1 Centrifugar amb *brake off* a 3.000 rpm, 30 minuts, 4ºC i eliminar el sobrenadant. Mirar a contrallum d´una font de llum directa. Treure el màxim de sobrenadant possible sense arrossegar gaire precipitat (no cal treure´l tot). Tenir en compte que en alguns casos el precipitat pot ser molt inestable.

5.3.2 En cas que sigui necessari deslipidar més, repetir els passos 5.3.1 i 5.3.2. En aquests casos, tant el sobrenadant con el precipitat presenten un color grogós, no blanc, degut al component lipídic.

5.3.3 En cas que la deslipidació sigui completa (pellet blanc), un cop extret el sobrenadant, afegir 3 ml èter, agitar amb vòrtex i deixar 4ºC O/N. Mirar que el precipitat es desfaci bé i que no es quedi per les parets del tub. L´èter es guarda a 4ºC.

5.3.4 Centrifugar amb *brake off* a 3.000 rpm, 30 minuts, 4ºC i eliminar el màxim de sobrenadant. Mirar a contrallum d´una font de llum directa. Treure el màxim

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de sobrenadant possible sense arrossegar gaire precipitat (no cal treure´l tot). Tenir en compte que en alguns casos el precipitat por ser molt inestable.

5.3.5 Assecar els tubs sota una campana d´extraccions per eliminar del tot les restes de dissolvents.

5.3.6 Tapar els tubs de vidre, posar-hi parafilm i guardar-los a –70ºC.

6. Bibliografia

Bloor WR. A method for the determination of fat in small amounts of blood. J Biol Chem. 1914.17;377.

Egusa G, Brady DW. et al. Isopropanol precipitation method for the determination of apo B-100 specific activity and plasma concentrations during metabolic studies of the VLDL and LDL apo B-100. Lipid Res. 1983.24:1261-67.

Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957.226;497-509.

http://www.cyberlipid.org/extract/extr0002.htm#

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ANNEXE VI

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Unitat de Recerca de Lípids (URL)	Data: 10/07/04
DESALACIÓ PER CROMATOGRAFIA DE FILTRACIÓ EN GEL	Pàg. 1 de 4

1. Fonament i objectiu de la prova

La cromatografia en filtració en gel o d´exclusió per tamany es basa en la separació de barreges de compostos segons la seva mida o pes molecular a mida que aquests travessen una fase estacionària amb una determinada mida de porus.
Les mostres dissoltes en solucions amb elevades concentracions de compostos hidrofílics, com ara sals o sacarosa, poden ser desalades per cromatografia de filtració en gel. Això permet eliminar les restes de solvents que, en alguns casos, poden interferir en posteriors determinacions de les mostres.

2. Espècimen

Mostres dissoltes en solucions amb elevades concentracions de sals, com ara la fracció lipoproteica HDL aïllada per ultracentrifugació seqüencial.

3. Reactius i materials

3.1. Solucions de desalació
- NaCl (JT Baker, ref. 0278)
- NaH2PO4 (Fluka, ref. 71500)
- Na2HPO4 (Fluka, ref. 71638)
- HCl (Carlo Erba, ref. 403871)
- aigua destil·lada

3.2. Material
- tubs de plàstic de 5 ml (Sarstedt, ref. 55.475)
- pipetes Pasterur de plàstic (Sarstedt, ref. 86.1171)
- col·lumnes PD-10 (Amersham Pharmacia Biotech, ref. 17-0851-01)

4. Instrumentació
- pHmetre (micropH2001, Crison)
- balança electrònica (FX-400, AND Electronic Balance)

5. Procediment

5.1 Desalació de mostres

5.1.1 Col·locar les col·lumnes en posició vertical en un suport (veure Annex).

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5.1.2 Equilibrar les col·lumnes afegint-hi 25 ml H_2O destil·lada (veure Annex).

5.1.3 Empaquetar-les afegint 3 ml de PBS. Per preparar PBS : 9.4 g/l NaCl + 1.2 g/l NaH_2PO_4 + 1.8 g/l Na_2HPO_4 (pH 7.4).

5.1.4 Afegir 2 ml de mostra i 1 ml de PBS per fer-la baixar.

5.1.5 Afegir 2 ml PBS per poder recollir la mostra en un tub de plàstic. En cas de mostres HDL analitzades posteriorment en un GC/MS, es mesura la proteïna total pel mètode de Bradford (veure PNT *Bradford*) per utilitzar-ne 800 μg. En aquest cas, el volum corresponent s´afegeix a tubs de vidre de 10 ml prèviament rentats amb àcid nítric-aigua. Guardar les mostres a 4ºC fins el seu posterior processament. Les col·lumnes es poden reutilitzar fins a tres vegades. Perquè es mantinguin bé, no deixar-les assecar (omplir-les amb una mica de H_2O destil·lada o PBS abans de guardar-les tapades).

6. Bibliografia

Ausubel FM. et al. Current Protocols in Molecular Biology. Wiley, Inc.1994. Vol.2.

Segrest, JP, Albers JJ. Methods in Enzymology. Academic Pess, Inc. 1986. Vol 128.

http://www.chromatography.amershambiosciences.com

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ANNEX

Instructions

PD-10 Desalting column

Amersham Biosciences PD-10 Desalting columns are prepacked, disposable columns containing Sephadex™ G-25 Medium for group separation of high (M_r > 5000) from low molecular weight substances (M_r < 1000) by desalting and buffer exchange. Columns are delivered in a package which can be converted into a convenient desalting stand – the PD-10 Desalting Workmate.

Table 1. PD-10 Desalting column characteristics.

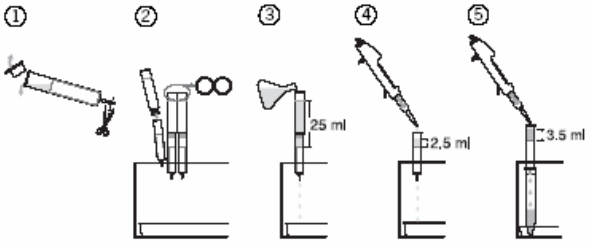
Matrix	Sephadex G-25 Medium
Particle size range	85–260 µm
Bed volume:	8.3 ml
Bed height:	5 cm
Rec. sample volume	2.5 ml
Exclusion limit	M_r 5 000
Chemical stability	All commonly used buffers
Working pH range	2–13
Storage temperature	+4 to +30°C
Supplied in	Distilled water containing 0.15% Kathon™ CG/ICP Biocide

ⓘ 52-1308-00 Edition A0

Amersham Biosciences

PD-10 Desalting Workmate and LabMate Buffer Reservoir

PD-10 Desalting Workmate is designed to fulfil the needs of a column stand for PD-10 Desalting columns. The packaging has been used to construct a simple stand with a plastic tray. The plastic tray is used for collecting waste liquid and holding tubes. If tubes with diameters less than that of the tray holes are used, cover the holes with tape and cut new holes to size. To simplify the use of PD-10 columns, use LabMate™ Buffer Reservoirs (Code No. 18-3216-03).



1. Cut off bottom cap, remove top cap and pour off excess liquid.
2. If available mount the LabMate Buffer Reservoir on top of the PD-10 column and place the columns in the PD-10 Desalting Workmate.
3. Equilibrate the column with approximately 25 ml elution buffer. Discard the flow-through (you can use the plastic tray to collect the flow-through)
4. Add sample of a total volume of 2.5 ml. If the sample is less than 2.5 ml, then add buffer until the total volume of 2.5 ml is achieved. Discard the flow-through.
5. Elute with 3.5 ml buffer and collect the flow-through. A typical chromatogram is showed in Figure 1.

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Operation

A typical chromatogram obtained is shown in Figure 1.

Yield and purity

Using the method described in the sub-section "Operation", protein yield is typically greater than 95% with less than 4% salt (low molecular weight) contamination.
Note that the dilution factor is only 1.4.

Note Small air bubbles along the plastic wall of the column and on the bottom filter may occur. This does not affect the performance of the column.

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ANNEXE VII

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 10/07/04
Liofilitzador	Pàg. 1 de 4

Freeze Dryer ALPHA 1-4	Codi inventari URV: 012395

Fabricant:
Martin Christ

Funcions:
Liofilitzar mostres a temperatura i pressió controlades.

Instal·lació:
Cal connectar-lo al corrent elèctric i a una bomba de buit.

Instruccions d'ús:
Manual d´accessoris que es troba en la carpeta Manuals d' Aparells.

http://www.martinchrist.de.

1. Per posar en marxa

1.2 Encendre l'aparell amb el botó situat darrera l´aparell, en la part inferior esquerra (veure Annex).

1.3 Posar la tapa.

1.4 Encendre la bomba de buit. Deixar funcionant una bona estona perquè s´escalfi i faci millor el buit. Tancar la vàlvula d´entrada-sortida d´aire perquè no es formin gasos (veure Annex).

1.5 Prèmer el botó *KM1/RM1* del panell frontal de control. Esperar què la temperatura baixi fins uns –29ºC (panell de control *Temperature ºC*). La temperatura tarda un parell d´hores fins arribar als –29ºC.

1.5 Fer forats al tub o *eppendorf* que contingui la mostra (prèviament congelada a –70ºC). Tapar el tub que contingui la mostra amb parafilm, fer-hi foradets perquè es pugui evaporar l'aigua. Si la mostra està en un tub *eppendorf*, fer uns foradets al tap.

1.6 Col·locar les mostres en un suport dins l'aparell.

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1.7 Tapar l'aparell. Assegurar-se que la clau de sortida de l´aire, situada darrera del liofilitzador, està tancada (veure Annex).

1.8 Per fer el buit dins el liofilitzador, prémer el botó *Vak pumpe/Vac pumpe* i *MV Druck/ MV Pr.con* del panell frontal de control i fer pressió a la tapa perquè tanqui l´aparell. Pot costar bastant fer el buit i és necessari fer força a la tapa. Assegurar que l´aparell fa el buit mirant l´indicador *Vakuum mbar* del panell de control (pressió de buit <1).

1.9 Deixar liofilitzar les mostres durant 24h o més segons sigui necessari.

2. Per desconnectar l'aparell

2.5 Desfer el buit, girant lentament la clau de sortida d´aire del liofilitzador.

2.6 Obrir la tapa i treure les mostres.

2.7 Apagar l'aparell i la bomba del buit.

2.8 Si queden restes d'aigua dins el liofilitzador, eixugar amb paper absorbent suau.

Instruccions d'ús abreujades

1. Encesa del liofilitzador

1.1 Encendre l'aparell prement el botó *ON/OFF*.

1.2 Connectar la bomba de buit, amb la clau de sortida d´aire tancada.

1.3 Prémer el botó *KM1/RM1* perquè baixi la temperatura del liofilitzador.

1.4 Quan la temperatura sigui de -29ºC, col·locar les mostres i posar la tapa.

1.5 Prémer el botó *Vak pumpe/Vac pumpe* i *MV Druck/ MV Pr.con* perquè l´aparell faci el buit.

2. Desconnexió de l'aparell

2.1 Desfer el buit girant la clau de sortida de l´aire del liofilitzador.

2.2 Treure les mostres i apagar l'aparell i la bomba de buit.

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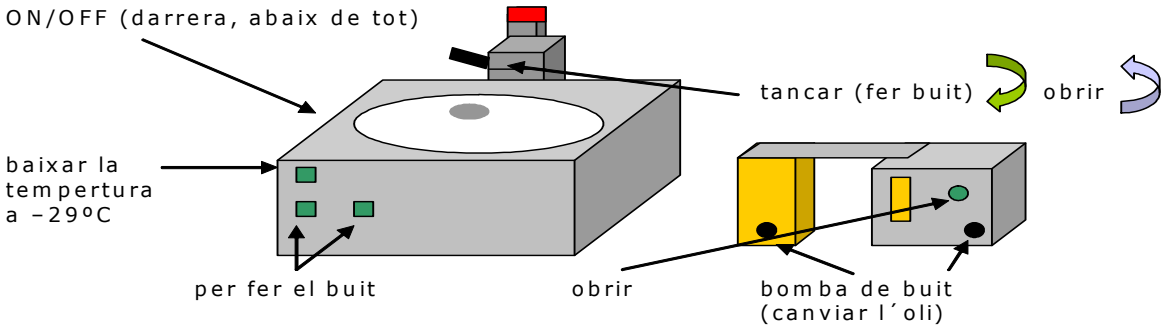
Autor: Katia Uliaque Cugat
ISBN: 978-84-690-6746-8 / DL: T.1183-2007

ANNEX

Fig.1. Imatge i esquema del liofilitzador.



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Annex Bomba de buit

Model: RZ-2

Fabricant: Vacuubrand

Instal·lació: connectar al corrent elèctric.

Instruccions d' ús: existeix un manual d'usuari proporcionat per la casa comercial que es troba en la carpeta Manuals d'Aparells.

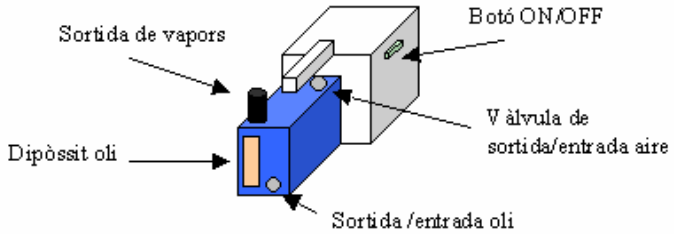
http:// www.vacuubrand.de

Breus instruccions d´ús:

1. Per posar en marxa
   1.1 Prèmer el botó ON/OFF situat al costat dret de l´aparell.

2. Per apagar l´aparell
   2.1 Prèmer el botó ON/OFF.

Fig.1. Esquema i imatge de l´aparell.



Manteniment Intern: per canviar l´oli, seguir les indicacions del manual d´usuari (Vaccumbrad, ref. 687010).

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ANNEXE VIII

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 10/07/04
DESLIPIDACIÓ D´HDL	Pàg. 1 de 2

1. Fonament i objectiu de la prova

El procés de deslipidació permet eliminar els lípids de les partícules de lipoproteïnes i obtindre les apoproteïnes per posteriors determinacions, evitant-se així interferències. Per deslipidar lipoproteïnes es fan servir dissolvents polars com ara barreges d´alcohols i/o cloroform.

En el cas de les HDL un primer procés de liofilització afavoreix la seva posterior deslipidació. D´aquesta manera s´obté un precipitat amb les apoproteïnes característiques de les HDL lliures del component lipídic.

2. Espècimen

Fracció lipoproteica HDL aïllada per ultracentrifugació seqüencial.

3. Reactius i materials

3.1. Solucions de deslipidació
- metanol (Carlo Erba, ref. 412383 / Merck, ref. 1.06007.2500)
- cloroform (Carlo Erba, ref. 438601)
- àcid nítric (HNO3 60%, Panreac, ref. 131036)

3.2. Material
- tubs de vidre de 8 ml (Sarstedt, ref. 55.467)
- parafilm (Parafilm®, ref. PM-996)
- pipetes Pasteur de vidre (Normax, ref. 5426015)
- pipetes calibrades de vidre de diferents volums

4. Instrumentació

- campana d´extraccions (F+, Vallés)
- evaporador de N2 (g) (Liebisch)
- font de llum directa
- vòrtex (D-051, Dinko)
- pipetus automàtic (Bibbyjet, Bibby)
- dispensador de volums (Dispensette, Brand, ref. 4700140, ref. 4700130)
- centrífuga (H-103R S, Kokusan)

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5. Procediment

5.1 Preparació de les mostres

5.1.1 Les HDL desalades es guarden a −70ºC, en tubs de vidre de 10 ml, sense tap i tapades amb parafilm. En cas de mostres per anàlisi posteriorment en un GC/MS, es mesura la proteïna total de les HDL dialitzades pel mètode de *Bradford* (veure PNT *Bradford*) per liofilitzar-ne 800 µg. En aquest cas, el volum corresponent s´afegeix a tubs de vidre de 10 ml prèviament rentats amb àcid nítric-aigua.

5.1.2 Quan les mostres estiguin congelades, es treuen els tubs i es fan un parell forats al parafilm amb ajut d´una agulla. Posar al liofilitzador durant unes 24 hores (veure PNT liofilitzador). Si les mostres no s´han liofilitzat bé, tornar a resuspendre-les amb aprox. 1 ml de PBS, congelar a −70ºC i tornar a liofilitzar més temps.

5.2 Deslipidació d´ HDL amb cloroform:metanol

5.2.1 Afegir a les mostres liofilitzades 2 ml de cloroform:metanol (3:1). Mirar que el precipitat es desfaci bé i que no es quedi per les parets del tub. El cloroform:metanol (3:1) es guarda a 4ºC.

5.2.2 Agitar amb vòrtex i deixar 1 h a 4ºC. Millor si es barreja de tant en tant.

5.2.3 Afegir 5 ml de metanol, agitar amb vòrtex i deixar 30 minuts a 4ºC.

5.2.4 Centrifugar les mostres amb *brake off* a 2.500 rpm, 10 minuts, 4ºC.

5.2.5 Treure el sobrenadant, amb compte de no agafar restes del precipitat flotant, amb una pipeta Pasteur de vidre. Mirar a contrallum d´una font de llum directa. Treure el màxim de sobrenadant possible sense arrossegar gaire precipitat (no cal treure´l tot). Tenir en compte que en alguns casos el precipitat pot ser molt inestable.

5.2.6 Assecar els tubs sota una campana d´extraccions amb N2 (g) per eliminar del tot les restes de dissolvents (veure PNT Evaporació amb nitrogen gas).

5.2.7 Tapar els tubs i guardar-los amb parafilm a −70ºC.

6. Bibliografia

Bloor WR. A method for the determination of fat in small amounts of blood. J Biol Chem. 1914.17;377.

Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957.226;497-509.

Segrest, JP, Albers JJ. Methods in Enzymology. Academic Pess, Inc. 1986. Vol 128.

http://www.cyberlipid.org/extract/extr0002.htm#

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ANNEXE IX

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 10/07/04
AÏLLAMENT DE L´ APOPROTEÏNA A-I i A-II PER ELECTROFORESI DESNATURALITZANT SDS-PAGE AMB GELS NuPAGE®	Pàg. 1 de 4

1. Fonament i objectiu de la prova

La separació de proteïnes en electroforesis SDS-PAGE es basa en l´acció del SDS sobre aquestes proteïnes. El SDS és un detergent que desnaturalitza les proteïnes, unint-se a elles i conferint-ne una càrrega negativa homogènia. Segons els requisits, també s´hi pot afegir 2-β-mercaptoetanol o ditiotreitol per reduir els ponts disulfur. Les proteïnes tractades es desplacen per un gel al aplicar-hi un camp elèctric i es separen segons el seu pes molecular. Finalment, poden ser visualitzades al tenyir-se amb colorants que s´ uneixen a proteïnes.

Per separar específicament l´apoA-I i A-II de la resta d´apoproteïnes característiques de l´HDL es fa servir aquest mètode d´electroforesi. Així es poden obtindre de forma ràpida y sencilla les apoproteïnes desitjades al ser separades segons el seu pes molecular.

2. Espècimen

Fracció lipoproteica HDL aïllada per ultracentrifugació seqüencial, un cop desalades i deslipidades.

3. Reactius i materials

3.1. Solucions

- ApoA-I (Sigma-Aldrich, ref. A-0722)
- ApoA-II (Sigma-Aldrich, ref. A-0972)
- Marcador de pes molecular de proteïnes (Bio-Rad Laboratories, ref. 161-0304)
- Tris o trizma (Sigma-Aldrich, ref. T-1503)
- Glicerol (Sigma-Aldrich, ref. G-5516)
- SDS o lauril sulfat (Sigma-Aldrich, ref. L-3771)
- Bromofenol Blue (BioRad Laboratories, ref. 161-0404)
- SDS Running Buffer (NuPAGE®, Invitrogen, MES X 20, ref. NP0002)
- aigua destil·lada

3.2. Material

- gel
- pipetes Pasteur de vidre (Normax, ref. 5426015)
- pipetes automàtiques per dispensar diferents volums (Eppendorf Research, Eppendorf)
- puntes de pipeta (Eppendorf)
- eppendorfs de 1.5 ml (Sarstedt)
- tubs de vidre de 10 ml
- gels NuPAGE 10% (NuPAGE® Bis-Tris Gel, Invitrogen, ref. NP0302)
- solució colorant per tenyir proteïnes (GelCode Blue Stain Reagent, Pierce, ref. 24592)

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4. Instrumentació

- tanc d´electroforesi (XCell SureLock™ Mini-Cell, Invitrogen)
- font d´electroforesi (Electrophoretic Power Supply EPS3500, AmershamPharmaciaBiotech)
- bany d´aigua (Heater Unitronic 320 OR, Selecta)
- centrífuga (H-103R S, Kokusan)
- centrífuga (EBA 12R, Hettich Zentrifugen)
- balança electrònica (FX-400, AND Electronic Balance)

5. Procediment

5.1 Preparació de les mostres

5.1.1 Col·locar les mostres HDL deslipidades en gel. És important tenir en compte la gran labilitat de les proteïnes i evitar al màxim la seva possible degradació.

5.1.2 Afegir-hi 100 µl de Sample Buffer i agitar una mica el tub. Per poder aïllar el dímer d´apoA-II es treballa en condicions no reductores, sense β-mercaptoetanol. Assegurar-se que el precipitat estigui ben resuspés i sempre en contacte amb gel. Fer alíqüotes de 500µl i guardar-les a –20ºC.

SAMPLE BUFFER 8 ml total		
Tris-HCl 0.5M (pH 6.8)	1 ml →	Tris-HCl 62.5 mM
Glicerol	800 µl →	Glicerol 10%
SDS 10%	1.6 ml →	SDS 2%
Bromofenol Blue	poquíssim (cop espàtula)	
H2O des.	fins a 8 ml	

5.1.3 Escalfar les mostres en un bany d´aigua a 95ºC, 5 minuts. Destapar una mica els tubs, sempre ben identificats. Tenir en compte que el vapor pot esborrar el permanent.

5.1.4 Col·locar immediatament les mostres en gel i donar als tubs un cop de centrífuga a 4ºC.

5.1.5 Recollir les mostres dissoltes en el Sample Buffer amb una pipeta Pasteur de vidre i trasferir-les a eppendorfs de 0.5 µl correctament identificats. A partir de que la mostra està desnaturalitzada no cal treballar en gel.

5.1.6 Les mostres es guarden a 4ºC. Les mostres també es conserven bé a temperatura ambient o bé emmagatzemades a –20ºC.

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5.2 Preparació dels gels i condicions de l´electroforesi

5.2.1 Preparar els gels i montar-los en el tanc d´electroforesi (veure Annex). Treure els gels NuPAGE de l´interior de les bosses de plàstic i rentar-los amb aigua destil·lada. Treure amb compte les pintes i rentar també els pous amb aigua destil·lada. Treure la cinta adhesiva de la part inferior dels gels perque hi pugui passar el corrent.

5.2.2 Preparar el SDS Running Buffer i omplir el tanc per la part exterior i interior. Preparar 400 ml totals de MES x1. Omplir primer el tanc interior entre els dos gels (fins que quedin coberts els pous) i després l´exterior (fins que quedi cobert l´elèctrode).

5.2.3 Carregar les mostres en els gels. Calcular el volum de mostra a carregar. En cas de mostres a analitzar posteriorment en un GC/MS, carregar 100 μg de proteïna total per mostra i pou (12.5 μl). En cas que no es conegui la banda de proteïna d´interés, fer servir una marcador de pes mol·lecular conegut com a referència. Apuntar quin és l´ordre de càrrega de les mostres respecte a la numeració dels pous (1 a 12).

5.2.4 Posar la tapa al tanc d´electroforesi i connectar-lo a la font d´electroforesi Pharmacia (veure PNT Fonts d´electroforesis). Anar amb compte de col·locar els gels i els elèctrodes en la posició correcta segons la seva polaritat.

5.2.5 Programar la font a 80V, 80 mA, 8W (x2 gels) i deixar còrrer l´electroforesi durant 3h 30 min. Vigilar en tot moment les condicions de carrera, la compactació de les bandes, etc perquè la electroforesi es desenvolupi adequadament i les proteïnes es separin bé. Per córrer un gel programar a 80V, 43 mA, 3W. Tot i així, el mA i els W es poden variar si el V no arriba a 80.

5.2.6 Un cop acabada la carrera, desmontar els gels del tanc d´electroforesi i treure els gels. Es trenca el suport de plàstic dels gels fent palanca per tos els cantons amb una pala.

5.2.7 Hidratar els gels durant 20 minuts amb H_2O destil·lada. Els gels es poden rentar més temps si és necessari (un màxim de 30 minuts).

5.2.8 Tenyir els gels durant 1h amb el colorant per proteïnes. És millor si abans de fer-lo servir es deixa el *Code Blue Stain Reagent* a temperatura ambient durant una estona. Afegir-hi uns 80 ml per cada 2 gels. Els gels es poden tenyir més temps si és necessari (un màxim de O/N).

5.2.9 Deixar destenyir els gels un parell de dies amb H_2O destil·lada (anar canviant freqüentment) fins que es vegin bé les bandes d´apoA-I i A-II (veure Annex).

6. Bibliografia

Ausubel FM et al. Current Protocols in Molecular Biology. Wiley, Inc.1994. Vol.2.

Segrest JP, Albers JJ. Methods in Enzymology. Academic Pess, Inc. 1986. Vol 128.

http://www.invitrogen.com

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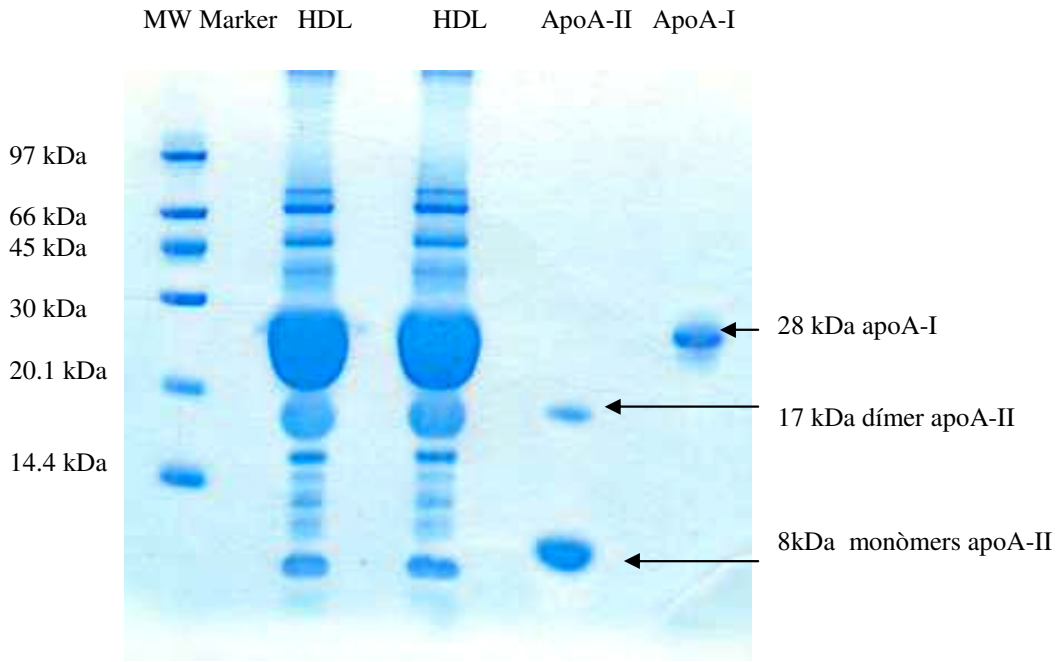
ANNEX

Figure 1. Gel NuPAGE® tenyit amb Code Blue Stain Reagent: separació d´apoA-I i A-II.



NuPAGE® Novex Bis-Tris Gels

QUICK REFERENCE CARD

Instructions are provided below for electrophoresis of NuPAGE® Novex Bis-Tris Gels using the XCell *SureLock*™ Mini-Cell. For detailed instructions, refer to the NuPAGE® Technical Guide available at www.invitrogen.com or contact Technical Service.

Prepare Samples

Reagent	Reduced Sample	Non-reduced Sample
Sample	x µl	x µl
NuPAGE® LDS Sample Buffer (4X)	2.5 µl	2.5 µl
NuPAGE® Reducing Agent (10X)	1 µl	--
Deionized Water	to 6.5 µl	to 7.5 µl
Total Volume	10 µl	10 µl

Heat samples at 70°C for 10 minutes.

Prepare 1X Running Buffer

Prepare 1X SDS Running Buffer by adding 50 ml 20X NuPAGE® MES or MOPS SDS Running Buffer to 950 ml of deionized water.

Load Sample

Load the appropriate concentration of your protein sample on the gel.

Load Buffer

Fill the Upper Buffer Chamber with 200 ml 1X NuPAGE® SDS Running Buffer. For reduced samples, use 200 ml 1X NuPAGE® SDS Running Buffer containing 500 µl NuPAGE® Antioxidant. Fill the Lower Buffer Chamber with 600 ml 1X NuPAGE® SDS Running Buffer.

Run Conditions

Voltage:	200 V constant
Run Time:	35 minutes (MES Buffer), 50 minutes (MOPS Buffer)
Expected Current:	100-125 mA/gel (start); 60-80 mA/gel (end)

1600 Faraday Avenue • Carlsbad • CA 92008
Toll Free: 800 955 6288 • F: 760 602 6500
tech_service@invitrogen.com

Invitrogen life technologies

Contact Information for Other Countries:
See our website www.invitrogen.com

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ANNEXE X

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 10/07/04
HIDRÒLISI ÀCIDA DE PROTEÏNES	Pàg. 1 de 3

1. Fonament i objectiu de la prova

La hidròlisi àcida mitjançant HCl de proteïnes és el primer pas per la obtenció de cadascun del seus aminoàcids característics per la posterior determinació i/o anàlisi d´aquests per tot un seguit de tècniques, com ara l´espectrofotometria de masses.

2. Espècimen

Tot tipus de proteïnes, com ara l´apo B-100 (obtinguda a partir de les fraccions lipoproteiques VLDL, IDL i LDL) i/o l´apoA-I i A-II (obtingudes a partir de la fracció lipoproteica HDL).

3. Reactius i materials

3.1. Solucions
- HCl 37% (Merck, ref. 100317.1000)
- àcid nítric HNO3 60% (Panreac, ref. 131036)
- etanol (Panreac, ref.121086)
- aigua bidestil·lada

3.2. Material
- pipetes calibrades de vidre
- pipetes Pasteur de vidre (Normax, ref. 5426015)
- tubs de vidre (*Schott* Duran®, nº ref. 26 135 11 55)
- tubs de vidre de 10 ml
- bisturí
- pinces

4. Instrumentació
- campana d´extraccions (F+, Vallés)
- vòrtex (D-051, Dinko)
- pipetus automàtic (Bibbyjet, Bibby)
- dispensador de volums (Dispensette, Brand, ref. 4700140, ref. 4700130)
- bloc de temperatura (Multiplaces, Selecta)
- termòmetre
- transil·luminador (UVT-20M/W, Herolab)

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5. Procediment

5.1 Hidròlisi àcida de l´apo B-100

5.1.1 Afegir 1 ml d´HCl 6N als precipitats de les mostres. En cas de mostres analitzades posteriorment en un GC/MS, preparar la dilució 1/2 d´àcid en una ampolla rentada amb àcid nítric- aigua, aigua i aigua destil·lada. Per fer la dilució fer servir aigua bidestil·lada fresca del dia. Rentar un parell de pipetes de vidre de 5-10 ml amb àcid nítric- aigua per dispensar el volum d´àcid.

5.1.2 Tapar bé els tubs i agitar amb vòrtex.

5.1.3 Deixar les mostres 24h en un bloc a 110ºC. Encendre el bloc una estona abans per que arribi a 110ºC, controlant la temperatura amb un termòmetre. Treballar sempre a la campana d´ extraccions. Vigilar que els tubs estiguin ben tapats, sense cap cop o esberla que faci que es puguin trencar al moment de posar-los al bloc.

5.1.4 Les mostres queden llestes per ser evaporades. Les mostres es poden guardar a 4ºC.

5.2 Hidròlisi àcida de l´apoA-I i l´apoA-II

5.2.1 En un transil·luminador, tallar les bandes dels gels corresponents a l´apoA-I i l´apoA-II per cada mostra (veure PNT aïllament d´apoA-I i A-II per electroforesi desnaturalitzant SDS-PAGE) i col·locar-les en tubs Schott. En cas de mostres a analitzar posteriorment en un GC/MS, rentar prèviament els tubs amb àcid nítric-aigua, aigua, aigua destil·lada i deixar-los assecar. Rentar també la superfície del transil·luminador, bisturí i pinces amb etanol. Utilitzar guants de làtex i netejar-los també amb etanol.

5.2.2 Afegir 1 ml de HCl 6N a les mostres i tapar bé els tubs. En cas de mostres a analitzar posteriorment en un GC/MS, preparar la dilució 1/2 d´àcid en una ampolla rentada amb àcid nítric-aigua, aigua i aigua destil·lada. Per fer la dilució fer servir aigua bidestil·lada fresca del dia. Rentar un parell de pipetes de vidre de 5-10 ml amb àcid nítric-aigua per dispensar el volum d´àcid. Comprovar que les bandes queden al fons del tub, ben cobertes per l´àcid.

5.2.3 Deixar les mostres 24h en un bloc a 110ºC. Encendre el bloc una estona abans per què arribi a 110ºC, controlant la temperatura amb un termòmetre. Treballar sempre a la campana d´extraccions. Vigilar que els tubs estiguin ben tapats, sense cap cop o esberla que faci que es puguin trencar al moment de posar-los al bloc.

5.2.4 Col·locar les mostres en un rac i deixar-les 20 minuts a –20ºC.

5.2.5 Centrifugar amb brake off a 3.000 rpm, 5 minuts, 4ºC.

5.2.6 Recollir el sobrenadant amb pipetes Pasteur de vidre i traspassa a tubs de vidre de 10 ml. Mirar a contrallum d´ una font de llum directa. Treure el màxim de sobrenadant possible sense arrossegar gaire precipitat. Compte que en alguns casos el precipitat de poliacrialamida pot ser inestable. En cas de mostres a analitzar posteriorment en un GC/MS, rentar prèviament els tubs amb àcid nítric-aigua, aigua, aigua destil·lada i deixar-los assecar .

5.2.7 Les mostres queden llestes per la purificació dels aminoàcids per bescanvi iònic. Les mostres es poden guardar a 4ºC.

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6. Bibliografia

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Segrest JP, Albers JJ. Methods in Enzymology. Academic Pess, Inc. 1986. Vol 182.

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Autor: Katia Uliaque Cugat
ISBN: 978-84-690-6746-8 / DL: T.1183-2007

ANNEXE XI

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 10/07/04
PURIFICACIÓ D´ AMINOÀCIDS PER BESCANVI IÒNIC	Pàg. 1 de 5

1. Fonament i objectiu de la prova

La cromatografia de bescanvi iònic és una tècnica que permet la separació de barreges de compostos al fer-los passar dissolts en una fase mòbil i interaccionar amb una fase estacionària. En la fase estacionària s´hi troben grups amb càrrega que interaccionen amb els diferents compostos a separar al presentar càrrega oposada.

Els aminoàcids obtinguts després de la precipitació de les proteïnes del plasma o bé a partir de gels de poliacrilamida es purifiquen per cromatografia de bescanvi iònic per evitar possibles interferències amb altres determinacions.

2. Espècimen

Aminoàcids obtinguts de la precipitació amb àcid triclor acètic (TCA) o hidrolitzats de proteïnes aïllades de gels de poliacrilamida, com el cas de l´ apoA-I i l´ apoA-II.

3. Reactius i materials

3.1. Solucions

- HCl (Carlo Erba, ref. 403871)
- NH4OH 25% (Fluka, ref. 09860)
- HNO3 60% (Panreac, ref. 131036)
- aigua bidestil·lada

3.2. Material

- xeringues de 20ml
- paper Whatman
- reïna AG®50W-X8 (BioRad, ref. 142-1441)
- paper de pH (Lyphan®)
- empty reservoir and frits (Varian, Inc, ref. 1213-1014)
- tubs de vidre de 10-15 ml
- tubs de plàstic 8 ml (Sarstedt, ref. 55.467)
- gots de precipitat de diferents volums
- pipetes Pasteur de plàstic (Sarstedt, ref. 86.1171)
- pipetes de vidre calibrades
- parafilm (Parafilm®, PM-996)
- espàtula
- iman

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4. Instrumentació

- pipetes automàtiques de diferents volums (Eppendorf Research, Eppendorf)
- puntes de pipeta (Eppendorf)
- Vac-Elut™ 20 manifold (Varian, Inc, ref. 1223-4103)
- Agitador magnètic (HC1202, Bibby)

5. Procediment

5.1 Preparació de la reïna de bescanvi iònic

5.1.1 Preparar el sistema de filtrat de la reïna (veure Annex). Agafar 2 xeringues de 20ml per filtrar la reïna. Tallar per cada xeringa 7 cercles de 2 cm de diàmetre de paper Whatman per què facin de filtre. Col·locar-los un per un amb compte amb ajut de l´ èmbol.

5.1.2 Afegir en un got de precipitat la reïna i hidratar-la una mica amb aigua bidestil·lada. Utilizar 2 ml de reïna per columna. Fer servir aigua bidestil·lada fresca del dia.

5.1.3 Remenar-ho tot bé amb una pipeta Pasteur de plàstic fins obtenir una pasta homogènia i abocar-ho a les xeringues.

5.1.4 Rentar la reïna per filtrat.

5.1.4.1 Col·locar les xeringues en el VacElut (veure PNT VacElut). Recollir l´aigua filtrada de la reïna en un tub de plàstic de 8-10 ml. Mantenir el buit fins que la reïna quedi eixuta del tot.

5.1.4.2 Desconnectar el Vac Elut (veure PNT VacElut). Quan s´hagi filtrat tota l´aigua de la reïna, desfer el buit amb compte.

5.1.4.3 Mesurar el pH de l´aigua filtrada de la reïna. Tornar a hidratar la reïna i repetir els rentats (pas 5.1.4) tantes vegades com faci falta fins que el pH sigui neutre.

5.1.5 Treure la reïna deshidratada de les xeringues amb ajut d´una espàtula. Intentar recuperar el màxim de reïna possible.

5.1.6 Tornar-la al got de precipitat i hidratar-la de nou amb una mica d´aigua.

5.2 Preparació de les columnes de bescanvi iònic

5.2.1 Col·locar un filtre en cada columna. Ajudar-se d´una vareta de vidre.

5.2.2. Agitar la reïna hidratada amb un iman i deixar-ho remenar una estona fins tenir una barreja homogènia.

5.2.3 Preparar les columnes, afegint-hi 2 ml de reïna (veure Annex). La reïna queda a uns 2 cm d´altura de la xeringa. Afegir un altre filtre i compactar-ho tot amb ajuda d´ una vareta de vidre. Afegir a les columnes aigua bidestil·lada. Deixar gotejar l´ aigua i anar-hi afegint per què no s´assequi la reïna.

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5.2.4 Equilibrar el pH de les columnes amb HCl 1N. Preparar una dilució 1/12 de HCl, afegir-hi 2 ml a cada columna i mirar que el pH=1. Rentar les columnes amb aigua destil·lada tantes vegades com sigui necessari fins que el pH torni a ser neutre.

5.2.5 Guardar les columnes a temperatura ambient, tapades amb parafilm per la part inferior i superior fins que es facin servir. Millor preparar-les d´ un dia per l´ altre. Descartar pel seu ús les columnes que gotegin molt a poc a poc o no gens.

5.3 Purificació d´aminoàcids per bescanvi iònic

5.3.1 Acondicionar les columnes de bescanvi iònic. Preparar les columnes en un rac, treure el tap i deixar eluir l´aigua destil·lada. Descartar les que no elueixin bé.

5.3.2 Afegir a les columnes els aminoàcids recollits en solució.

5.3.3 Quan ja no s´elueixi res, rentar les columnes x3 amb aigua destil·lada.

5.3.4 Eluir els aminoàcids units a la columna. Afegir 3 ml de NH4OH 4M a les columnes i deixar eluir, recollir en tubs de vidre. En cas de mostres a analitzar posteriorment en un GC/MS, rentar prèviament els tubs amb àcid nítric -aigua, aigua, aigua destil·lada i deixar-los assecar. Preparar en el moment de fer servir el NH4OH en una ampolla rentada amb àcid nítric- aigua. Rentar amb àcid nítric- aigua un parell de pipetes de vidre de 5-10 ml. Per preparar la dilució 1/1.625 d´NH4OH fer servir agua bidestil·lada fresca del dia. Anar amb compte amb mostres dissoltes en àcids forts (l´àcid pot reaccionar amb la reïna i fer que aquesta perdi la seva estructura).

5.3.5 Col·locar les columnes al sistema de buit VacElut per eluir les restes de aminoàcids. Posar dins el sistema de buit els tubs de vidre de 10 ml, on s´acaben de recollir les restes d´aminoàcids de les columnes. Quan s´hagi eluït tot el volum, fer el buit i esperar a què la reïna quedi ben eixuta (veure PNT VacElut). La reïna es pot reutilitzar fins a 3 vegades (es pot guardar durant períodes llargs una mica hidratada amb etanol o antibacterià). Les columnes i els filtres també es reutilitzen.

5.3.6 Guardar les mostres a 4ºC.

6. Bibliografia

Ausubel FM et al. Current Protocols in Molecular Biology. Wiley, Inc.1994. Vol.2.

Segrest JP, Albers JJ. Methods in Enzymology. Academic Pess, Inc. 1986. Vol 128.

www.bio-rad.com

http://www.ls.huji.ac.il/~purification/PDF/IonExchange/AMERSHAM_ion_exchangeManual.pdf

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ANNEX

AG® 50W and AG MP-50 Cation Exchange Resins

Instruction Manual

BIO-RAD

Table 2. Summary of the Properties of AG 50 and AG MP 50 Resins

Active Group (X8 Resin)	Thermal Stability	Solvent Stability	Resistance to Oxidizing Agents	Resistance to Reducing
$R\text{-}SO_3^-$	Good to 150 °C	Very good	Slowly oxidizes in hot 15% HNO_3	Very good

Section 5
Instructions for Use

5.2 Column Method

The column method involves pouring a column with the resin and passing the sample through to achieve the separation. Particle size will determine the flow rate, which will affect the separation. The resin should be in the correct ionic form and equilibrated prior to adding the sample.

1. Calculate the amount of resin required based on the expected resin capacity and sample concentration. If the sample ionic concentration is unknown, begin with 5 grams of resin for 100 ml of sample, and then optimize the volumes after obtaining the results.
2. Insure that the resin is in the ionic form which will allow the sample ions to be exchanged onto the resin. If conversion of the resin into another ionic form is necessary, use the guidelines described above for resin conversion (see Table 5).
3. Prepare the initial buffer, so that the pH and ionic concentration will allow the sample ions to be exchanged onto the column. For unknown solutions, use deionized water.

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4. Slurry and pour the resin into the column. Equilibrate the resin in the initial buffer using 3 bed volumes of buffer. Poorly equilibrated resin will not give reproducible results. Alternatively, equilibration can be done by the batch technique, prior to pouring the column. First, convert the resin to the appropriate form, then suspend it in the starting buffer. Check the pH with a pH meter while stirring continuously. Adjust the pH by adding acid or base dropwise to the buffer until the desired pH is obtained. Then transfer the resin to the column, and pass 1 bed volume of the starting buffer through the column.
5. Add the initial buffer and allow excess buffer to pass through the column, leaving enough buffer to just cover the top of the resin bed.
6. Apply the sample dropwise to the top of the column without disturbing the resin bed. Drain the sample into the top of the bed and apply several small portions of starting eluant, being very careful to rinse down the sides of the column and to avoid stirring up the bed. Drain each portion to the level of the resin bed before the next portion is added. Never allow the liquid level to drain below the top of the resin bed.
7. The actual flow rate that is used will depend upon the application, the resin, and the column cross section. To obtain flow rates for any given size column, multiply the suggested flow rates in Table 6 by the column cross-sectional area. Table 6 gives typical flow rates of analytical grade resins.
8. If a cation-free solution is the goal, collect the effluent. If the concentrated cations are of interest, allow all of the sample to pass through the column, then elute the metals with a solution containing a counterion of higher selectivity than the bound cation.

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ANNEXE XII

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 10/07/04
VacElut o sistema d´elució de col·lumnes per buit	Pàg. 1 de 2

Vac-Elut 20 Manifold, Varian Inc	Codi inventari URV:

Fabricant:
Varian Inc.

Funcions:
Sistema per fer el buit aplicat a col·lumnes

Instal·lació:
Connectar a una font de sortida-entrada d´aigua per fer el buit

Instruccions d'ús:
Veure http://www.varianinc.com

1. Per posar en marxa

1.1 Col·locar les col·lumnes en el Vac Elut.

1.1.1 Connectar la col·lumna a la conexió del manifold i fer el buit (veure Annex). Per fer el buit, obrir la clau de pas de l´aigua (3). Arribar a una pressió 5-10 (2). Girar la clau de la connexió a la xeringa per obrir-la.

2. Per desconnectar l'aparell

2.1 Treure les col·lumnes del Vac Elut.

2.1.1 Desconnectar el Vac Elut (veure Annex) Tancar la clau de conexió del VacElut. Per desfer el buit, tancar la clau de pas de l´aigua (3) i aixecar la goma (1) que fa el buit al mateix temps.

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ANNEX

Figura 1. Preparació de la reïna. Sistema de buit VacElut.

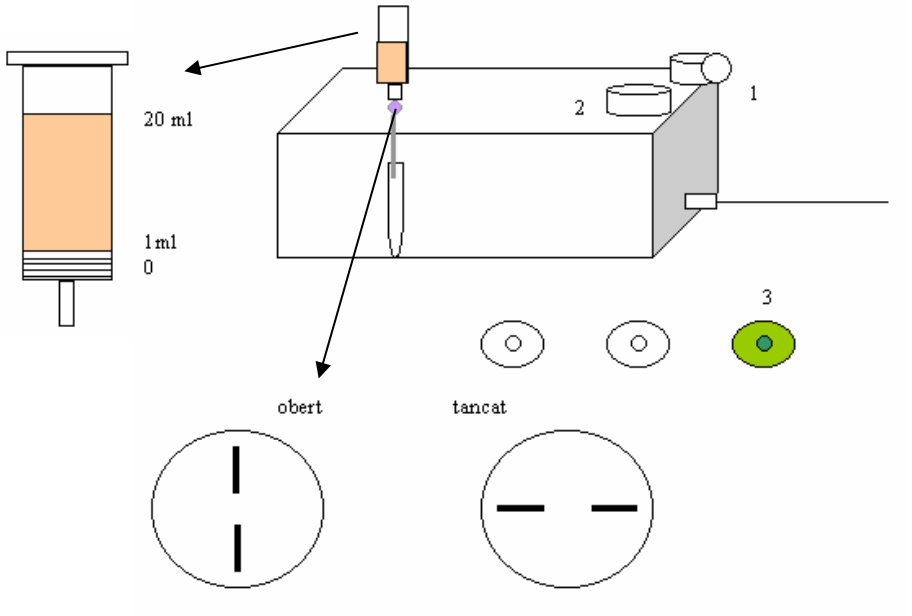
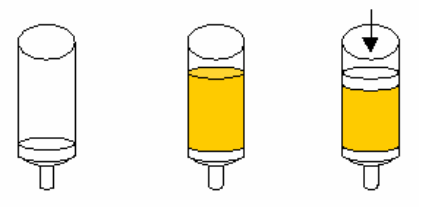
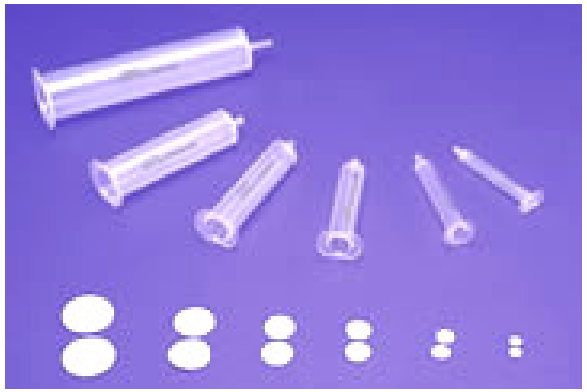


Figura 2. Preparació de les col·lumnes.





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ANNEXE XIII

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 10/07/04
OBTENCIÓ DELS AMINOÀCIDS DEL PLASMA O PLASMA METHOD	Pàg. 1 de 2

1. Fonament i objectiu de la prova

Afegint determinats compostos a les solucions aquoses de proteïnes es pot aconseguir que les molècules d´aigua s´allunyin dels grups polars de les proteïnes, afavorint la seva precipitació. La utilització de TCA (àcid triclor acètic) permet la precipitació de les proteïnes i altres components del plasma per obtenir els aminoàcids que s´hi troben de forma lliure.

2. Espècimen

Plasma.

3. Reactius i materials

3.1. Solucions
- TCA (Merck, ref. 1.00807.0100)
- aigua bidestil·lada

3.2. Material
- tubs de plàstic de 5ml (Sarstedt, ref. 55.475.005)
- pipetes Pasteur de plàstic (Sarstedt, ref. 86.1171)
- pipetes automàtiques de diferents volums (Eppendorf Research, Eppendorf)
- puntes de pipeta (Eppendorf)

4. Instrumentació

- vòrtex (D-051, Dinko)
- centrífuga (H-103R S, Kokusan)

5. Procediment

5.1 Desproteinització de mostres de plasma

5.1.1 Afegir a 1 ml de plasma, 1 ml de TCA 10%. Descongelar les mostres de plasma una estona abans. Preparar les solucions de treball en al moment de fer-les servir.

5.1.2 Agitar amb vòrtex i deixar reposar 15-30 minuts a temperatura ambient.

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5.1.3 Centrifugar a 3.000 rpm, 30min, 4ºC.

5.1.4 Recollir el sobrenadant en tubs de plàstic. Les mostres es poden guardar a 4ºC.

6. Bibliografia

Segrest JP, Albers JJ. Methods in Enzymology. Academic Pess, Inc. 1986. Vol 182.

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ANNEXE XIV

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 10/07/04
Evaporació al buit de mostres en solució	Pàg. 1 de 2

1. Fonament i objectiu de la prova

Les centrífugues d´evaporació al buit són una manera ràpida i eficient d´eliminar els dissolvents de les mostres. Per la evaporació de mostres en solució són necessaris sistemes que incloguin bombes de buit eficaces, condensadors a molt baixes temperatures i trampes per àcids o bases per evitar que aquests solvents facin malbé el sistema.

2. Espècimen

Mostres en solució com ara hidrolitzats de proteïnes, com els aminoàcids obtinguts de l´apo B-100 resuspesos en àcid o de l´apoA-I i apoA-II resuspesos en bases.

3. Reactius i materials

3.2. Material

- vials Chromacol (Supelco-Sigma, ref. 27333)
- taps (SnapCap, Supelco-Sigma, ref. 508357)
- pipetes Parteur de plàstic (Sarstedt, ref. 86.1171)

4. Instrumentació

- bomba de buit (RZ-2, Vacuubrand)
- filtre d´àcids i/o bases (Jouan)
- condensador (Jouan RCT 90)
- centrífuga evaporadora (Jouan RC 10.09)

5. Procediment

5.1 Evaporació de mostres

5.1.1 Encendre el sistema d´evaporació (veure PNT Evaporador de buit).

5.1.2 Deixar les mostres a temperatura ambient durant una estona abans de col·locar-les al rotor de l´evaporador. Equilibrar els tubs entre si. Fer servir el rotor per a tubs de 10-15 ml. Si els tubs són massa estrets, només omplir la part interior del rotor.

5.1.3 Evaporar les mostres els temps necessari segons el seu volum.

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5.1.3.1 En cas de mostres analitzades posteriorment en un GC/MS, quan el volum dels tubs s´hagi evaporat fins uns 0.5 µl, trasbassar aquest volum a vials Chromacol. En cas de mostres a analitzar posteriorment en un GC/MS, els vials s´han de rotular amb el nom del pacient, fracció lipoproteica i número de vial (escriure en una llista a quina mostra correspon cada nº de vial, veure Annex). Guardar els tubs originals de vidre juntament amb les pipetes Pasteur fins que no s´acabi tot el procés (per si s´ha de recuperar mostra). Les mostres es poden guardar tapades a 4ºC per continuar evaporant-se el dia següent si és necessari. Em cas que el volum s´evapori del tot abans de ser transferit al vials Chromacol, es pot tornar a resuspendre el precipitat amb la mateixa solució en la qué estaven resuspesos.

5.1.3 Continuar evaporar les mostres fins la seva evaporació total. Posar les mostres en el rotor pels vials. Les mostres es poden guardar tapades a 4ºC per continuar evaporant-se el dia següent si és necessari.

5.1.4 Un cop evaporat tot el volum, tapar els vials i guardar-los a temperatura ambient.

5.1.5 Apagar el sistema de evaporació (veure PNT Evaporador de buit).

6.- Bibliografia

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ANNEXE XV

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Unitat de Recerca de Lípids (URL)	Data: 17/02/06
Evaporador de buit	Pàg. 1 de 4

Bomba de buit RZ-2 Vacuubrand Condensador Jouan RCT 90 Evaporador de buit Jouan RC 10.09 Campana d´extracció de gasos Valles F+	Codi inventari URV:

Fabricant:
Jouan

Funcions:
Evaporar mostres dissoltes en solucions àcides i/o bàsiques, com ara les solucions d´aminoàcids de l´apo B-100-100, apoAI i/o apoAII.

Instal·lació:
Connectar el sistema al corrent elèctric.

Instruccions d'ús:
Manuals d' instruccions proporcionats per la casa comercial que es troben en la carpeta Manuals d'Aparells.

http://www.jouaninc.com

1. Per posar en marxa

1.1 Primer de tot, tancar la connexió (2) i deixar-la així durant tot el procés (veure Annex).

1.2 Encendre la bomba de buit i el condensador. Després, encendre la centrífuga i deixar que es vagi escalfant a 60ºC.

1.3 Obrir la vàlvula de la bomba de buit per què entri aire (veure Annex). Deixar la bomba en funcionament una estona per eliminar vapors i netejar l´oli de possibles impureses

1.4 Encendre la campana d´extraccions. Per eliminar vapors.

1.5 Deixar en funcionament la bomba una bona estona fins que estigui molt calenta. Funcionant al màxim farà molt millor el buit .

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1.6 Esperar que s´encengui el llum verd del condensador per què aquest funcioni al màxim. La temperatura de treball és d´uns –90ºC.

1.7 Tancar la vàlvula de la bomba de buit per què no entri més aire.

1.8 Col·locar les mostres balancejades al rotor.

1.9 Abaixar la tapa de la centrífuga i col·locar-la bé per què faci el buit.

1.10 Tancar la connexió (1) i comprovar que es fa correctament el buit.

1.11 Deixar rodar els tubs el temps necessari per què s´evapori el contingut.

2. Per desconnectar l'aparell

2.1 Per aturar la centrífuga, primer treure el buit desconnectant la connexió (1). Després, prèmer botó STOP de la centrífuga

2.2 Obrir la tapa i treure les mostres.

2.3 Apagar la bomba del buit, el condensador, l´evaporador i la campana d´extraccions.

2.4 Netejar tot el sistema.

3. Per netejar tot el sistema

3.1 Col·locar un parell de tubs de vidre plens d´aigua destil·lada en el rotor i deixar l´aparell funcionant durant 1h o més, segons el temps que s´hagui fet servir anteriorment. (veure passos 1 i 2)

3.2 Obrir la tapa i treure les mostres.

3.3 Un cop apagat tot, netejar els rotors que s´haguin fet servir amb aigua destil·lada i deixar assecar. Passar una mica d´etanol si queden restes de sals.

3.4 Buidar l´ampolla de vidre del condensador. Amb molt de compte, omplir-la amb aigua, netejar l´interior i tirar el contingut. Eixugar-la una mica i tornar-la a col·locar. Eixugar també la malla de coure.

3.5 Treure la peça on va ajustat el rotor amb una clau. Posar una mica d´oli a la part inferior de la peça.

3.6 Netejar amb metanol l´interior i exterior de l´evaporador.

3.7 Tornar a muntar-ho tot (veure manual d´instruccions).

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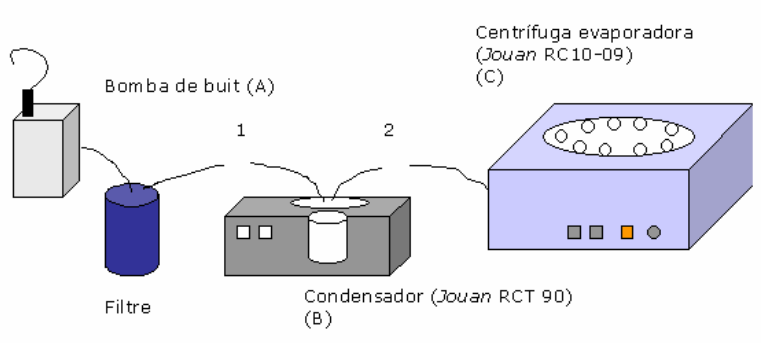
ANNEX: EVAPORADOR DE BUIT

Figura 1. Imatge i esquema del sistema d´evaporació per buit.



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ANNEX BOMBA DE BUIT

Model: RZ-2

Fabricant: Vacuubrand

Instal·lació: connectar al corrent elèctric.

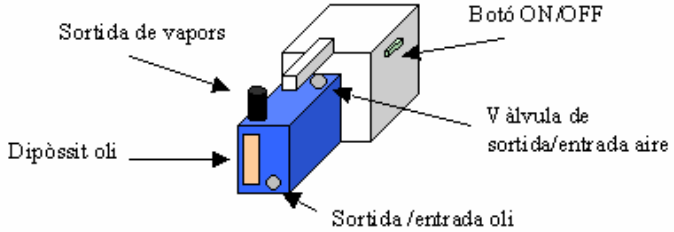
Instruccions d'ús: manual d' instruccions proporcionat per la casa comercial.
www.vacuubrand.de.

Breus instruccions d´ús:

1 Per posar en marxa
1.1 Prémer el botó ON/OFF situat al costat dret de l´aparell.

2. Per apagar l´aparell
2.1 Prémer el botó ON/OFF.

Fig.1. Esquema i imatge de l´aparell.



Manteniment Intern: per canviar l´oli, seguir les indicacions del manual d´usuari (Vaccumbrad, ref. 687010).

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ANNEXE XVI

Facultat de Medicina i Ciències de la Salut	Revisió: 0
Unitat de Recerca de Lípids (URL)	Data: 17/02/06
DETERMINACIÓ DE PROTEÏNES PEL MÈTODE DE LOWRY	Pàg. 1 de 6

1. Fonament i objectiu de la prova

La determinació de proteïnes pel mètode de Lowry és efectiu per mostres solubles en aigua. La formació de color depén principalment de la reducció del reactiu de *Folin-Ciocalteu* per proteïnes, unides al coure. La reacció de reducció es realitza en dos passos diferents. En el primer, la mostra de proteïna es barreja amb ions de coure en medi alcalí. En el segon pas, el reactiu de *Folin-Ciocalteu* (fosfomolibdat fosfotúngstic) és reduït per les proteïnes unides al coure.

2. Espècimen

Proteïnes totals d´ una mostra (diferents fraccions lipoproteiques, extractes cel·lulars, precipitats de proteïna, etc).

3. Reactius i materials

3.1. Solucions

- Na2CO3 (Panreac, ref.131638)
- NaOH (Fluka, ref.71690)
- CuSO45H2O (Probus, ref. 2381)
- Folin Ciocalteu´s phenol solution (Merck, ref.1090010100)
- NaK Tartrat (Panreac, ref. 131729)
- albúmina liofilitzada (Sigma, ref. P-7656)
- SDS o lauril sulfat (Sigma, ref. L-3771)
- aigua destil·lada

3.2. Material

- tubs de plàstic de 5ml (Sarstedt, ref. 55.475)
- pipetes automàtiques per dispensar diferents volums (Eppendorf Research, Eppendorf)
- puntes de pipeta (Eppendorf)
- cubetes espectrofotòmetre 3 ml, 1cm pas de lum (Afora, ref.KA2940)

4. Instrumentació

- espectrofotòmetre (Kontron)
- vòrtex

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5. Controls

La corba de calibració no cal que es faci sencera en cada determinació (està grabada en l´espectrofotòmetre Kontron). S´ha de comprovar afegint dos punts de la mateixa, que es llegeixen amb les mostres.

Com a control en mesures de lipoproteïnes es fa servir un *pool* de LDL. Es fan 10 determinacions per calcular la mitjana i la desviació estàndard. Es guarden alíqüotes a –70ºC. Aquest control es referencia amb la determinació d´apo B-100-100 per inmunoturbidimetria en un autoanalitzador (Cobas-Mira). S´ha d´afegir un control en cada assaig.

Com a control en la resta de mesures es fa servir una solució d´ albúmina sèrica bovina (BSA) de concentració coneguda.

6. Procediment

6.1 <u>Per determinar proteïnes totals en fraccions lipoproteiques:</u>

Preparar sempre mostres, blancs, controls i standars per duplicat com a mínim.

6.1.1 Solucions de treball

- solució A (preparar al moment de fer servir): 2% Na2CO3, 0.1M NaOH (2g+0.4g/100ml)
- solució B: 1% Cu SO45H2O (1g/100ml)
- solució C: 1 % NaK Tartrat (1 g/100ml)

NOTA: protocol per cinètiques! solució C: 2 % NaK Tartrat (2 g/100ml)

- solució alcalina de coure per lipoproteïnes: barrejar les soluciones A, B, C en una proporció 100:1:1
- solució de Folin (preparar al moment de fer servir): barrejar el reactiu de Folin amb H2O destil·lada en una proporció 1:1 (calcular la quantitat que es necessita segons el nombre de mostres)

6.1.2 Preparació de la corba estàndard

Preparar una solució stock d´albúmina sèrica bovina (BSA) a 500µg/ml (diluir el liofilizat amb H2O destil·lada).

6.1.2.1 Corba estàndard per lipoproteïnes

Pipetejar els següents volums en tubs de plàstic de 5 ml. Afegir primer l´H2O destil·lada.

	ST1	ST2	ST3	ST4	ST5	ST6
Sol. Stock	10µl	20µl	50µl	100µl	150µl	200µl
H2O	190µl	180µl	150µl	100µl	50µl	0µl
Conc. µg	5µg	10µg	25µg	50µg	75µg	100µg

A continuació segueixen el mateix processament que les mostres.

6.1.3 Blancs:

Es preparen dos blancs: pipetejar 200µl de H_2O destil·lada en un tub de plàstic de 5ml.

A continuació segueixen el mateix processament que les mostres.

6.1.4 Volums de mostra

Qm = 200µl
VLDL= 200µl
IDL = 50 µl
LDL = 20µl
HDL = 20µl

NOTA: Per mesurar proteïnes en mostres amb triglicèrids (ex: VLDL)!

Les mostres amb triglicèrids molt alts s´han de dislipidar (la turbidesa deguda als lípids pot falsejar el resultat). En aquest cas s´ha d´introduir aquesta modificació al protocol estàndard de Lowry per poder fer una mesura correcta.

6.1.4.1 Procediment per deslipidar :

- trasvasssar els 2,4ml finals que tenim en tubs de 5ml en tubs de vidre de 10ml.
- afegir 2ml de cloroform
- agitar enèrgicament
- centrifugar 5 minuts a 2000 rpm
- agafar el sobrenadant per fer la lectura de l´absorvància

6.1.4.2 Procediment per deslipidar (estudis cinètics) :

- modificar la solució alcalina de coure per lipoproteïnes

afegir 1 ml de SDS 10% a la solució alcalina de coure o reactiu de Biuret (100 ml sol. A : 1 ml sol. B : 1 ml sol. C).
seguir el protocol de processament de mostres, controls, blancs i corba sense afegir més modificacions.

6.1.5 Processament de les mostres, blancs, controls i corba estàndard

- pipetejar el volum corresponent de cada fracció lipoproteica en tubs de 5 ml
- afegir H2O, en cas que sigui necessari com en las fracciones IDL, LDL i HDL, fins un volum final de 200µl
- afegir 2ml de la solució alcalina de cobre

- barrejar amb vòrtex
- deixar 10 minuts a temperatura ambient
- afegir 200µl de la solució de Folin (1:1) immediatament als tubs en agitació en un vòrtex
- deixar a temperatura ambient mínim 30 minuts (en lloc fosc)
- llegir a 650 nm (trasvassar el contingut dels tubs a una cubeta de plàstic)

NOTA: protocol per cinètiques! llegir a 750 nm (trasvassar el contingut dels tubs a una cubeta de plàstic)

- insertar en la corva de calibració les absorvàncies de les mostres. Els resultats es donen en mg/dl (s´ha de tenir en compte els µl que s´han agafat de cada fracció i multiplicar cadascuna d´elles pel factor de dilució corresponent).

6.2 Per determinar proteïnes totals en extractes cel·lulars:

Preparar sempre mostres, blancs, controls i estàndards per duplicat com a mínim.

6.2.1 Solucions de treball

- solució A (preparar al moment de fer servir): 2% Na2CO3 (2g/100ml)

- solució B: 1% Cu SO45H2O (1g/100ml)
- solució C: 1% NaK Tartrat (1g/100ml)

- solució alcalina de coure per lipoproteïnes: barrejar les soluciones A, B, C en una proporció 100:1:1
- solució de Folin (preparar al moment de fer servir): barrejar el reactiu de Folin amb H2O destil·lada en una proporció 1:1 (calcular la quantitat que es necessita segons el nombre de mostres).

6.2.2 Preparació de la corba estàndard

Preparar una solució stock d´albúmina sèrica bovina (BSA) a 500µg/ml (diluir el liofilizat amb H2O).

6.2.2.1 Corba estàndard per extractes cel·lulars

Pipetejar els següents volums en tubs de plàstic de 5 ml.

	ST1	ST2	ST3	ST4	ST5	ST6
Sol. Stock	10µl	20µl	50µl	100µl	150µl	200µl
H2O	490µl	480µl	450µl	400µl	350µl	300µl
Conc. µg	5µg	10µg	25µg	50µg	75µg	100µg

A continuació segueixen el mateix processament que les mostres.

Important: afegir la solució alcalina de coure que té NaOH (apartat 6.1.1).

6.2.3 Blancs:

Es preparen dos blancs: pipetejar 500µl de H2O destil·lada en un tub de plàstic de 5ml. A continuació segueixen el mateix processament que les mostres però afegint 2 ml de la solució alcalina de coure amb NaOH (apartat 6.1.1).

6.2.4 Volums de mostra

- pipetear 500µl d´extracte cel·lular (ja té NaOH propi) en un tub de 5ml de plástic
- afegir 2ml de la solució alcalina de coure per extractes (sense NaOH)
- barrejar amb vòrtex
- deixar reposar 10 minuts a temperatura ambient
- afegir 200µl de la solució de Folin (1:1) immediatament als tubs en agitació en un vòrtex
- deixar a temperatura ambient mínim 30 minuts en un lloc fosc.
- deixar a temperatura ambient mínim 30 minuts (en lloc fosc)
- llegir a 650 nm (trasvassar el contingut dels tubs a una cubeta de plàstic)
- insertar en la corba de calibració les absorvàncies de les mostres. Els resultats es donen en mg/dl (s´ha de tenir en compte els µl que s´han agafat de cada fracció i multiplicar cadascuna d´elles pel factor de dilució corresponent).

6.3 Per determinar proteïnes totals dissoltes en dissolvents orgànics (tipus isopropanol):

Preparar sempre mostres, blancs, controls i estàndards per duplicat com a mínim.

6.3.1 Solucions de treball

- solución A (preparar al moment de fer servir): 2% Na2CO3, 0.1M NaOH (2g+0.4g/100ml)
- solución B: 1% Cu SO45H2O (2g/100ml)
- solución C: 2% NaK Tartrat (2g/100ml)

- solució alcalina de coure per lipoproteïnes: barrejar les solucions A, B, C en una proporció 100:1:1
- solució de Folin (preparar al moment de fer servir): barrejar el reactiu de Folin amb H2O destil·lada en una proporció 1:1 (calcular la quantitat que es necessita segons el nombre de mostres).

6.3.2 Preparació de la corba estàndard

Seguir el procediment apartat 6.1.2. Preparar la corba estàndard cada vegada que es mesurin mostres.

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6.3.3 Blancs:

Seguir el procediment apartat 6.1.3.

6.3.4 Volums de mostra

Afegir als tubs 200µl de mostra.

6.3.5 Processament de les mostres, blancs, controls i corba estàndard

- pipetejar 200µl de mostra en tubs de 5 ml
- afegir 2ml de la solució alcalina de coure
- barrejar amb vòrtex
- deixar 10 minuts a temperatura ambient
- afegir 200µl de la solución de Folin (1:1) immediatament als tubs en agitació en un vòrtex
- deixar a temperatura ambient mínim 30 minuts (en lloc fosc)
- llegir a 750 nm (trasvassar el contingut dels tubs a una cubeta de plàstic)
- insertar en la corba de calibració les absorvàncies de les mostres. Els resultats es donen en mg/dl (s´ha de tenir en compte els µl que s´han agafat de cada fracció i multiplicar cadascuna d´elles pel factor de dilució corresponent).

6.4 Per determinar proteïnes totals en precipitats de proteïna (precipitat d´apo B-100 deslipidada):

Preparar sempre mostres, blancs, controls i estàndards per duplicat com a mínim.

6.4.1 Solucions de treball

Seguir el procediment apartat 6.3.1.

6.4.2 Preparació de la corba estàndard

Seguir el procediment apartat 6.1.2.

6.4.3 Blancs

Seguir el procediment apartat 6.1.3.

6.4.4 Volums de mostra

Afegir 1 ml de NaOH 0.5 N als precipitats de proteïna (como ara d´apo B-100) i deixar que es dissolguin a 4ºC.
Afegir als tubs on es prepararà la reacció de Lowry 200µl de mostra.

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6.4.5 Procesament de les mostres, blancs, controls i corba estàndard
Seguir el procediment apartat 6.3.5 però ajustant el volum de la solució alcalina de coure a afegir a les mostres (les mostres ja porten NaOH propi).

6.4.5.1 Mostres precipitat apo B-100

Volum de mostra µl (resuspés NaOH 0.5 N)		Volum NaOH 0.1 N µl (solució alcalina de coure)	Volum H2O des.µl
VLDL1	200	968	1032
VLDL2	200	968	1032
IDL	200	968	1032
LDL	200	968	1032

7. Bibliografia

- Lowry O.H., Rosebrough N.J., Farr A. L., Randall R. J. J Biol. Chem. 1951.193; 265.

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Erratum to the PhD thesis of Katia Uliaque Cugat **IMPLEMENTATION OF STABLE ISOTOPE LIPOPROTEIN KINETIC STUDIES: EFFECTS ON HDL METABOLISM OF A MEDITERRANEAN-TYPE DIET RICH IN MUFAs FROM VIRGIN OLIVE OIL**

page 15, Section 1.2.1.1.1.2.2:

in sentence 1, "exogenous" should be "endogenous".

page 66, Section 1.2.1.1:

hydratation should be "hydration".

page 63:

"obtention" should be "isolation".

page 125:

the FTR for a lipoprotein = flux from compartment *i* to *j* divided by the mass of *i* (time 24h to give pools/d).

page 143, inside Figure 38:

% apoA-I (1).

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DOCTORAL THESIS
EUROPEAN DOCTORATE MENTION

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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 pobra 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 $^{2}H_3$-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 posat a punt i realitzat 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 tesis 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

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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 $VLDL_1$, $VLDL_2$, 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 las 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.

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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 $^{2}H_{3}$-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

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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.

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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 sugget 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 asymptomatically 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

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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 containg 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].

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Figure 1. Atherosclerotic lesion progression [5].

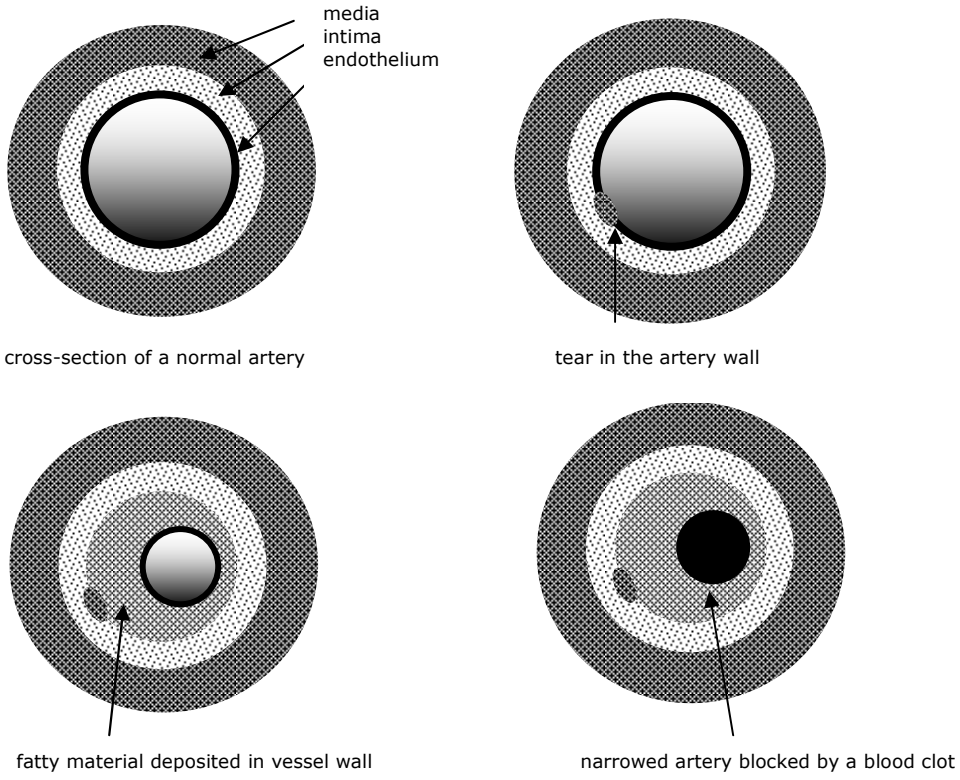

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Figure 2. Endothelial dysfunction, increased LDL permeability and atherosclerotic lesion formation [8].

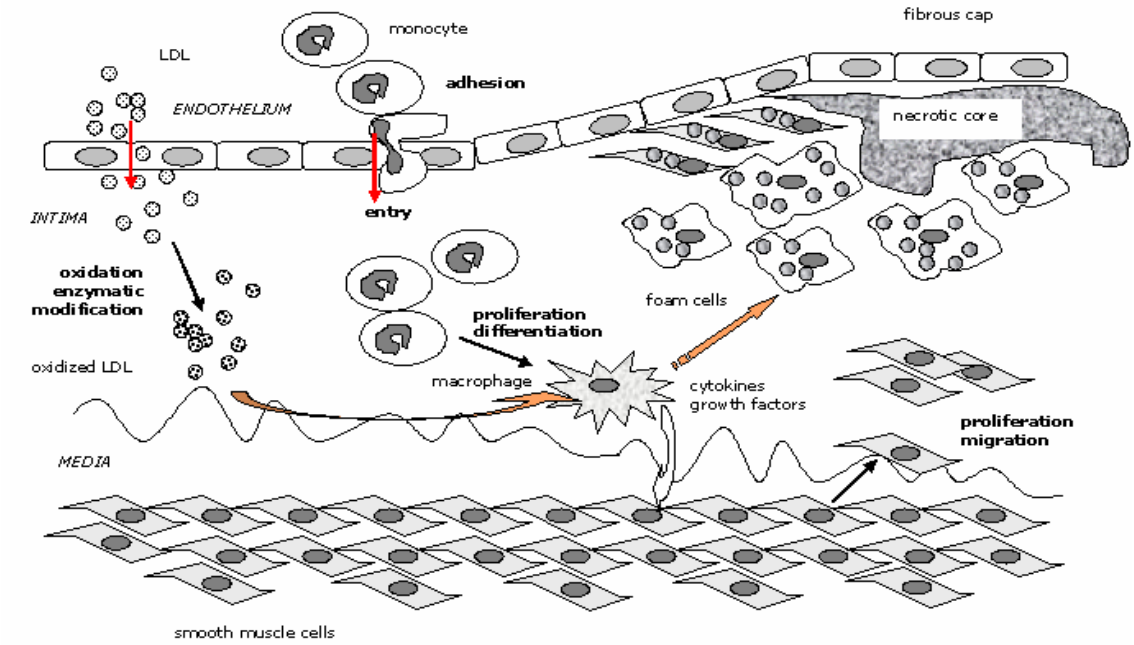
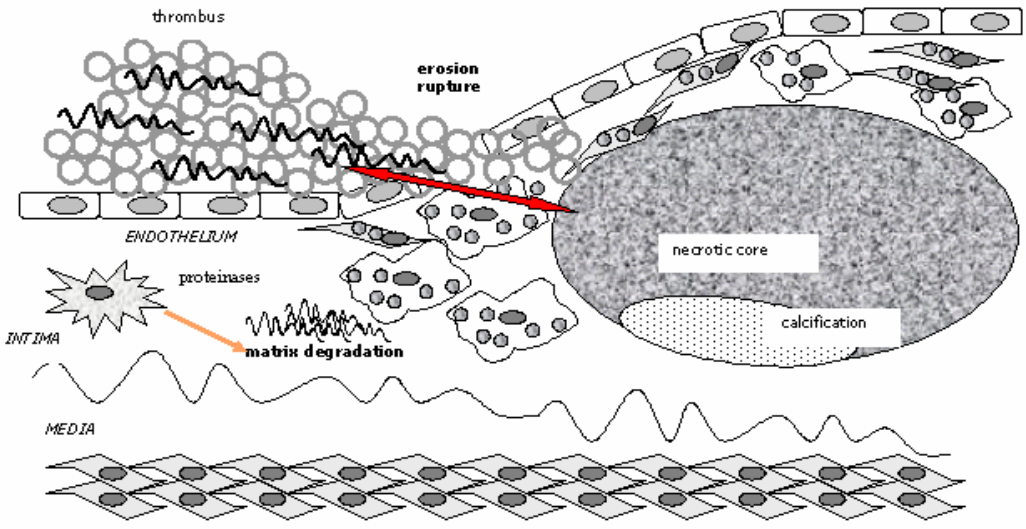


Figure 3. Advanced atherosclerotic lesion [8].



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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 nummerous 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 resistence, 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 ≥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/m^2), 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.

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* 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-sensivity 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

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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 ≥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 ≥5% instead of the previous ≥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].

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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 sightly 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].

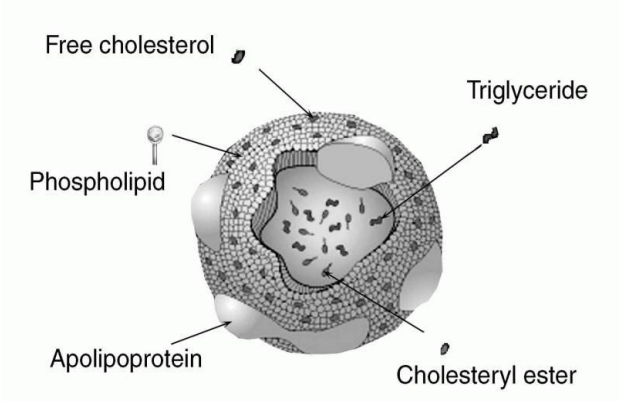

Table 1. Characteristics (A) and composition (B) of plasma lipoproteins particles [73, 74].

(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)

	Percentage composition				
Lipoprotein	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	Apos			
	Major	Other	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].

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1.2.1.1.1.2.1 Exogenous pathway or dietary lipid transport

The exogenous pathway of lipoprotein metabolism enables an efficient tranport 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 A_2). 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 incopored 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 liver [76-79].

1.2.1.1.1.2.2 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 lost of apo-C's, the remaining apos in IDL are predominantly apo B-100 and apo-E. IDL are converted either to LDL (by further lost of TG) or are taken-up directly by the liver.

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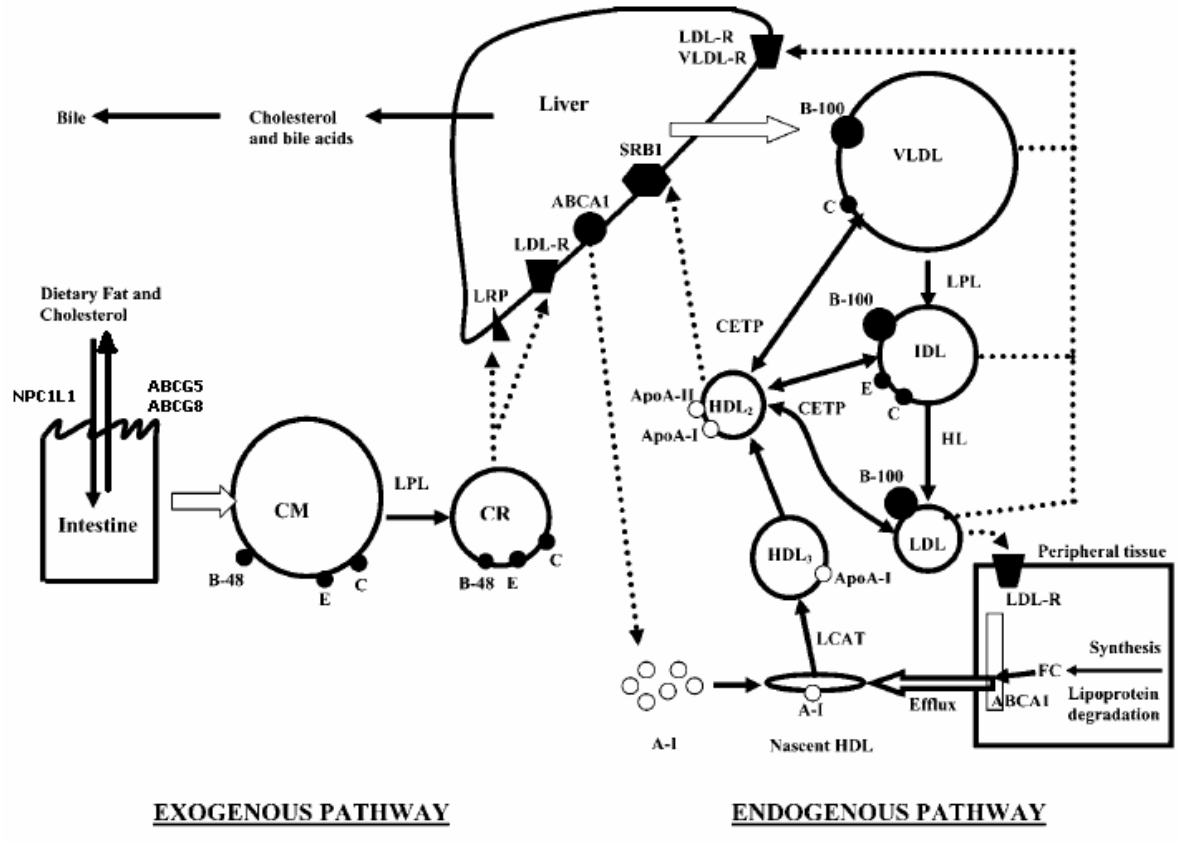
The liver takes-up VLDL remnants and IDL by VLDL- and/or LDL-receptor-mediated cell endocytosis. These interactions require the presence of apo-E [82-86].

The rest of the IDL are remodelled to form LDL by the action of hepatic lipase (HL). HL is a lipase synthetised 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].



CR=chylomicron renmants; 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.

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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 acids [72].

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 gold 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 risk [67-70].

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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.

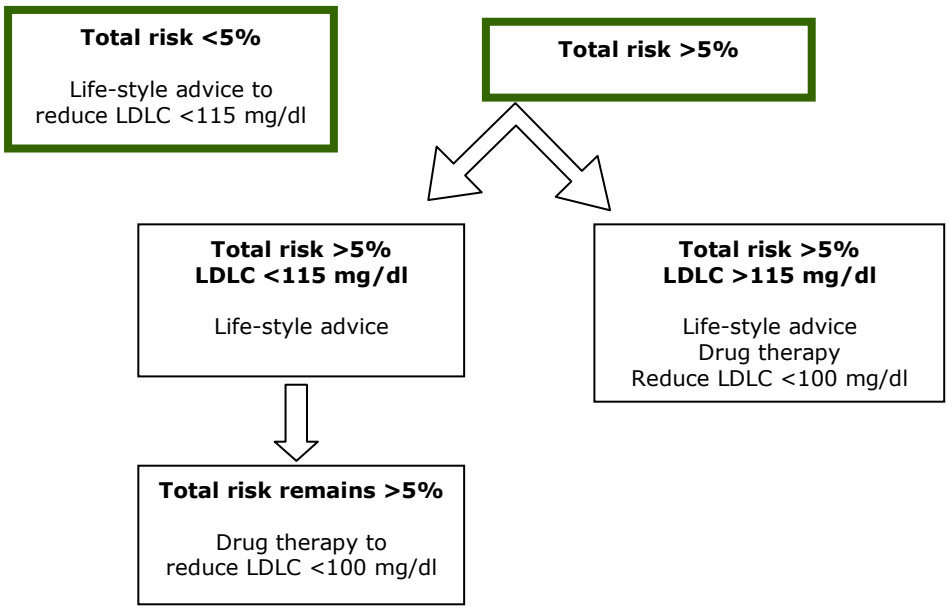
Table 4. Third Joint Task Force LDLC goals [68, 69].

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 Spain [69].

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].

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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 risk [67-70].

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 weight [127-131]. 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 mortality [132-136].

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 chain [72]. The two major groups are [137-142]:

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

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vegetables, walnuts, grains, various seeds and seed oils. α-Linolenic acid can be n-3 group precursor (α-linolenic acid is converted to eiocosapentaenoic 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 synthetised 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].

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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 cells [176-184].

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 demostrated, little is known on apoA-II function in HDL metabolism [72, 124, 154, 192-203].

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Table 5. Characteristics and distribution of the major apos [73, 74, 148-171].

Apo	Plasma (mg/dl)	Cm	Lipoprotein fraction VLDL	LDL	HDL2	HDL3	Tissue source	MW (Da)
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/).

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1.2.1.2 Life-style modifications

Dietary interventions are highly effective in the prevention of recurent 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 meal.
* consuming oily fish and n-3 FAs.
* achieving and maintaining an appropriate body weight (BMI 20-25 kg/m^2) and regular physical activity.
* reducing intake of salt.

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* 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

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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 schedding 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

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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 Coutries 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].

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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].

PHYSICAL TECHNIQUES
- mass spectrometry (MS)
- X-ray crystallography
- nuclear magnetic resonance (NMR)
- ultracentrifugation

PHOTOMETRIC TECHNIQUES
- spectrophotometry
- turbidimetry

CHROMATOGRAPHIC AND ELECTROPHORETIC TECNIQUES
- ion exchange chromatography
- gas chromatography (GC)
- gel electrophoresis

BIOCHEMICAL, CELLULAR AND MOLECULAR TECHNIQUES
- enzymology
  - immunochemical
    - enzyme linked immunosorbent assay (ELISA)
  - enzymatic assays
    - enzyme kinetics (Km)
- cell/tissue culture
- DNA/RNA structural and funtional analysis
  - genetic screening techniques
  - arrays
  - transgenic animal models
  - knockout animal models
- protein structural and functional analysis
  - Western Blot

BIOINFORMATIC TECHNIQUES

BIOSTATISTIC TECHNIQUES

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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

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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.

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The use of stable isotopes in lipoprotein kinetic studies was a development during the decade of the 1990's. It was acchieved, 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 occured 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 indistinguishabled 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 ^{2}H, ^{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 (^{2}H) 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 $^{2}H_2$ (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 $^{2}H_3$ which contains three atoms of ^{2}H 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 ^{2}H/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 ^{2}H 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 isoptopes 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)
^{2}H	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

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Table 9. Characteristics of hydrogen's isotopes [299].

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 $^{2}H_3$-L-leucine, ^{15}H-L-glycine and ^{13}C-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 $^{2}H_3$-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.

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* 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. $^{2}H_3$-L-leucine tracer) is commercially available.

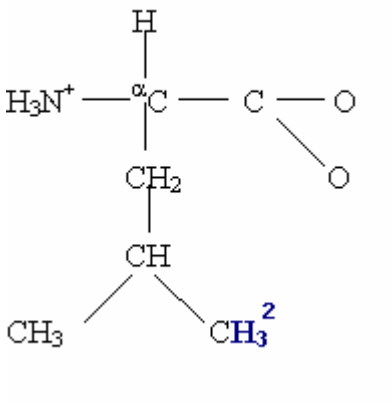
There need to be certain assumptions regarding $^{2}H_3$-L-leucine tracer [310]:

* 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 $^{2}H_3$- 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:

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

where *d% 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].

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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 them [309]. A "kinetic model" is developed taking into account what is known regarding the kinetics of naturally-occuring 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 collected [257].

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 element [264]. 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

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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 rates [265].

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 infusion [323] . 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.

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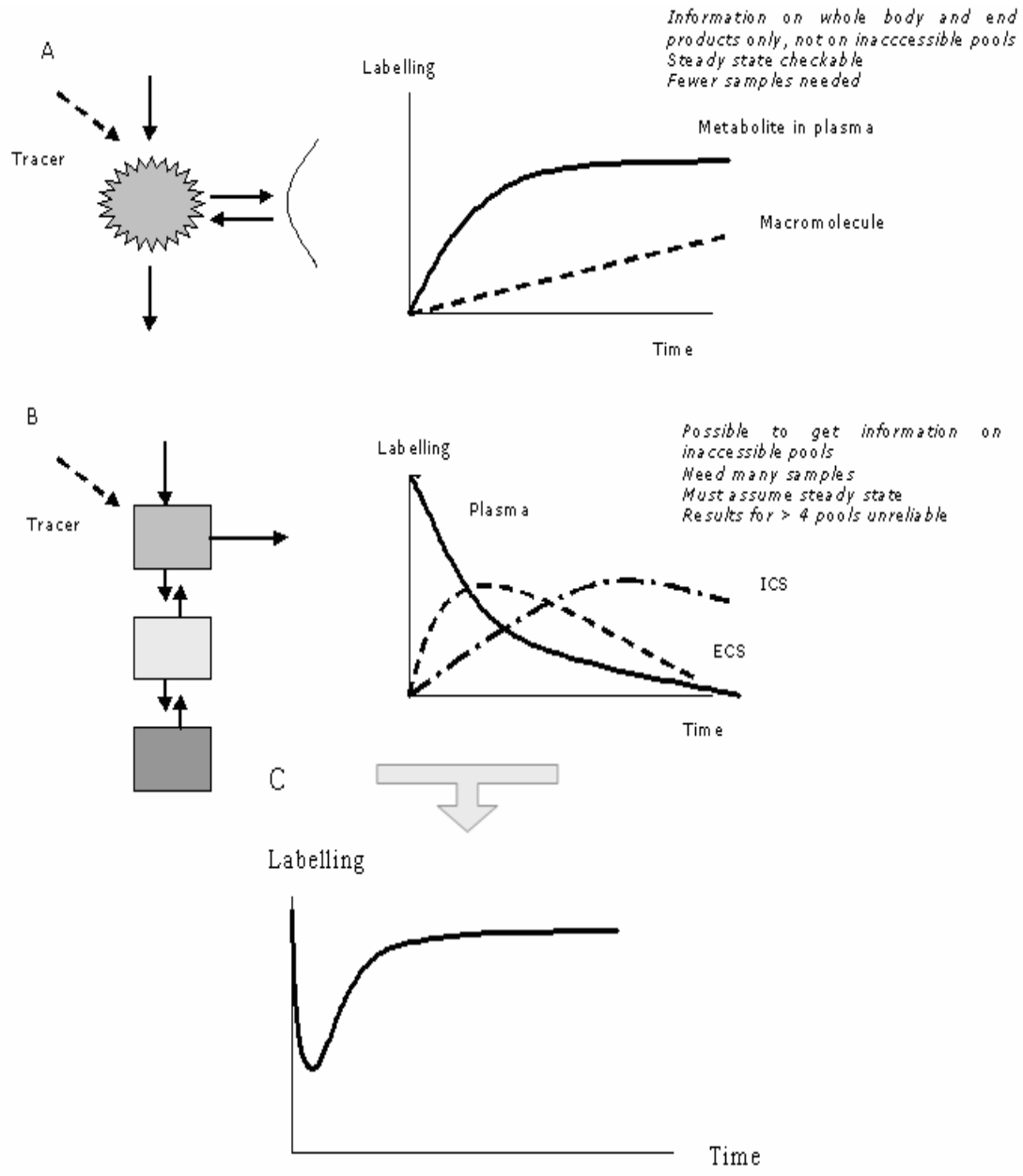
Figure 8. Constant infusion (A), bolus injection (B) and primed constant infusion (C) approaches for the administration of the tracer [313].



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

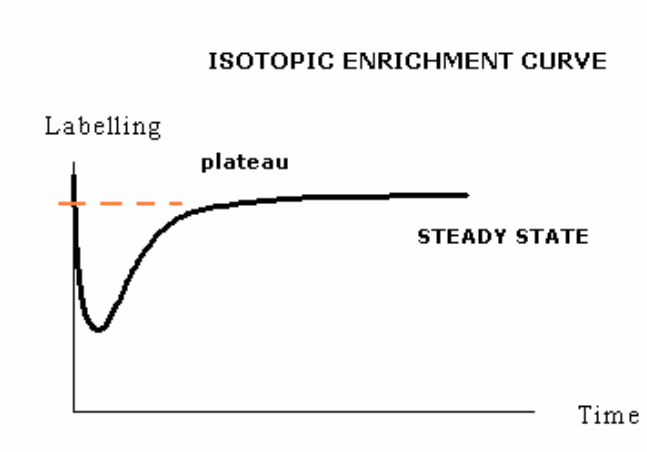
which can provide new insights into metabolic pathways. When using the modelling approach several aspects need to be taken into consideration. These include: experimental design, the number of comportments, certain tests to ensure that the model describes the experimental data and that the model parameters can be estimated with precision. Modelling programs provide an easy-to-use interface to develop and test multi-compartmental models against experimental models [257].

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 determined [325].

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 accesible 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 synthesis [267]. 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 tracee [304]. 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 acchieved 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 combinaton 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 occuring 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. appearence rate of the substance into a compartment and its disappearence 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 $^{2}H_3$-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 $VLDL_1$, $VLDL_2$, IDL and LDL compartments. The model also consideres 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 $^{2}H_3$-L-leucine entrance to the system via plasma and, from which, tracer is distributed to body protein pools, and an intracellular compartment [264]. 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.

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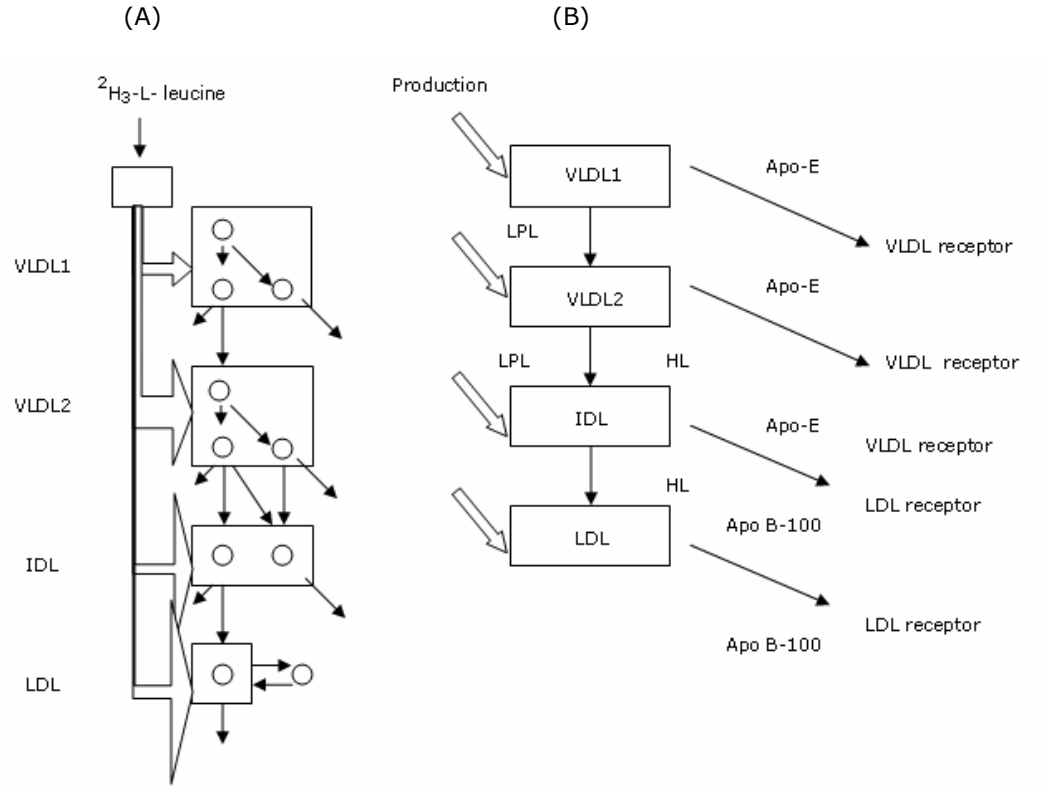
Figure 10. Model structure design: multi-compartmental model for apo B-100-rich lipoproteins metabolism used for analysis of stable isotope data (A) and diagram showing its physiological basis (B) [284].



O=representing each compartment;
LPL=lipoprotein lipase; HL=hepatic lipase.

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 transfered 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).

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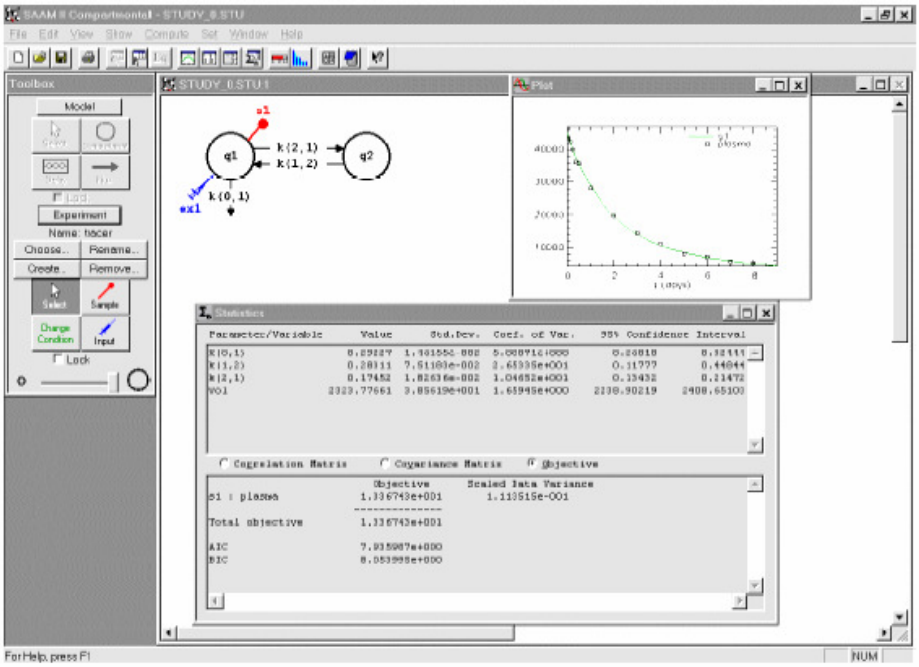
Figure 11. SAAM II program main window [336].



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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 $VLDL_1$, $VLDL_2$, 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

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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: $VLDL_1$, $VLDL_2$, IDL, LDL and HDL.
* obtention of apo B-100 from $VLDL_1$, $VLDL_2$, 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.

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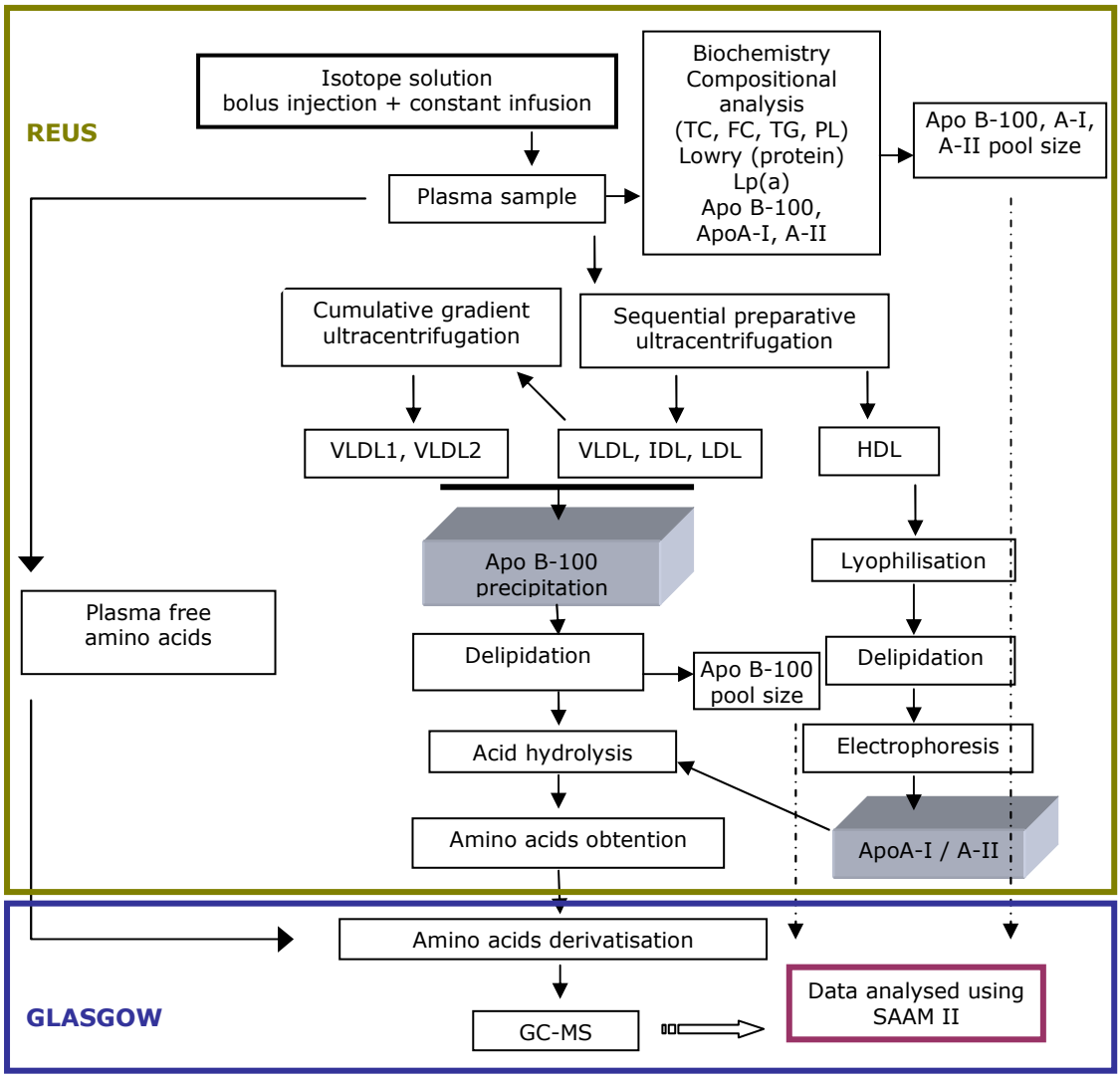
Figure 12. Flow diagram to illustrate lipoprotein kinetics methodology.



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

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

There were several new methodologies, laboratory techniques and major instruments used during the implemention 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].

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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 $VLDL_1$, $VLDL_2$, IDL, LDL and HDL were obtained from plasma samples. Isolation was by ultracentrifugation [345-350].

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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 hydratation 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 simulateously 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 $VLDL_1$ and $VLDL_2$ lipoprotein fractions

Isolation of $VLDL_1$ and $VLDL_2$ 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. $VLDL_1$ (S_f 60-400), $VLDL_2$ (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 surface [349].

Isolation of $VLDL_1$ and $VLDL_2$ 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).

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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, 10ºC
- 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, 10ºC
- 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, 10ºC
- 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, 10ºC
- 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

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Table 11.

Density solutions used in sequential preparative ultracentrifugation (Annexe II)

- Density solution 1.006 g/ml: 22.8 g NaCl, 0.2 g $EDTANa_2$, 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 $VLDL_1$

- ultracentrifuge at 39,000 rpm, 1.38h , 23ºC and brake off
- collect 1 ml $VLDL_1$ from the surface layer by aspiration up to the level of a 1 ml volumetric flask

Obtaining $VLDL_2$

- 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 $VLDL_2$ 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

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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 $VLDL_1$, $VLDL_2$, 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 insolubity in aqueous medium, which makes it a difficult molecule to manipulate. Isopropanol precipitation technique of Egusa is effective in isolating apo B-100 [351]. Isopropanol selectively precipates 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) precipation 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 preciptation is conducted at low temperature (4ºC) 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 4ºC
- centrifuge at 3,000 rpm, 30 minutes, 4ºC
- remove the isopropanol supernatant without disturbing the precipitated apo B-100

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1.2.2.2 Apo B-100 delipidation

$VLDL_1$, $VLDL_2$, 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 (4ºC) 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 4ºC to precipitated apo B-100
- close and tighten tube caps; vortex mix; leave overnight at 4ºC
- centrifuge at 3,000 rpm, 30 minutes, 4ºC
- 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 (4ºC) to the precipitated apo B-100
- close and tighten tube caps; vortex mix; leave overnight at 4ºC
- centrifuge at 3,000 rpm, 30 minutes, 4ºC
- remove supernatant without disturbing the precipitated apo B-100
- evaporate-off any remaining solvent into air

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1.2.3 Obtaining apoA-I and A-II

ApoA-I and A-II, the major HDL apos, 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 previosuly 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 analyses [358].

PD-10 columns (Amersham Pharmacia Biotech, Inc) are disposable desalting columns, prepacked with Sephadex™G-25 beads and equilibrated with distilled H_2O. 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.

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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 H_2O
- 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 4ºC)

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 determinig total protein concentration [359-362]. 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 un-protonated 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. Immunoglogin 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].

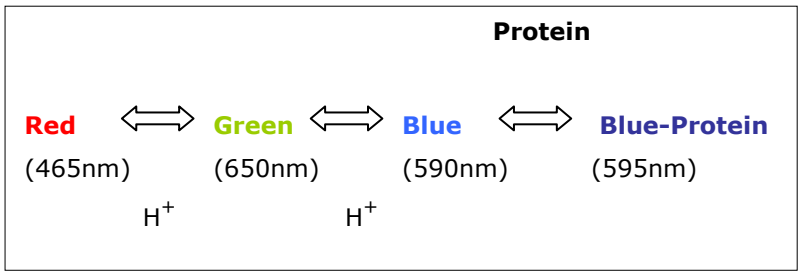

Table 16.

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 (µg/µl) according to absorbance

Preparing calibration curve

- BSA protein standard solution → 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

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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 vaccuum. Lyophilisation is performed at low temperatures (<0ºC) and low pressure (vacuum). Evaporation of water vapour directly from solid state (ice) is termed sublimation. Prior to lyophilisation process, samples are frozen (-70ºC) at normal pressure and then freeze-dried at -29ºC. 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)
- tranfer to tubes and close taps
- freeze HDL at –70º C
- open tubes and cover them with parafilm (make holes in the film cover to allow water evaporation)
- lyophilise samples (–29ºC, vacuum) over 24-48h (Freeze-Dryer ALPHA 1-4, Martin Christ, GmbH)
- close and tighten tube caps and store at –70ºC

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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 4ºC to samples
- vortex mix and allow to stand for 1h at 4ºC
- add 5 ml methanol at 4ºC
- vortex mix and allow to stand for 30 minutes at 4ºC
- centrifuge at 2,500 rpm, 10 minutes, 4ºC
- remove supernatant without disturbing the precipitated proteins
- dry under N_2 (g)
- store at –70ºC

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 gels [364]. 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 studies [366].

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 95ºC, 5 minutes
- place samples on ice
- pulse centrifuge samples at 4ºC
- recover samples dissolved in Sample Buffer and transfer to eppendorf tubes
- store at 4ºC

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_2O	up to 8 ml	

NOTE: do NOT use reducting 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 H_2O until proteins bands are seen clearly

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

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1.2.4 Acid hydrolysis of samples

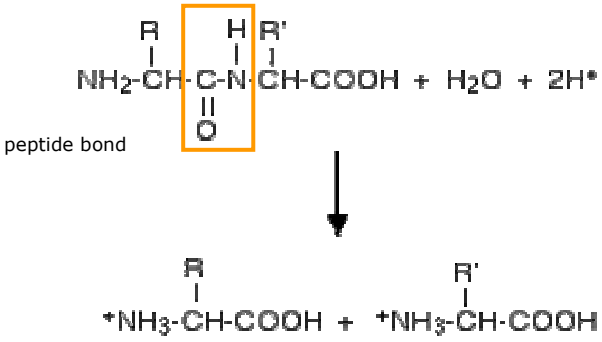
Isolated $VLDL_1$, $VLDL_2$, IDL and LDL apo B-100 samples and HDL apoA-I and A-II samples were hydrolysed with hydrochloric acid to obtain their amino acids content (Tables 21, 22, Annexe X) [367, 368].

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



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1.2.4.1 Apo B-100 acid hydrolysis

Apo B-100 from $VLDL_1$, $VLDL_2$, 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 110ºC

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 110ºC
- place samples for 20 minutes at –20ºC
- centrifuge at 3,000 rpm, 5 minutes, 4ºC
- recover supernatant and store at 4ºC until subsequent purification with ion exchange chromatography

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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 (NH_4OH) (Figure 15).

Figure 15. Cationic exchange resins [362, 369].

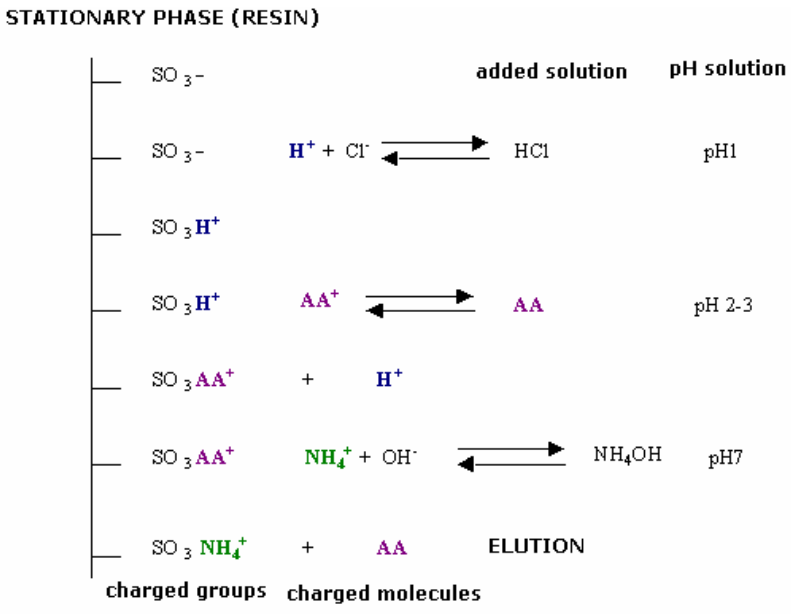

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Table 23.

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 H_2O
- 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 H_2O and shake
- fill columns with 2 ml resin
- add a second filter and allow to compact under gravity
- let distilled H_2O elute from columns
- add 2 ml 1N HCl (pH 1)
- wash columns with distilled H_2O until neutral pH
- fill columns with distilled H_2O to avoid resin dehydratation
- cover columns and store at room temperature

Processing samples

- add hydrolysed samples to columns
- wash columns x3 with distilled H_2O
- place columns in a vacuum manifold (Vac-Elut™, Analytichem International, Inc)
- elute amino acids with 3 ml 4M NH_4OH and collect in tubes
- store at 4ºC

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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].

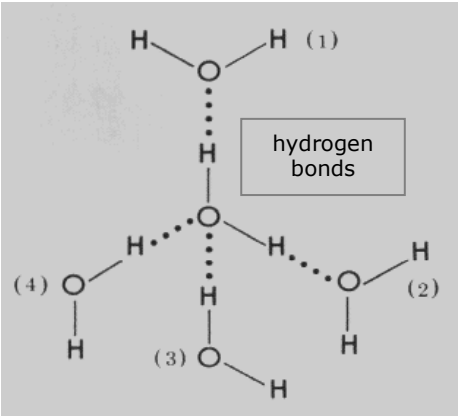

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Table 24.

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, 4ºC
- 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 H_20
- place columns in a vacuum manifold (Vac-Elut™, Analytichem International, Inc)
- elute amino acids with 3 ml 4M NH_4OH and collect in tubes
- store at 4ºC

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 NH_4OH 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).

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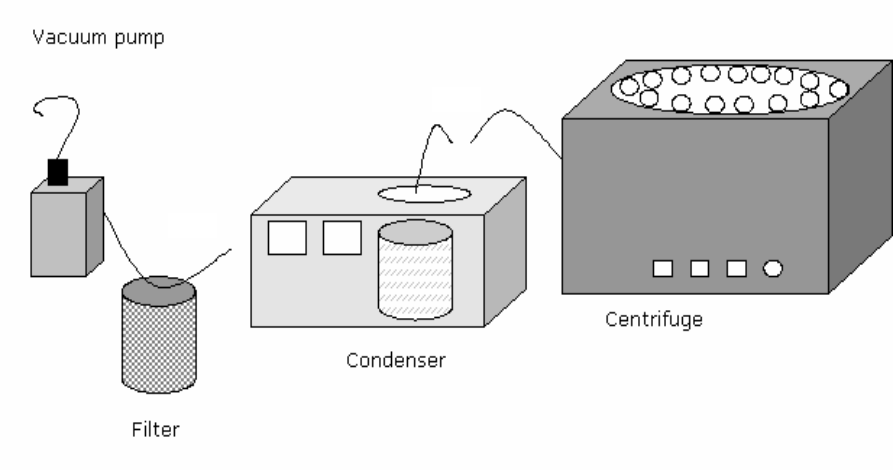
Table 25.

Vacuum centrifugal evaporation (Annexes XIV, XV) [368, 372]

- place sample tubes in a vacuum centrifugal evaporator (Jouan, Thermo Electron Corp)
- evaporate untill a final sample volume of 1-0.5 ml
- transfer contents to vials (Chromacol, Supelco-Sigma Aldrich Inc)
- continue evaporation untill 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 analysys; a system which is one of the most sensitive methods used in the detection of $^{2}H_3$-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].

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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 (µl)	Volume Acetonitrile (µl)
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 70ºC
- 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

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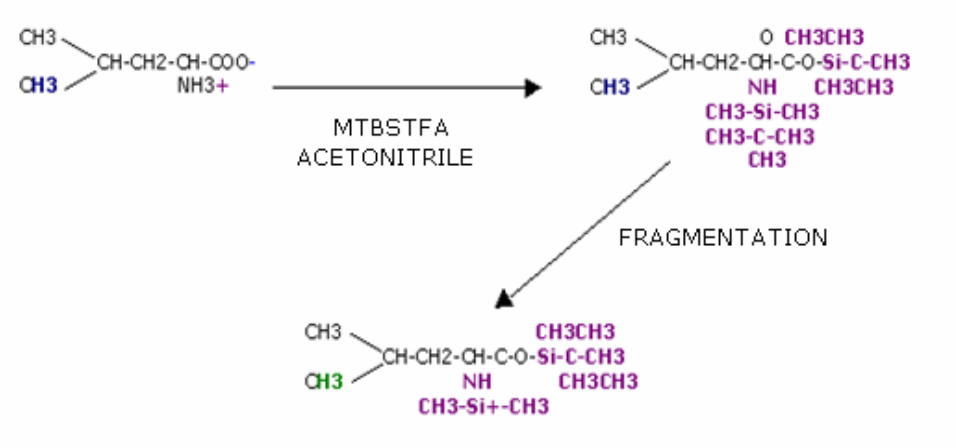
Figure 18. Amino acid $^{2}H_3$-L-leucine derivatisation and its t-MTBSTFA derivative [374-376].



1.2.8 Derivatised amino acid analysis in a GC-MS

Derivatised 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

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

Figure 19. A GC-MS mass spectra [377].

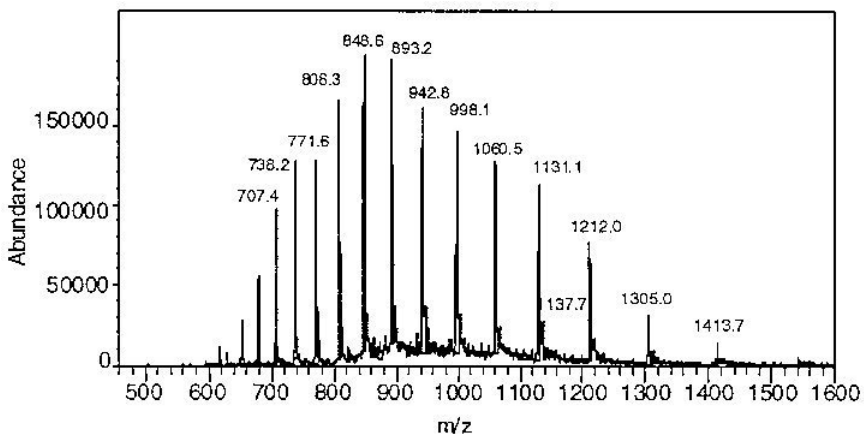
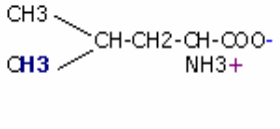


Figure 20. Leucine (A) and $^{2}H_3$-L-leucine (B) structure and m/z ratios [374-376].

(A) **LEUCINE**
m/z 131
C_{12} X 6 =72
H_1 X 13 =13
N_{14} X 14 =14
O_{16} X 2 =32



D_3-LEUCINE
m/z 134
C_{12} X 6 =72
H^2_1 X 3 =6
H_1 X 10 =10
N_{14} X 14 =14
O_{16} X 2 =32
(B)

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].

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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 (300ºC). The column oven, the external part of the equipment, can reach high temperatures of between 40ºC to 320ºC. 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].

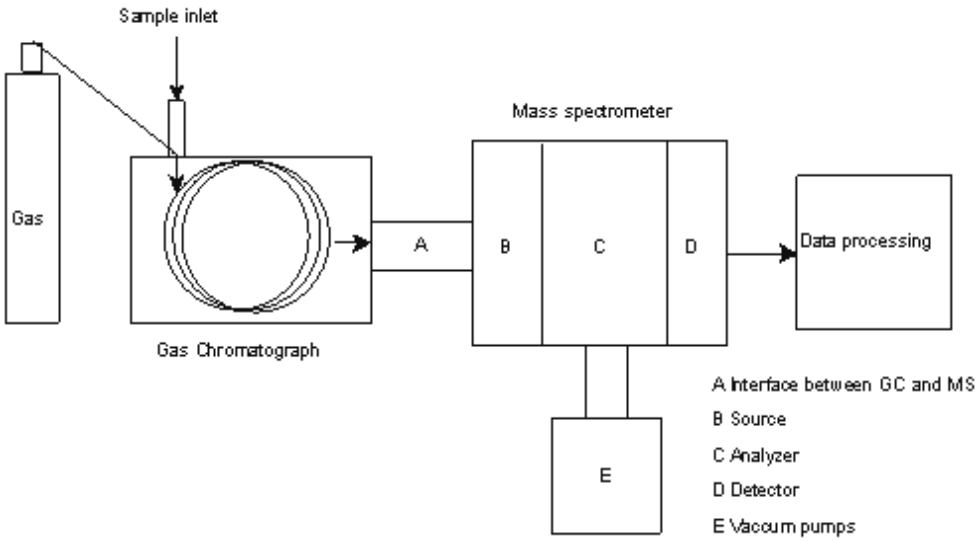
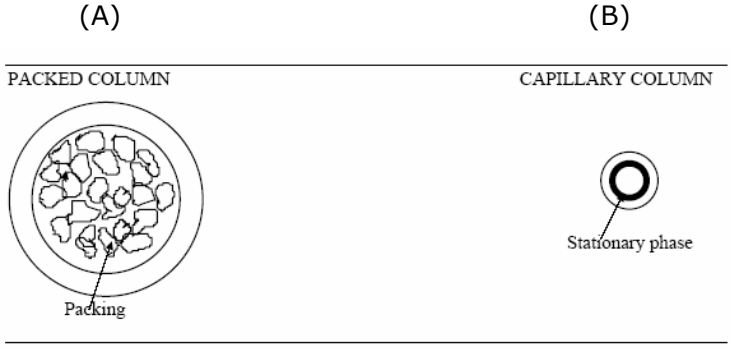


Figure 22. Columns used in GC: packed (A) and capillary (B) columns [377, 378].



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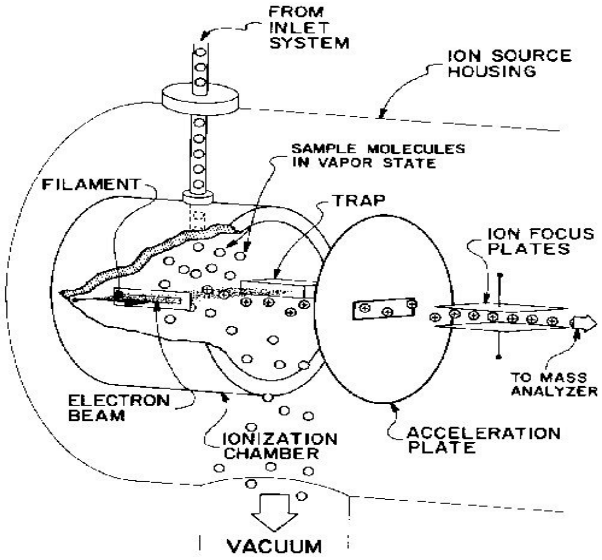
When analysing samples in a GC-MS, the minimum possible sample quantity injected has to be taken into account [377, 378]. The quantity of sample introduced into the GC-MS depends on the type of injection, splitless or split mode. It is preferable to use split mode and to choose the amount of sample reaching the detector. Split mode solves one of the inconveniencies 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 275ºC.
* inert gas helium pressure of 110 kPa.
* capillary column at a temperature of 150ºC and a temperature ramp for amino acid analyses of 110ºC for 1 minute and 20ºC/minute until 280ºC 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.

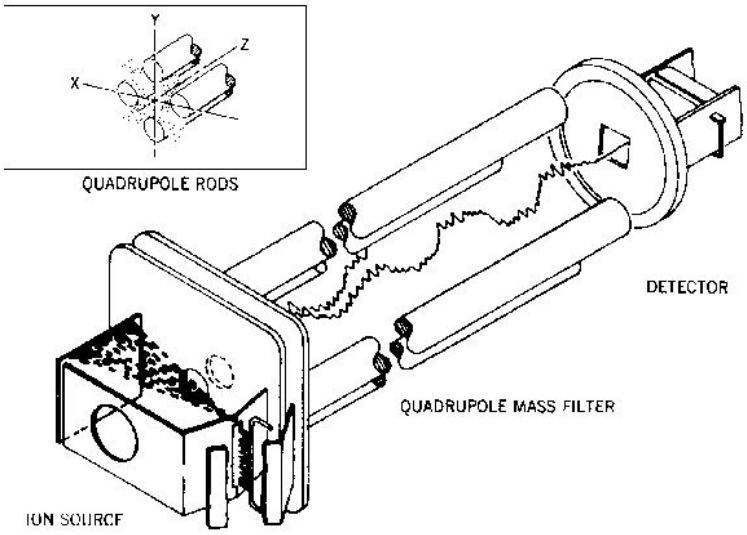
Figure 23. GC-MS injector and ion source [377, 378].



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-on-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.

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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 → 275ºC
  - inert gas (helium) pressure → 110 kPa
  - capillary column → 30 m x 0.25 mm x 0.25 µm DB5-MS (Agilent Technologies)
  - column temperature → 150ºC
  - temperature ramp used for amino acid analysis
    - 110ºC, 1 minute
    - 20ºC/minute until reaching 280ºC
  - 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 $^{2}H_3$-L-leucine specific enrichment was determined in free plasma amino acid samples and hydrolysed amino acids from apo B-100 $VLDL_1$, $VLDL_2$, 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 $^{2}H_3$-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 $^{2}H_3$-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

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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 $^{2}H_3$-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 $^{2}H_3$-L-leucine solution
  - mix Mock plasma solution volumes with different volumes of $^{2}H_3$-L-leucine solution to obtain calibration curve samples with different isotopic enrichments, or atomic percent excess (APE)

Samples	Volume Mock Plasma (ml)	Volume $^{2}H_3$-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 centrige and store at 4ºC

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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
Cycloleucine	20	129.20	0.0129

NOTE: use an analytical balance for weighing

Figure 25. $^{2}H_3$-L-leucine derivatisation to form t-MTBSTFA derivative: *m/z* ratios [374-376].

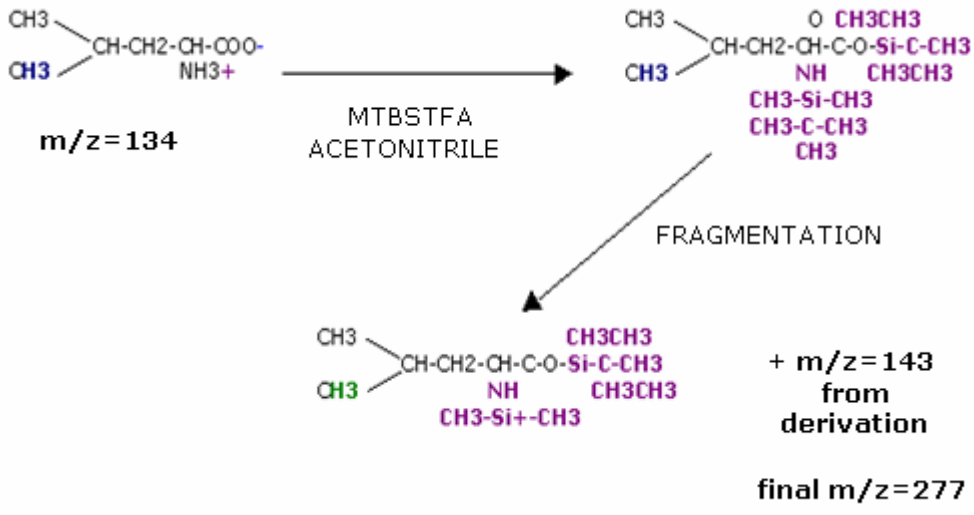

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Table 30.

Derivatisation of calibration curve samples [304, 374-376]

• follow method described in Table 26

Samples	Volume MTBSTFA (µl)	Volume Acetonitrile (µl)
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 (µl)	Volume Acetonitrile (µl)
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

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Table 31.

Derivatisation of amino acid samples [304, 374-376]

• follow method described in Table 26

Samples	Volume MTBSTFA (µl)	Volume Acetonitrile (µl)	Injection Volume in GC-MS (µl)
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 $^{2}H_3$-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:

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

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 $^2H_3$-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 $^2H_3$-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 dectected 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 $^2H_3$-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

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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 (Qi, 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 kij 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.

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

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-compatmental 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.

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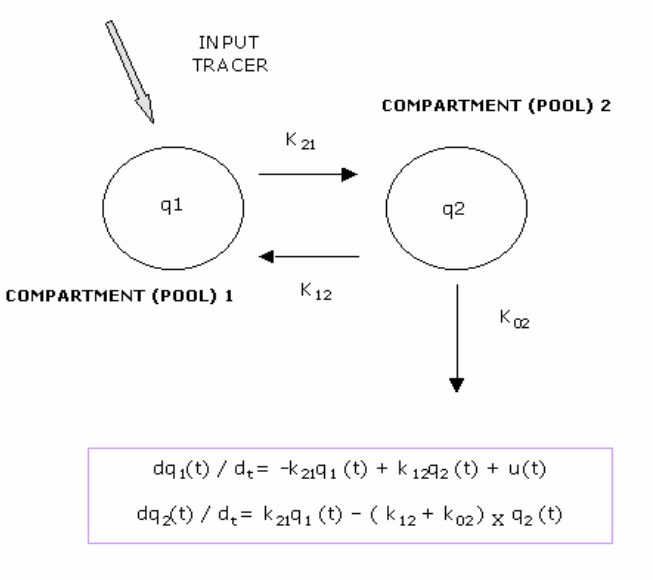
Figure 26. Diagram of a simple multi-compartmental model with two compartments, isotope injection site, and multi-exponential functions describing the kinetics [313, 334, 386].



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:

apo pool (mg)=apo concentration (mg/dl) x plasma volume (l) x 1 l /10 dl
plasma volume (l)=4.50% of body weight (kg)

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

leucine (mg)=apo pool (mg) x % leucine
% leucine in apo B-100=12.12
% leucine in apoA-I=15.68
% leucine in apoA-II=10.70

- 10% leucine (mg)=leucine (mg) x 0.90
- 10%=90%

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+ 10% leucine (mg)=leucine (mg) x 1.10

+ 10%=110%

* mg of $^{2}H_3$-L-leucine bolus injection:

$^{2}H_3$-L-leucine bolus (mg)=body weight (kg) x bolus $^{2}H_3$-L-leucine concentration

bolus $^{2}H_3$-L-leucine concentration (mg/kg)=0.7

* mg of $^{2}H_3$-L-leucine constant infusion:

$^{2}H_3$-L-leucine constant infusion (mg)=

[body weight (kg) x $^{2}H_3$-L-leucine constant infusion (mg/kg) x 16h] / real-time infusion (h)

constant infusion $^{2}H_3$-L-leucine concentration (mg/kg)=0.7

* time of constant infusion:

time (h)=16 (specifying exact hours and minutes)

* time of last collected blood sample during kinetic study:

time (h)=48 (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 $VLDL_1$, $VLDL_2$, IDL and LDL lipoprotein fractions [330, 400-405].

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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, $VLDL_1$, $VLDL_2$, 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 $VLDL_1$, $VLDL_2$, 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:

apo B-100 concentration (mg/dl)=
total protein pool 1 (mg/dl) – [2 x isopropanol protein mean pools 2, 3 (mg/dl)]

apo B-100 pool size (mg)=
apo B-100 concentration (mg/dl) x plasma volume (l) x 1 l/10 dl
plasma volume (l)=4.5% of body weight (kg)

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):

apo B-100 pool size (mg)=
apo B-100 concentration (mg/dl) x plasma volume (l) x 1 l/10 dl
apo B-100 $VLDL_1$, $VLDL_2$, IDL and LDL concentration (mg/dl) from direct determination (pool 1)

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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 $VLDL_1$, $VLDL_2$, IDL and LDL. After apo B-100 isopropanol precipitation, proteins present in the isopropanol supernatant were measured using the method of Lowry [400-405] (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.

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Table 32.
Lowry method for the determination of proteins present in isopropanol extract (Annexe XVI) [400-405]

- add 2 ml of alkaline solution (Na_2CO_3, NaOH, NaK Tartrate, $CuSO_4$ 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 H_2O
- 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 $VLDL_1$, $VLDL_2$, 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.

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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 disolve at 4ºC
- add 2 ml of alkaline solution (Na_2CO_3, NaOH, NaK Tartrate, $CuSO_4$ 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 H_2O 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 H_2O
- 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)

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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). $VLDL_1$, $VLDL_2$, 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

immunoturbidimetric 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 (Na_2CO_3, NaOH, NaK Tartrate, $CuSO_4$ 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 (Na_2CO_3, NaOH, NaK Tartrate, $CuSO_4$ 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 H_2O
- 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 stadard 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 mg/dl ≥LDLC≥ 189 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/m^2.

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).

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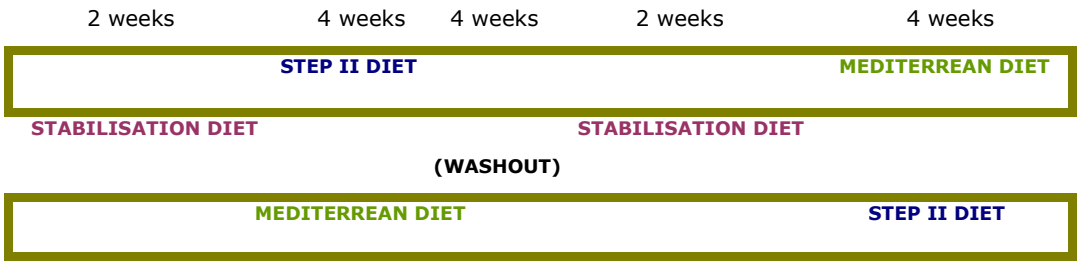
In this type of clinical trial, each volunteer acts as his own control.

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).

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

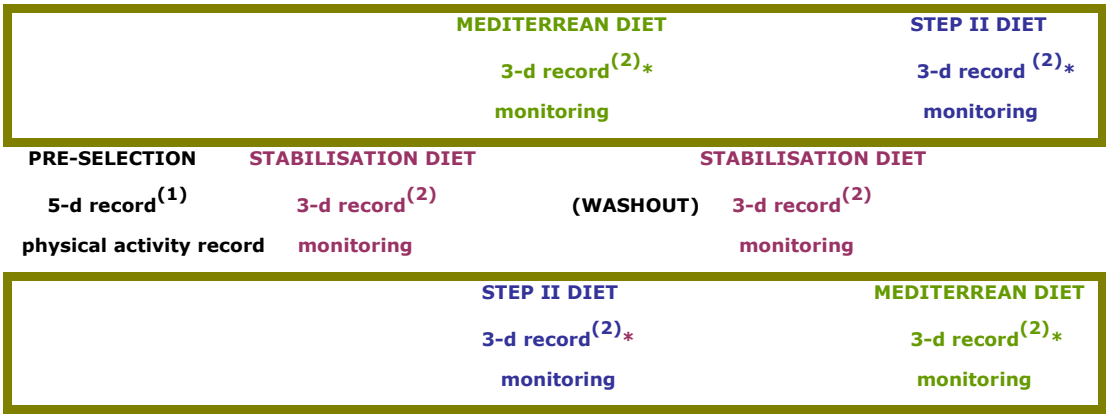
Food records provide a picture of a subject's typical eating patterns. These records register intake of food and beverages over a 3-day period. To provide accurate intake information, foods are to be weighed or volume measured and the methods of food preparation (baked, sautéed, steamed) described in 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 x weight in kg) + (5 x height in cm) - (6.8 x age in years)

The nutrient composition of the diet was calculated using the *DATABASE: Répertoire Géneral 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.

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2.2.2 Kinetic study protocol

2.2.2.1 Preparing the $^{2}H_3$-L-leucine solution

A stock solution of pyrogen-free deuterated leucine, $^{2}H_3$-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 lisate of amebocytes of *Limulus polyphemus* crab) to test for pyrogen content to ensure that the final product is pyrogen-free [420-423].

$^{2}H_3$-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 $^{2}H_3$-L-leucine/kg body weight and the constant infusion over a 16h period was at the rate of 0.7 mg $^{2}H_3$-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 $^{2}H_3$-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 $^{2}H_3$-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.

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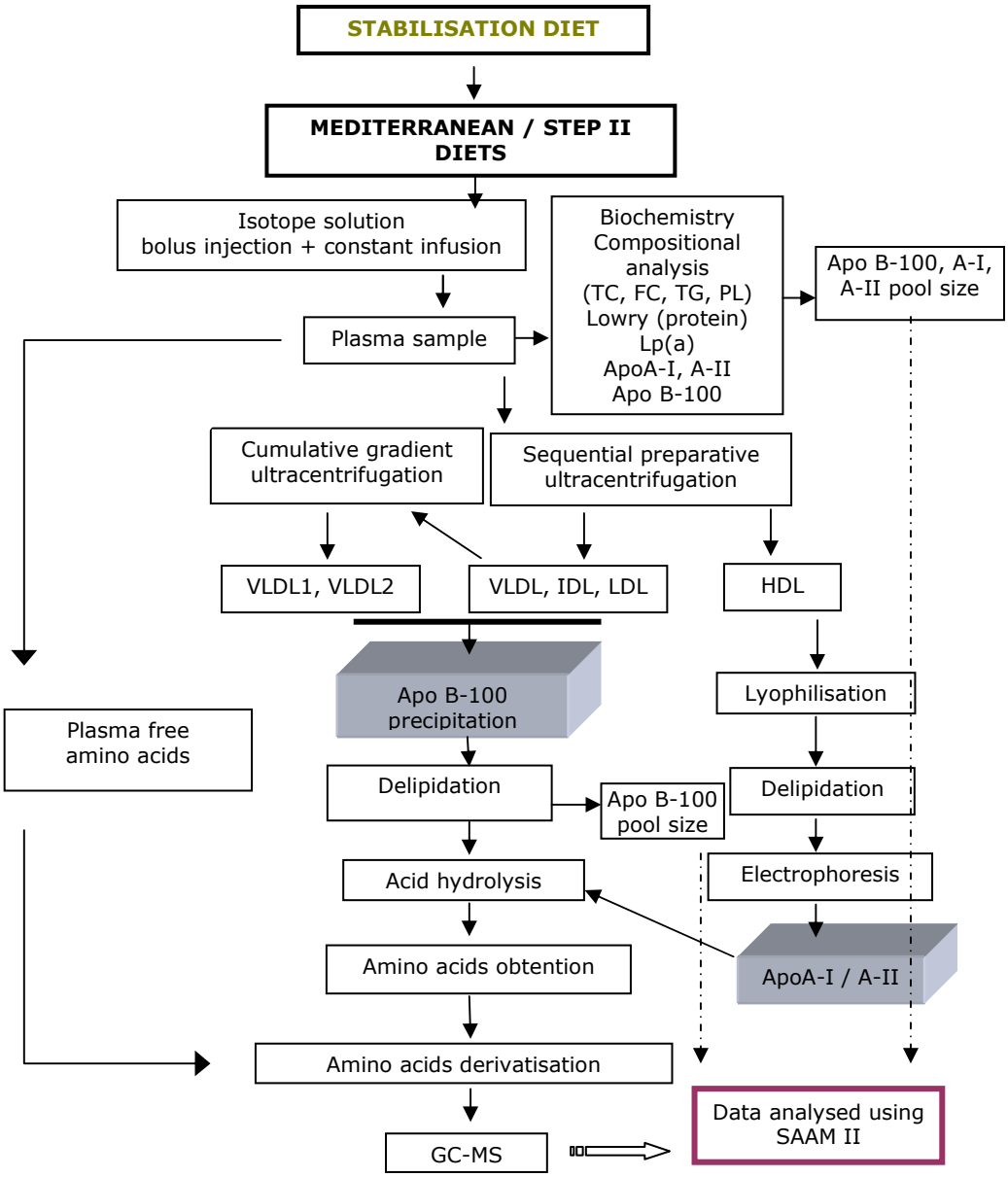
After 16h of $^{2}H_3$-L-leucine infusion, the volunteers went home and returned to the hospital in the morning (at 8:00 a.m.) in a fasting state for blood samples collection at 24 and 48h post infusion.

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.

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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, 4ºC) 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 comercial 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 phophate 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 comercial assay kits (Beckman Coulter Inc). A Quality Control was used throughout (Apo Synchron LX®, Beckman Coulter Inc).

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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 comercial assay kit (Beckman Coulter Inc). A Quality Control was used throughout (Synchron®Systems CAL5 PLUS, Beckman Coulter Inc).

  • interleukin 6 (IL-6), solubles 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 –70ºC 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 –70ºC 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=0h) (Figure 30) [264]:

* biochemical determinations (see page 107). Blood samples were taken into EDTA tubes, centrifuged at 2,500 rpm, 10 minutes, 4ºC (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):

* $VLDL_1$, $VLDL_2$, 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, 4ºC. From all plasma samples obtained during the 48h kinetic study, sample pools or plasma mixes were prepared in triplicate from

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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, $VLDL_1$, $VLDL_2$, 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, $VLDL_1$, $VLDL_2$, 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 $^2H_3$-L-leucine infusion, up until the end of kinetic studies at 48h post injection:

- samples were centrifuged at 2,500 rpm, 10 minutes, 4ºC 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 –70ºC 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].

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

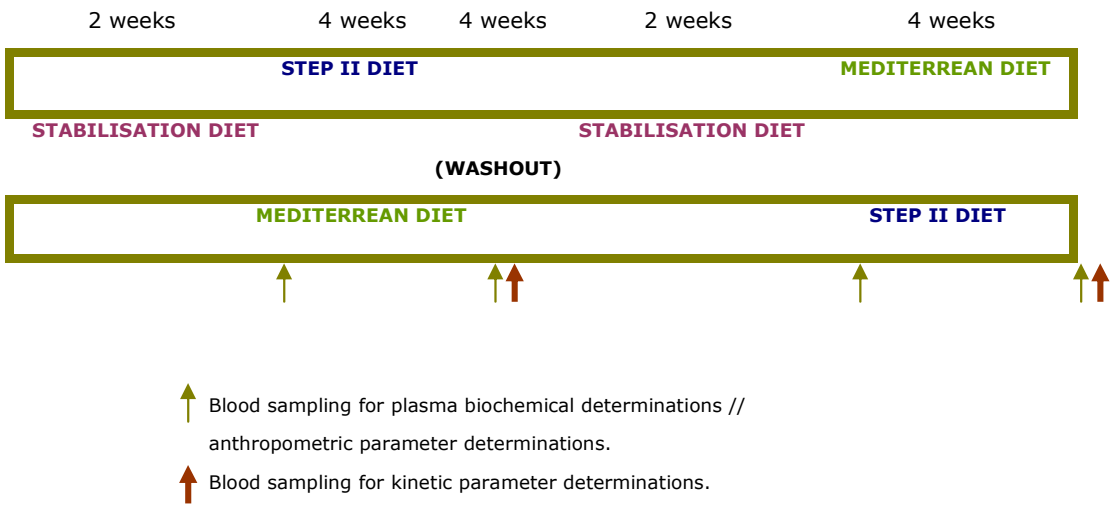

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Table 36. Blood sampling and determinations during kinetic studies [264].

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

POOLS 1, 2, 3 or PLASMA MIXES → 12.5 ml (0.5 ml from each plasma sample)	
ULTRACENTRIFUGATION	**ANALYSIS VLDL1, VLDL2, IDL, LDL, HDL**
2 ml	BIOCHEMISTRY

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 ± 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].

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RESULTS

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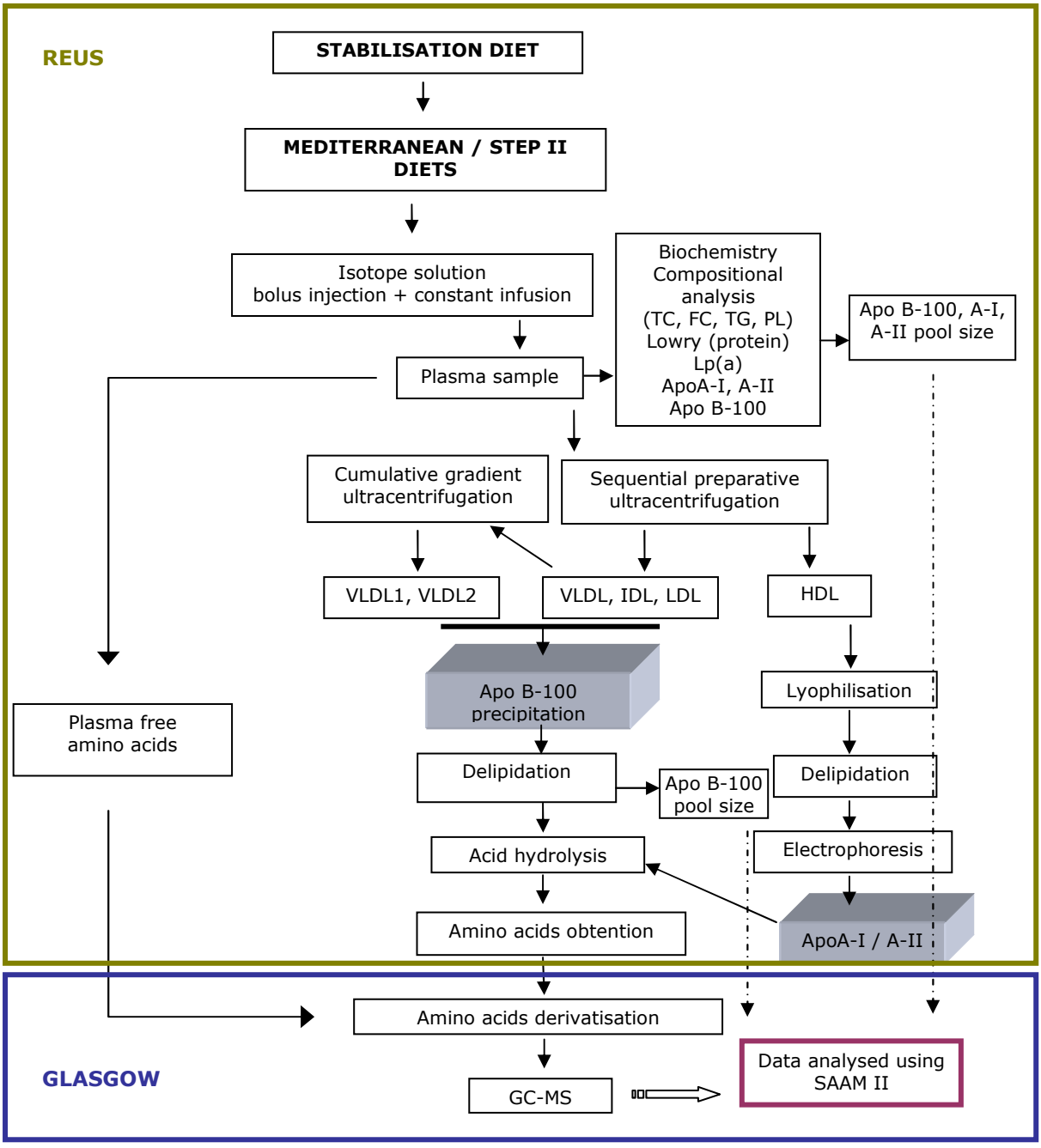
1. Results of the general objective: implementation of the methodology of lipoprotein kinetic studies using stable isotopes

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 calculated [264].

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 $VLDL_1$ or compartment *q5*, $VLDL_2$ 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 x k (1,2)
* k (13,14)=2.5 x 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 $VLDL_1$ to $VLDL_2$, transport rate from compartment *6* to *8* was divided by $VLDL_1$ apo B-100 mass (combined masses of compartments *5*, *6* and *7*):

$$FTR_{VLDL1,VLDL2}=k\,(8,6) / (q5+q6+q7)$$

- for $VLDL_2$ to IDL, transport rates from compartment *9* to *11* and *9* to *12* were combined and divided by $VLDL_2$ apo B-100 mass (combined masses of compartments *8*, *9* and *10*):

$$FTR_{VLDL2,\ IDL}=[k\,(11,9) + k\,(12,9)\,] / (q8+q9+q10)$$

- 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*):

$$FTR_{IDL,\ LDL}=k\,(13,11) / (q11+q12)$$

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

- for $VLDL_1$, by dividing the sum of outputs from compartments *6* and *7* by $VLDL_1$ apo B-100 mass:

$$dirFCR_{VLDL1}=[k\,(0,6) + k\,(0,7)] / (q5+q6+q7)$$

- for $VLDL_2$, by dividing the sum of outputs from compartments *9* and *10* by $VLDL_2$ apo B-100 mass:

$$dirFCR_{VLDL2}=[k\,(0,9) + k\,(0,10)] / (q8+q9+q10)$$

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

$$dirFCR_{IDL}=[k\,(0,11) + k\,(0,12)] / (q11+q12)$$

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

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$$dirFCR_{LDL} = k(0,13) / q13$$

* overall FCR (pools/day) were calculated:

  - for $VLDL_1$, $VLDL_2$ and IDL apo B-100 was the sum of FTR and dirFCR for each lipoprotein fraction:

$$FCR = FTR + dirFCR$$

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

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

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

$$RT = 1 / FCR$$

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

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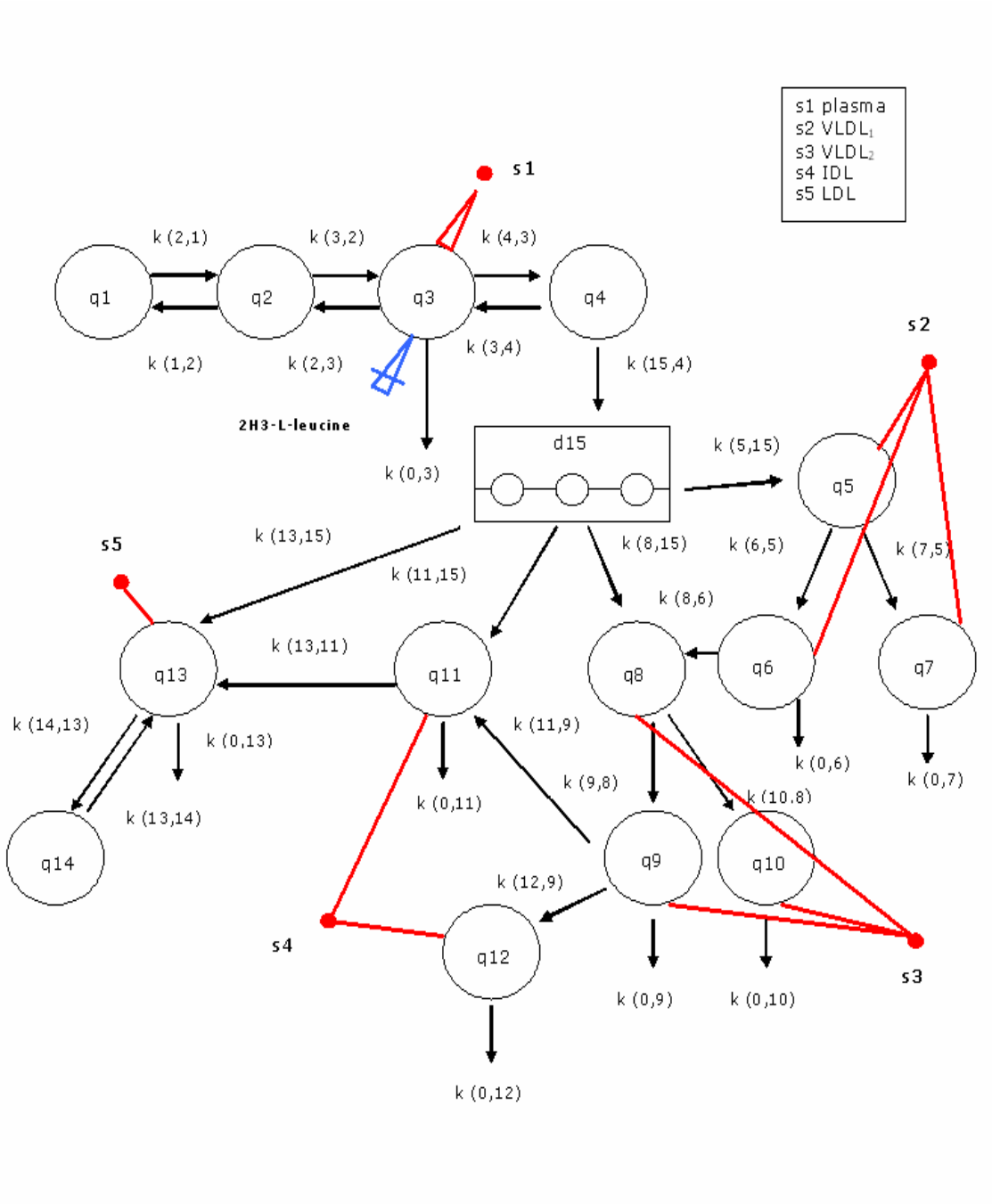
Figure 32. Multi-compartmental model for apo B-100-rich lipoproteins metabolism.



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.

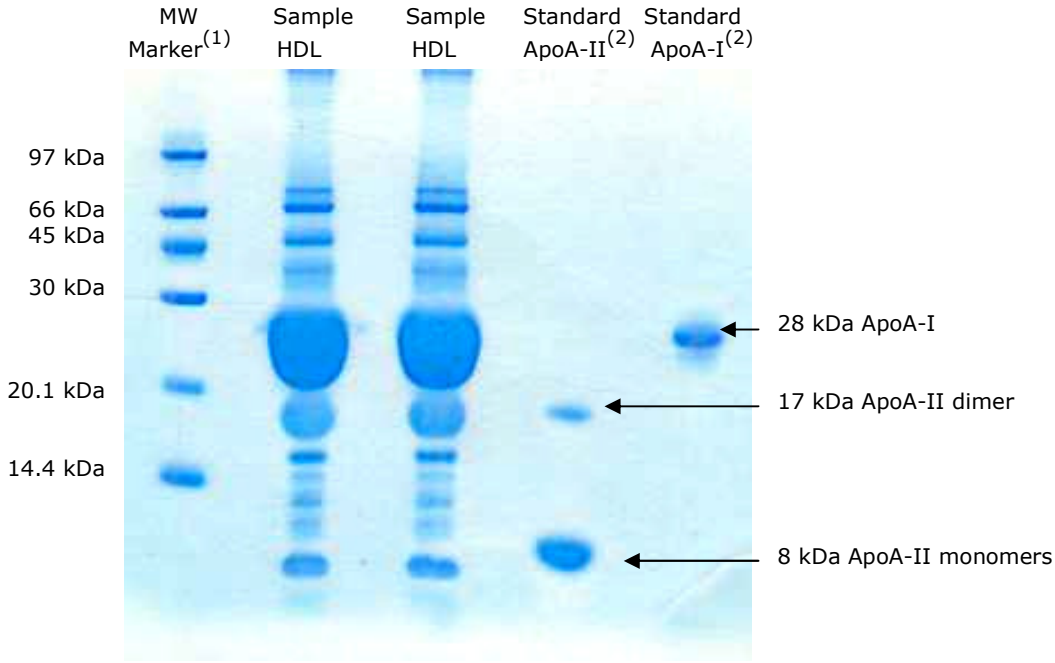
The results for apoA-I and A-II PAGE isolation, as performed in our Unit using commercial NuPAGE®Novex gels (Invitrogen, Corp), are presented in Figure 33. This variantion on the general PAGE technique made obtaining HDL apos easier and more reproducible than previous, handmade, gels.

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.

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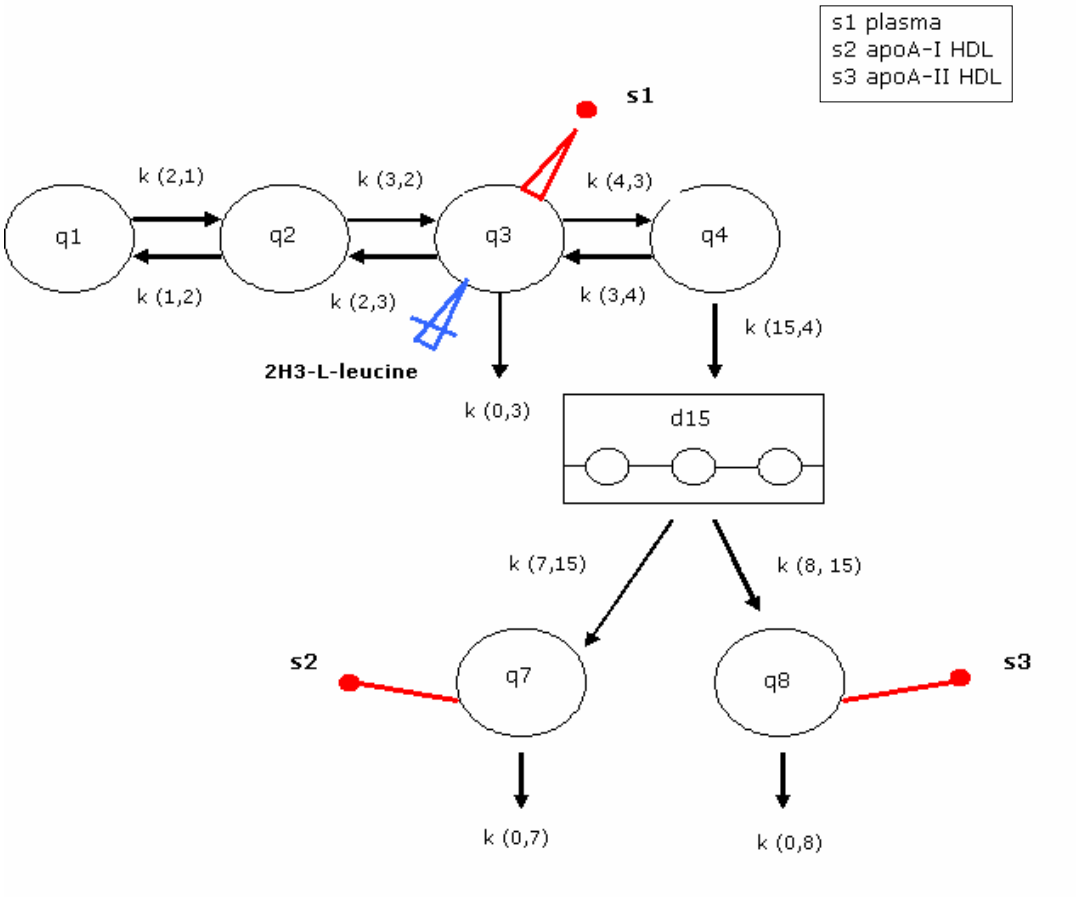
Figure 34. Multi-compartmental model for apoA-I and A-II HDL metabolism.



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 ± 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 ± 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).

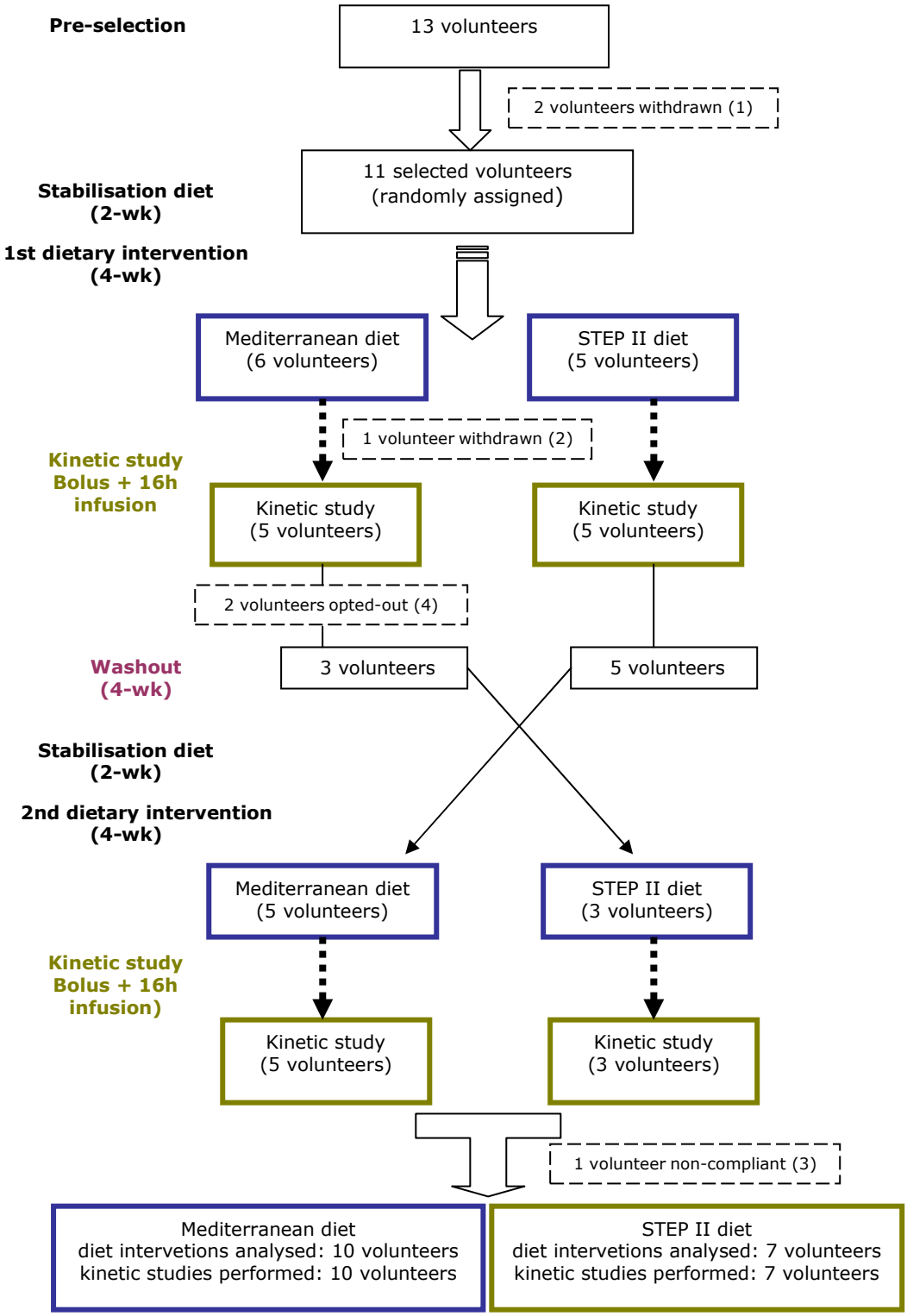
We had pre-selected a total of 13 volunteers. Two volunteers were withdrawn because they did not fulfil all of the inclusion criteria. In the first study period, 6 volunteers were randomly assigned to the Mediterranean diet, and 5 to the STEP II diet. The kinetic studies were performed after each dietary 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 moleucule 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.

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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.

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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.

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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 Mediterrenean 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/m^2 (*P*=0.04).

Comparing the Mediterranean versus the STEP II diet, the Mediterranean diet increased, albeit not significantly, body weight by +0.74 ± 0.07 kg (*P*=0.06) and BMI by +0.34 ± 0.3 kg/m^2 (*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% ± 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% ± 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% ± 16.73, *P*=0.29; -3.13% ± 12.35, *P*=0.37; -5.55% ± 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% ± 6.52, *P*=0.06) and significantly decreased plasma concentrations of apoA-I (-9.29% ± 5.08, *P*=0.01). Further, the STEP II diet significantly decreased the plasma concentrations of total C (-5.59% ± 6.25; *P*=0.05) and decreased, albeit not significantly, the plasma concentrations of LDLC (-4.51% ± 8.89; *P*=0.19). The plasma concentrations of apo B-100 were not significantly altered following the STEP II diet (-0.28% ± 19.01; *P*=0.71).

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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**
	Pre-	**Post-**	**Δ**	**Post- *vs.* Pre- *P***	**Pre-**	**Post-**	**Δ**	**Post- *vs.* Pre- *P***	***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.

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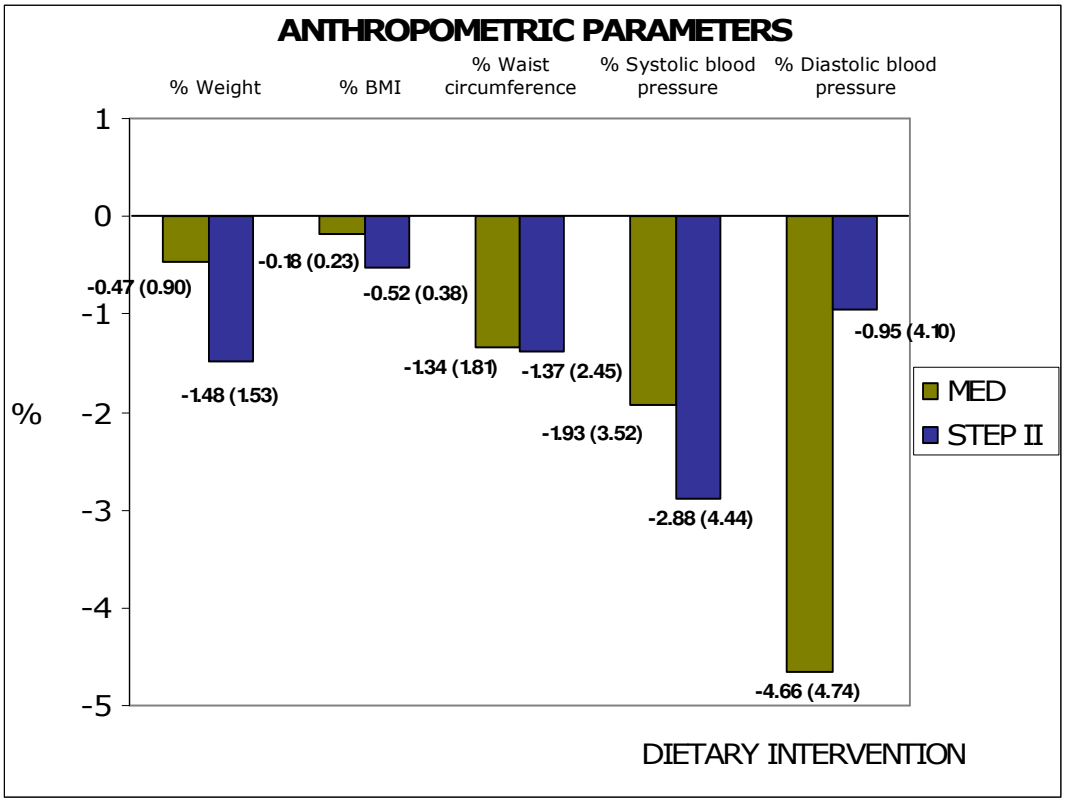
Figure 36. Comparison of anthropometric parameters; percentage change between dietary interventions.



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.

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(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 moleucule 1; hs CRP=high sensitivity C reactive protein; ox LDL=oxidised LDL; ox LDL-Ab=oxidised LDL antibodies.

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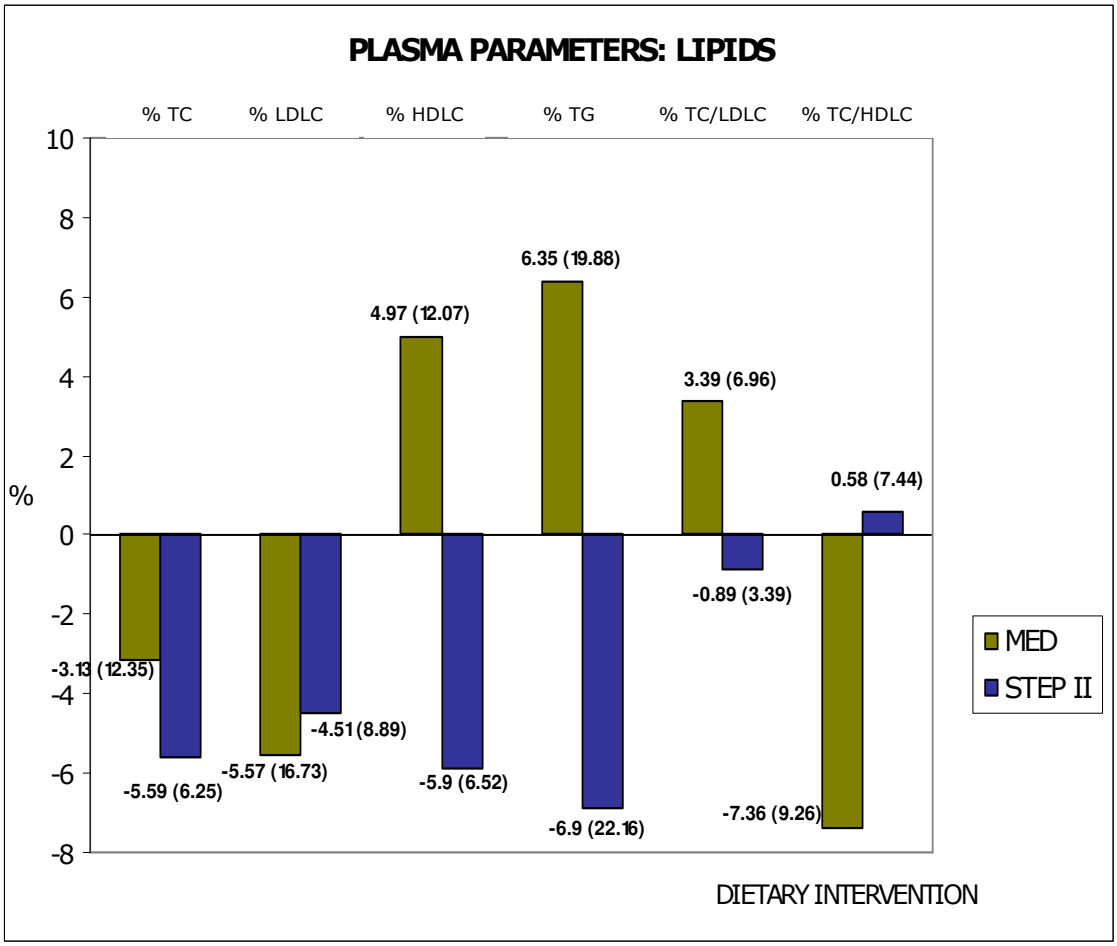
Figure 37. Comparison of plasma lipid parameters; percentage changes between dietary interventions.



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.

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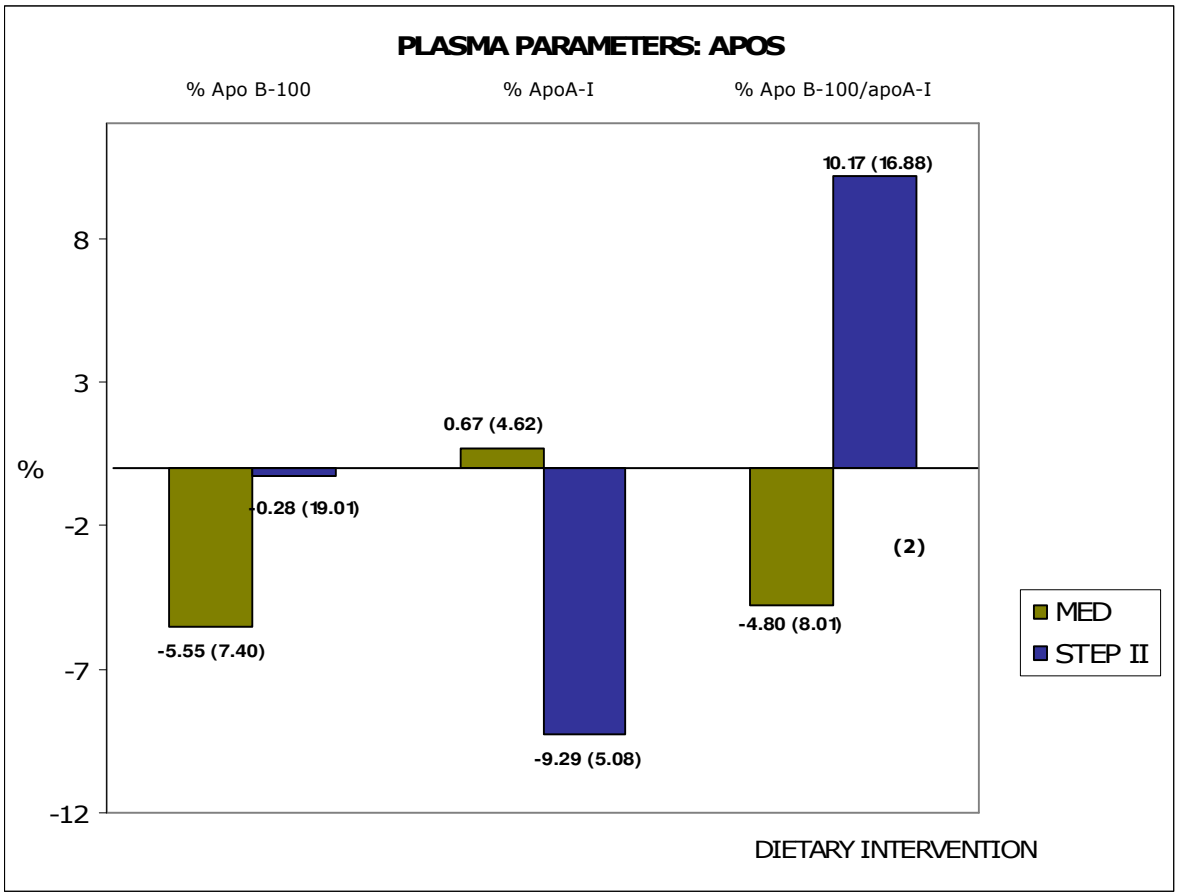
Figure 38. Comparison of plasma apo parameters; percentage changes between dietary interventions.



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.

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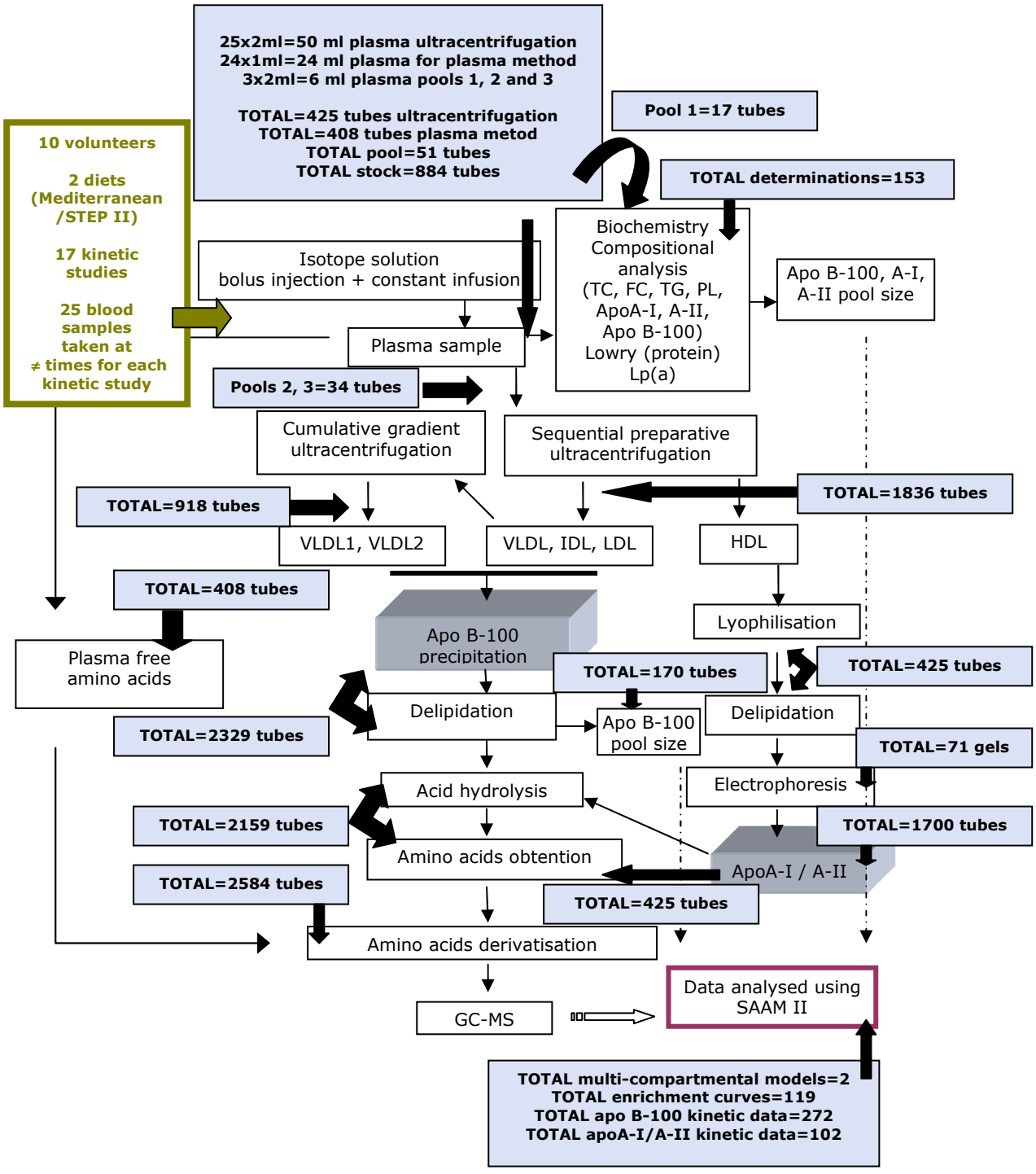
Figure 39. Flow diagram indicating the total number of samples generated in the course of the lipoprotein kinetic studies in all 10 volunteers.



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 Mediterrenean 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/m^2 (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% ± 0.29; P=0.04) and tended to increase the plasma concentrations of HDLC (+12.13% ± 11.31; P=0.08). Further, the Mediterranean diet increased, albeit not significantly, the plasma concentrations of LDLC (+2.66% ± 23.41; P=0.80), total C (+2.20% ± 19.29; P=0.76) and apo B-100 (+6.35% ± 0.41; P=0.67).
  - the STEP II diet *per se* significantly decreased the plasma concentrations of apoA-I (-4.87% ± 0.40; P=0.05) and tended to decreased the plasma concentrations of HDLC (-9.80% ± 4.83; P=0.09). Further, the STEP II diet decreased, albeit not significantly, the plasma concentrations of LDLC (-6.28% ± 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)				**STEP II DIET (N=3)**				**MED vs. STEP II**
	Pre-	**Post-**	**Δ**	***Post- vs. Pre- P***	**Pre-**	**Post-**	**Δ**	***Post- vs. Pre- P***	***P***
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.

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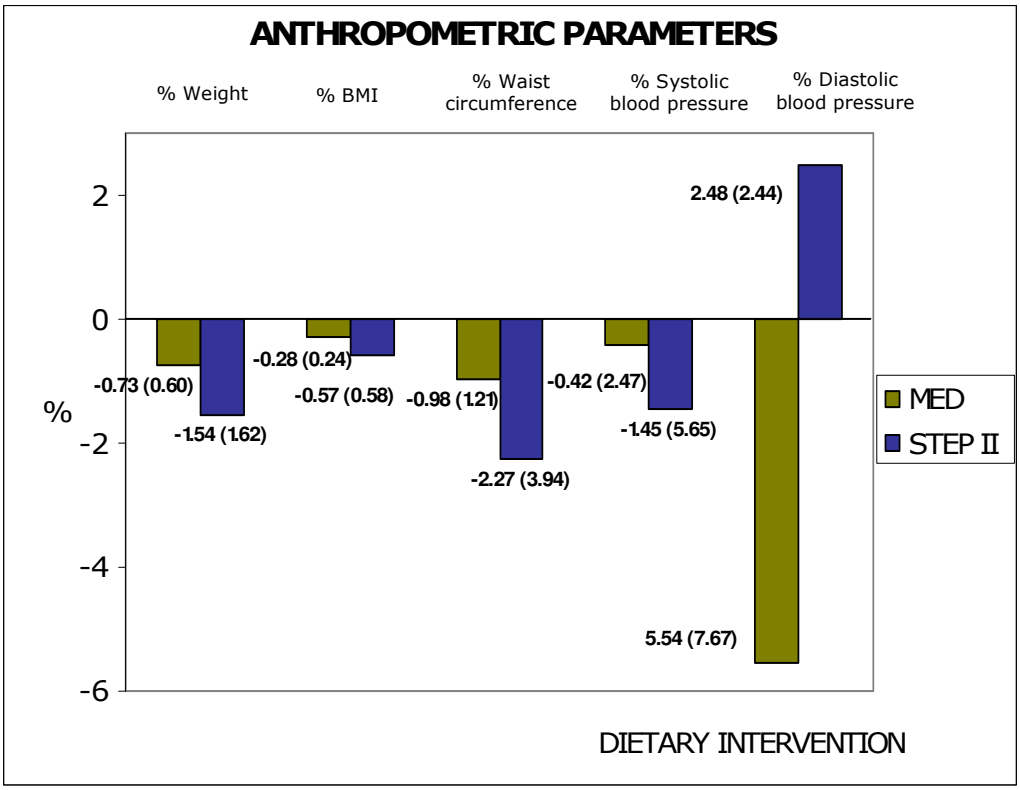
Figure 40. Comparison of anthropometric parameters; percentage changes in the kinetic subset of volunteers between dietary interventions.



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.

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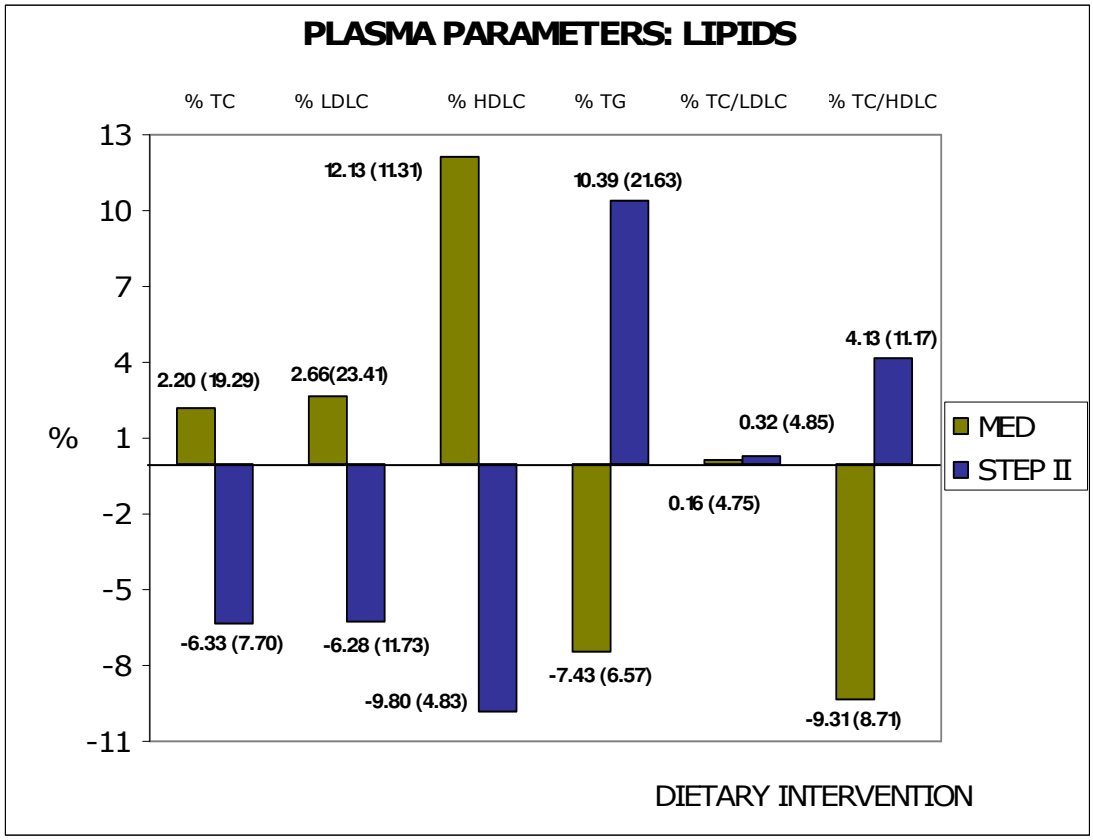
Figure 41. Comparison of plasma lipid parameters; percentage changes in the kinetic subset of volunteers between dietary interventions.



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.

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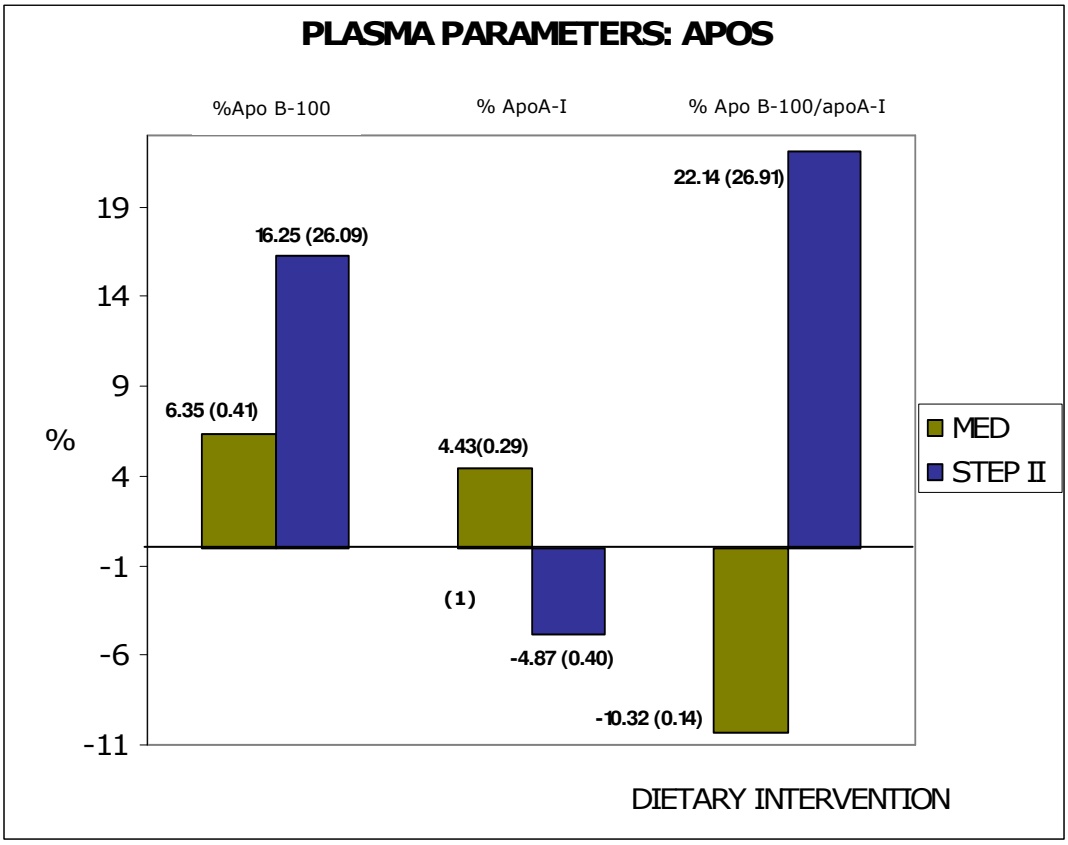
Figure 42. Comparison of plasma apo parameters; percentage changes in the kinetic subset of volunteers between dietary interventions.



MED (Mediterranean diet) N=4 / STEP II diet N=3.
Results expressed as mean (SD).
(1) *P*=0.03.

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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 $VLDL_1$, $VLDL_2$, 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 ≥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 compostition, especially PL content; the content of PL was significantly increased in $VLDL_2$ (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 $VLDL_1$ 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).

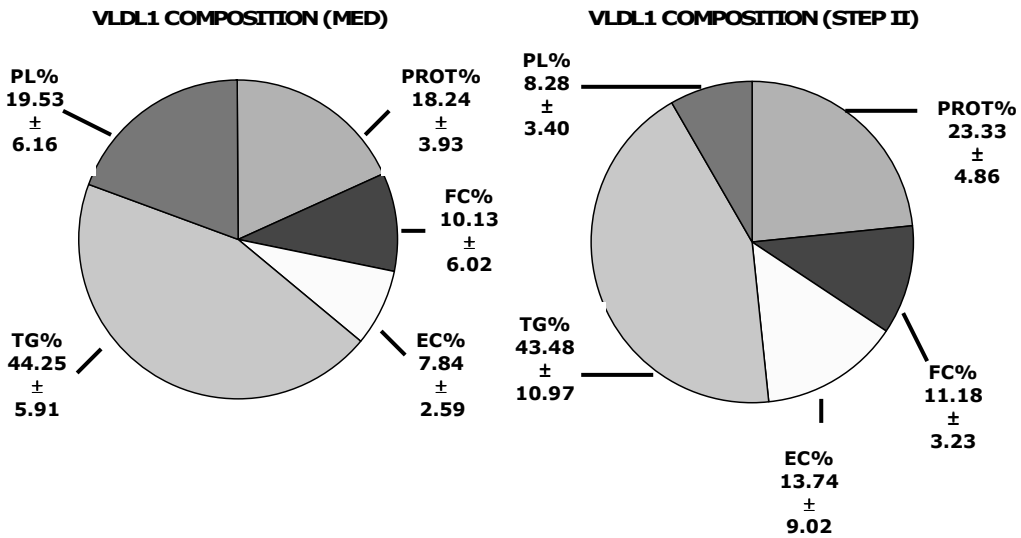
(B)

	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 $VLDL_1$ (A), $VLDL_2$ (B), IDL (C), LDL (D) and HDL (E) in the kinetic subset of volunteers (day of the kinetic study).

(A)



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.

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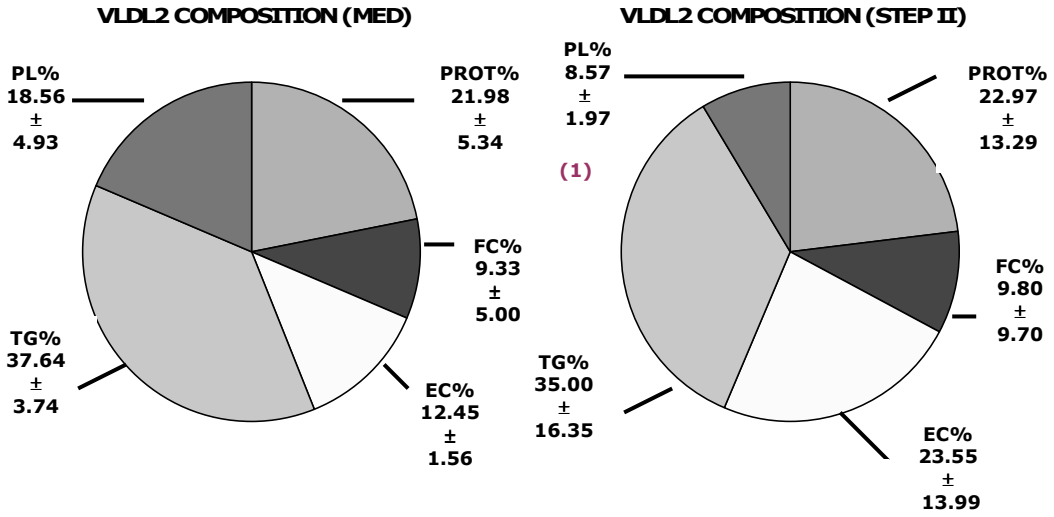
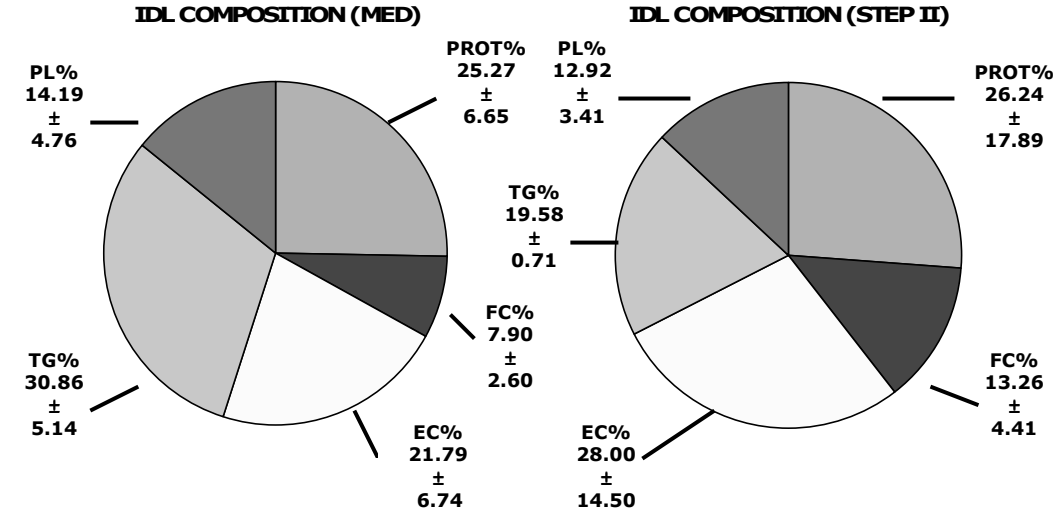
(B)

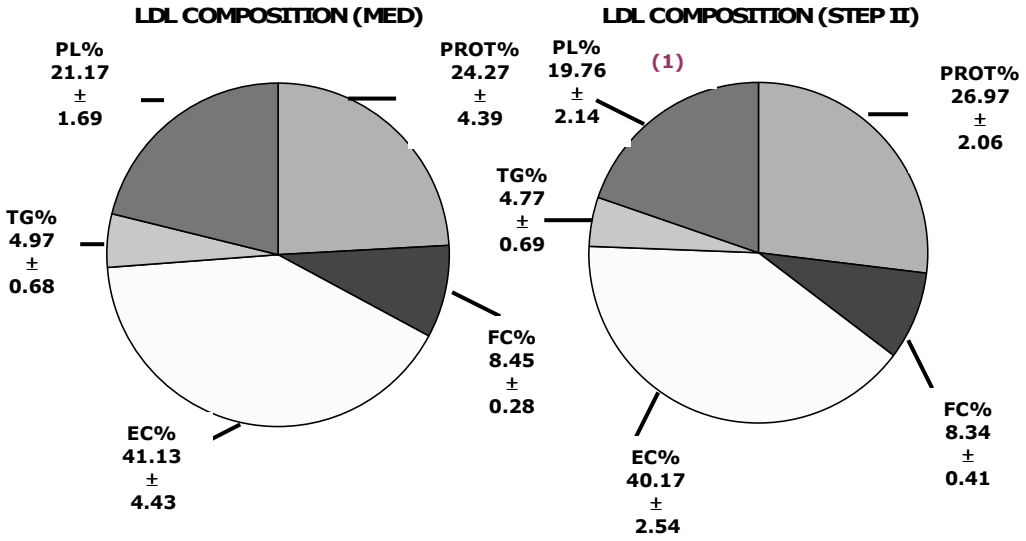


(C)

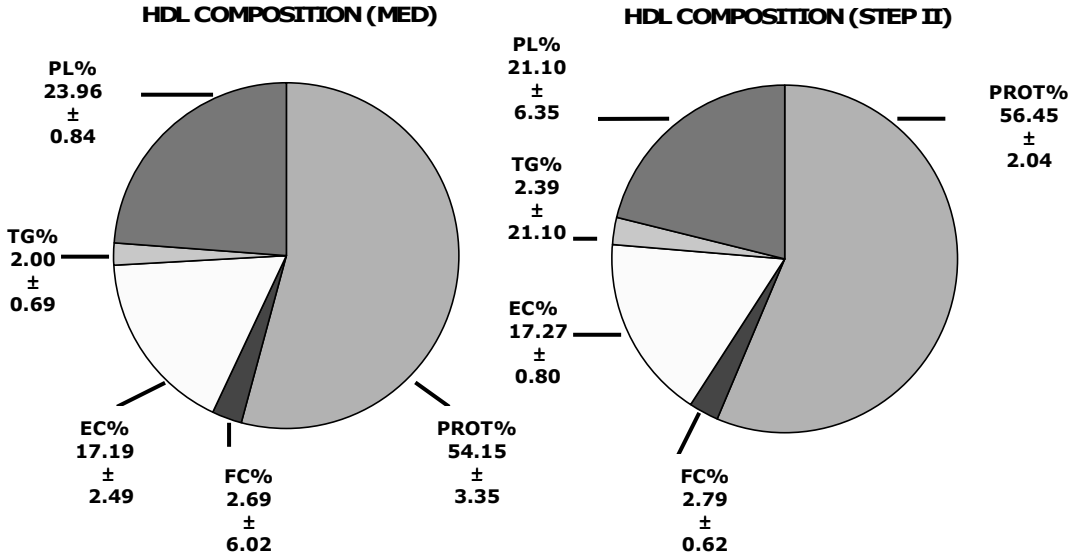


MED (Mediterranean diet) N=4 / STEP II diet N=3.
Results expressed as mean ± standard deviation (SD).
(1) *P*=0.03.
PROT=proteins; FC=free cholesterol; EC=esterified cholesterol;
TG=triglycerides; PL=phospholipids.

(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 $VLDL_1$, $VLDL_2$, 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.

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

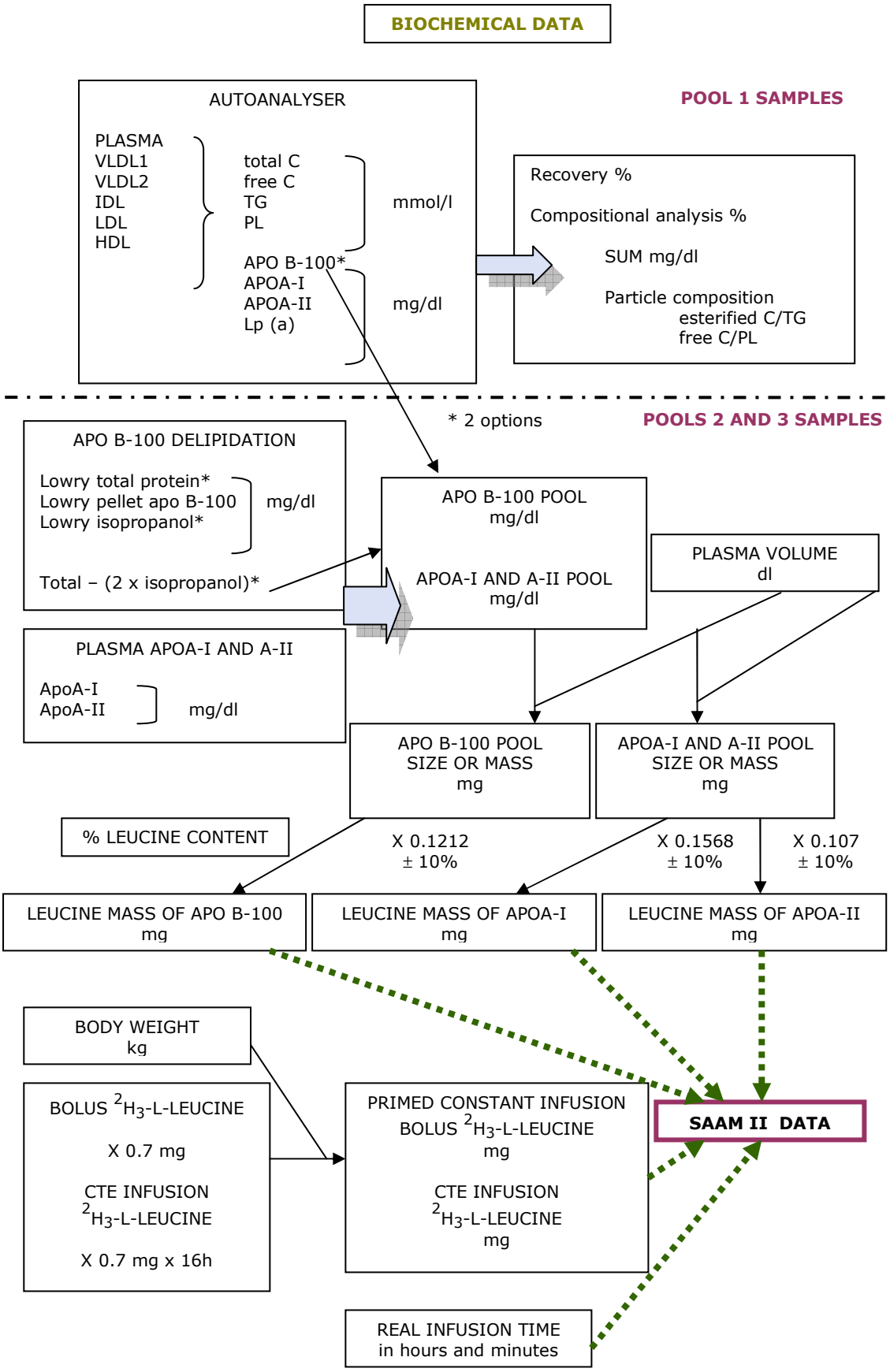

Table 48. Observed and SAAM II calculated apo B-100, and leucine masses for $VLDL_1$, $VLDL_2$, IDL and LDL.

	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.

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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).

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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 $VLDL_1 \rightarrow VLDL_2 \rightarrow IDL \rightarrow LDL$.
    • $VLDL_1$, $VLDL_2$ 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, $VLDL_1$, $VLDL_2$ 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).

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

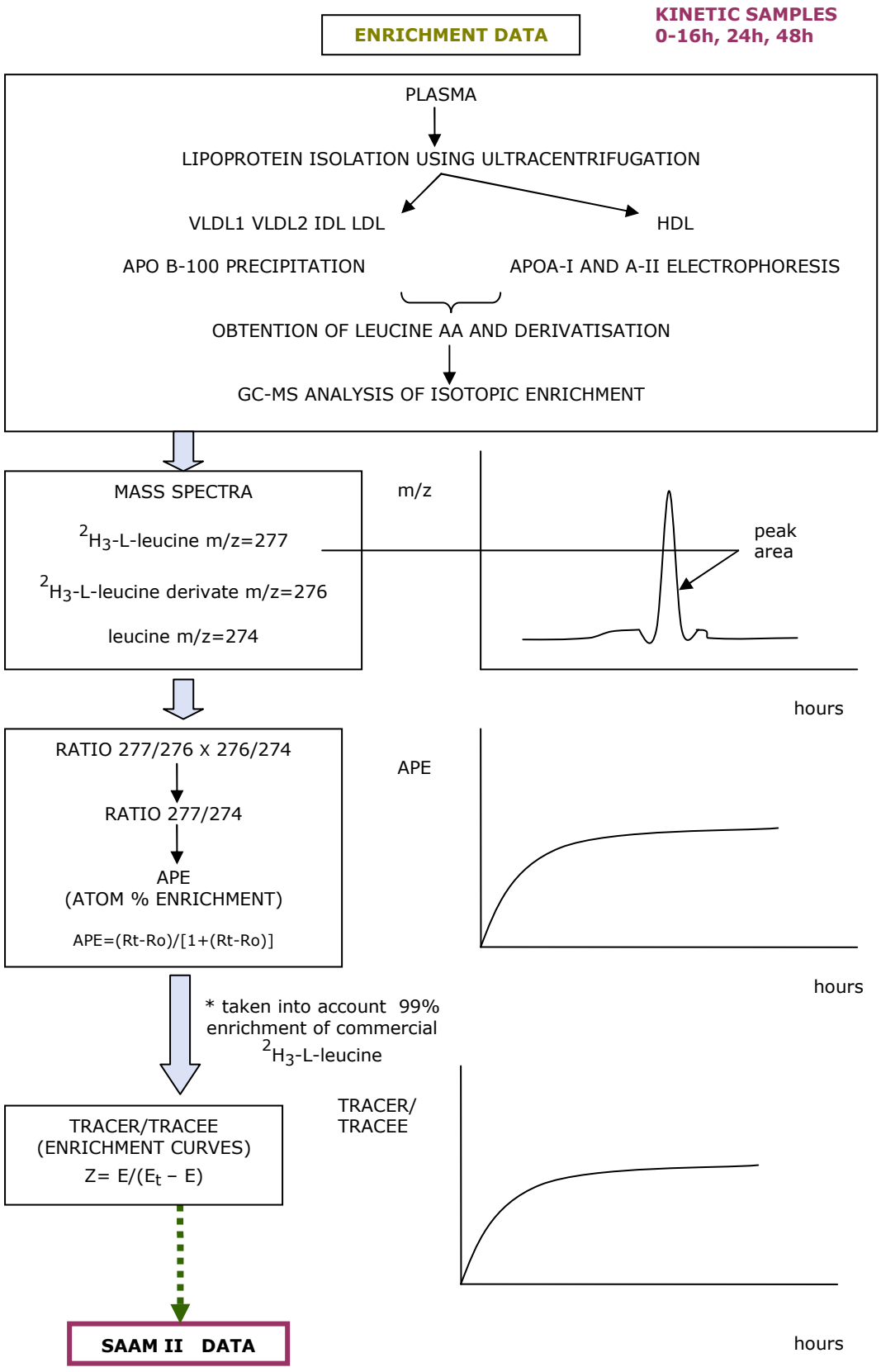

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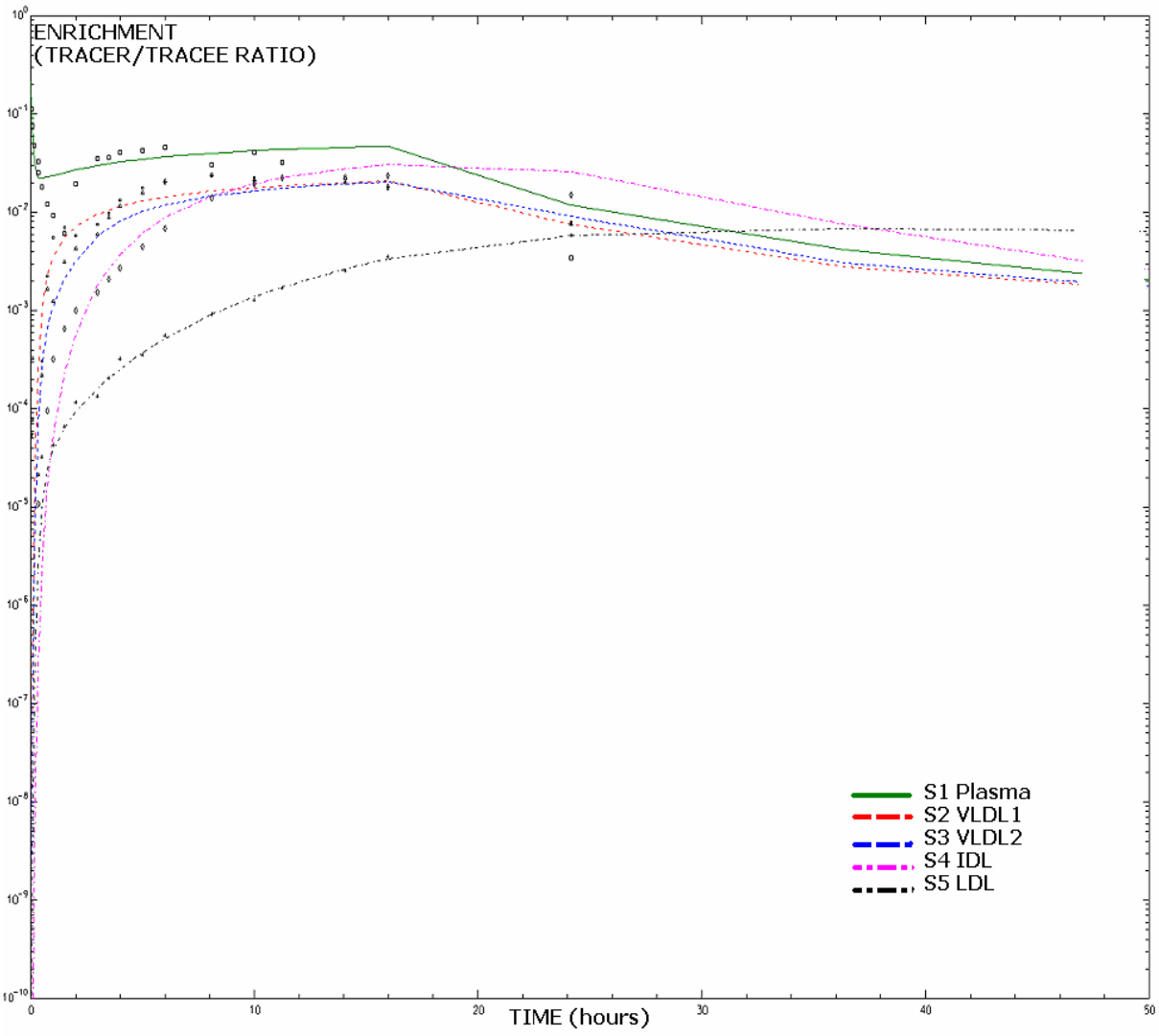
Figure 46. Apo B-100-rich lipoproteins enrichment curves for Mediterranean diet study.



N=1 volunteer.

° (curve points) observed tracer/tracee ratios.

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

s=site of determination.

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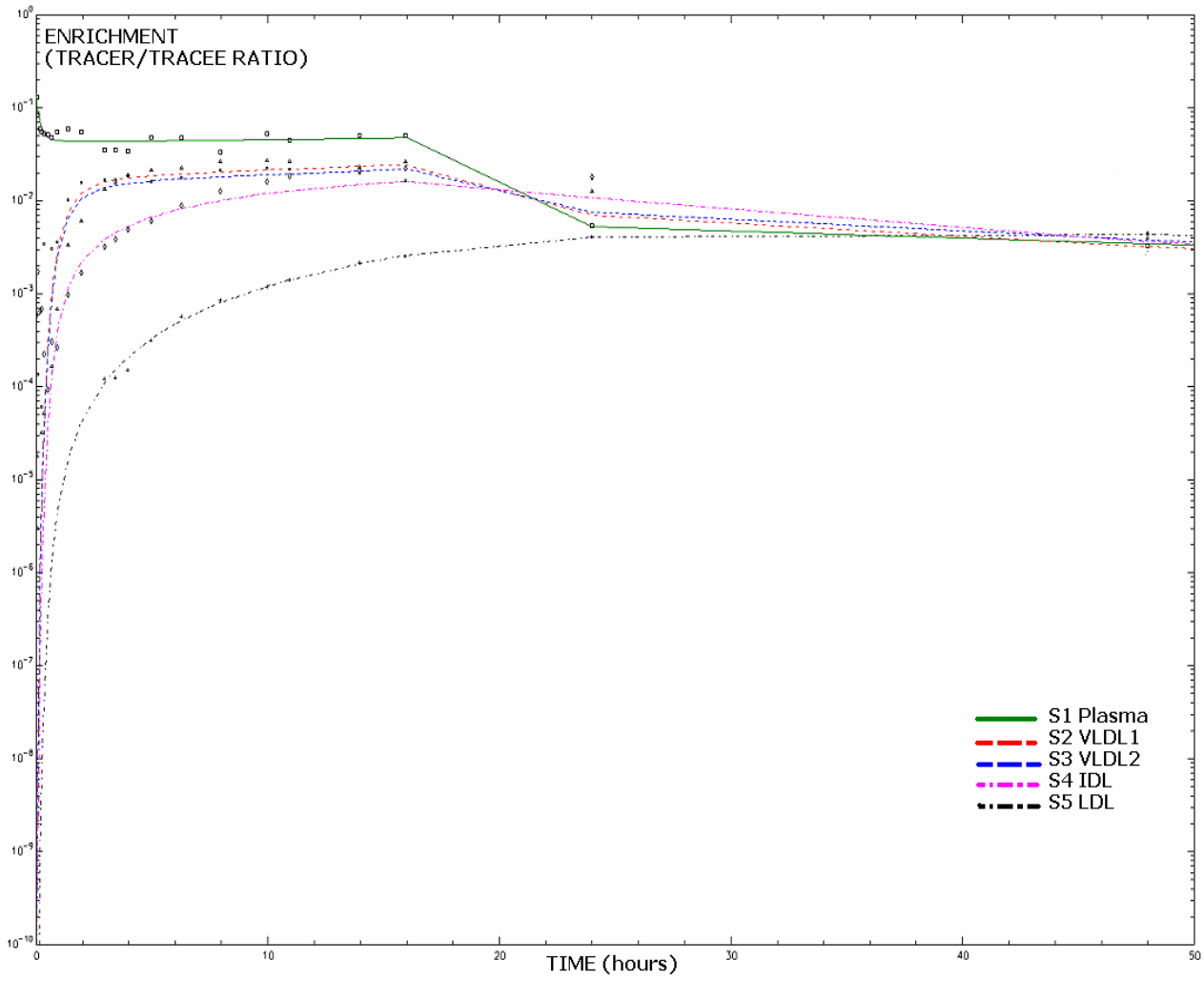
Figure 47. Apo B-100-rich lipoproteins enrichment curves for STEP II diet study.



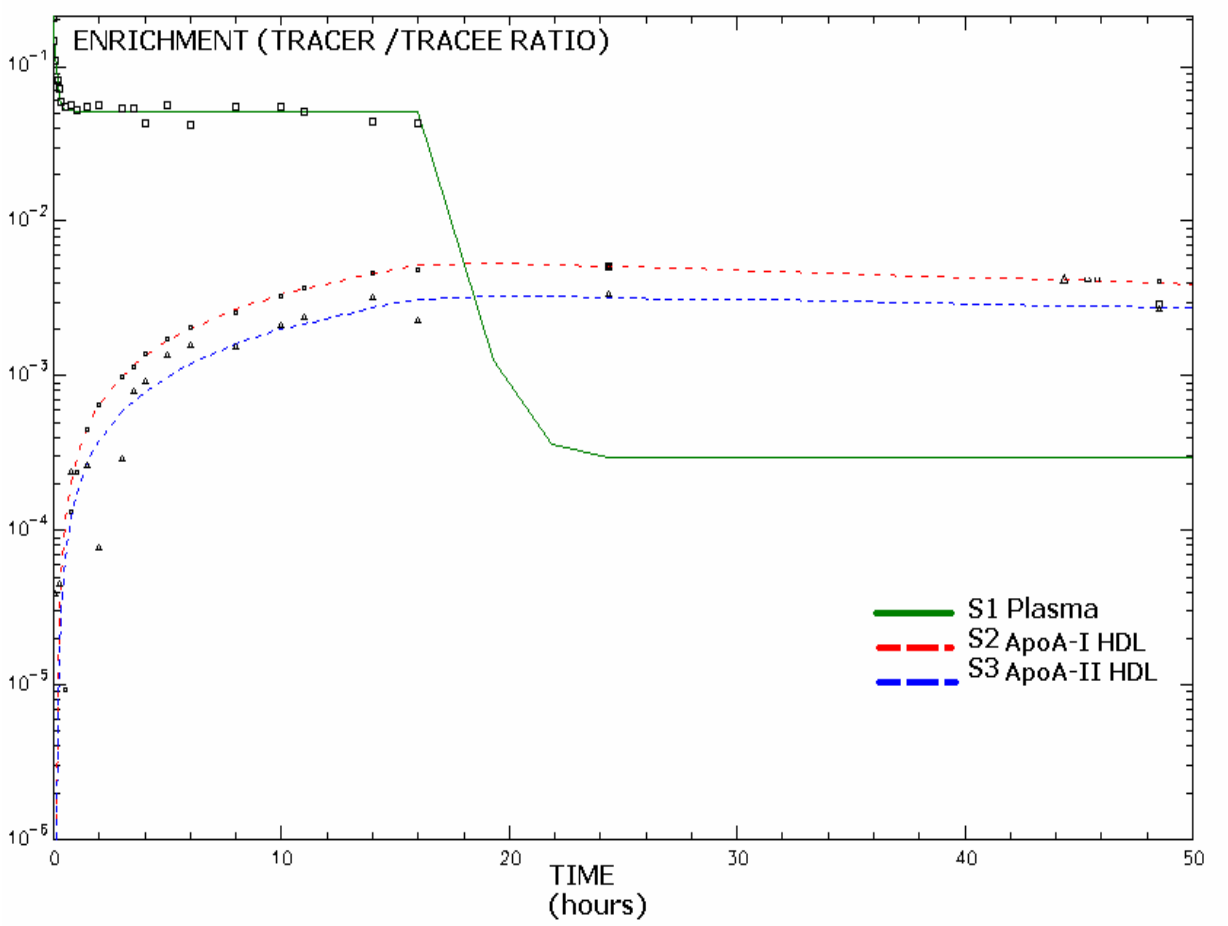
N=1 volunteer.

° (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.

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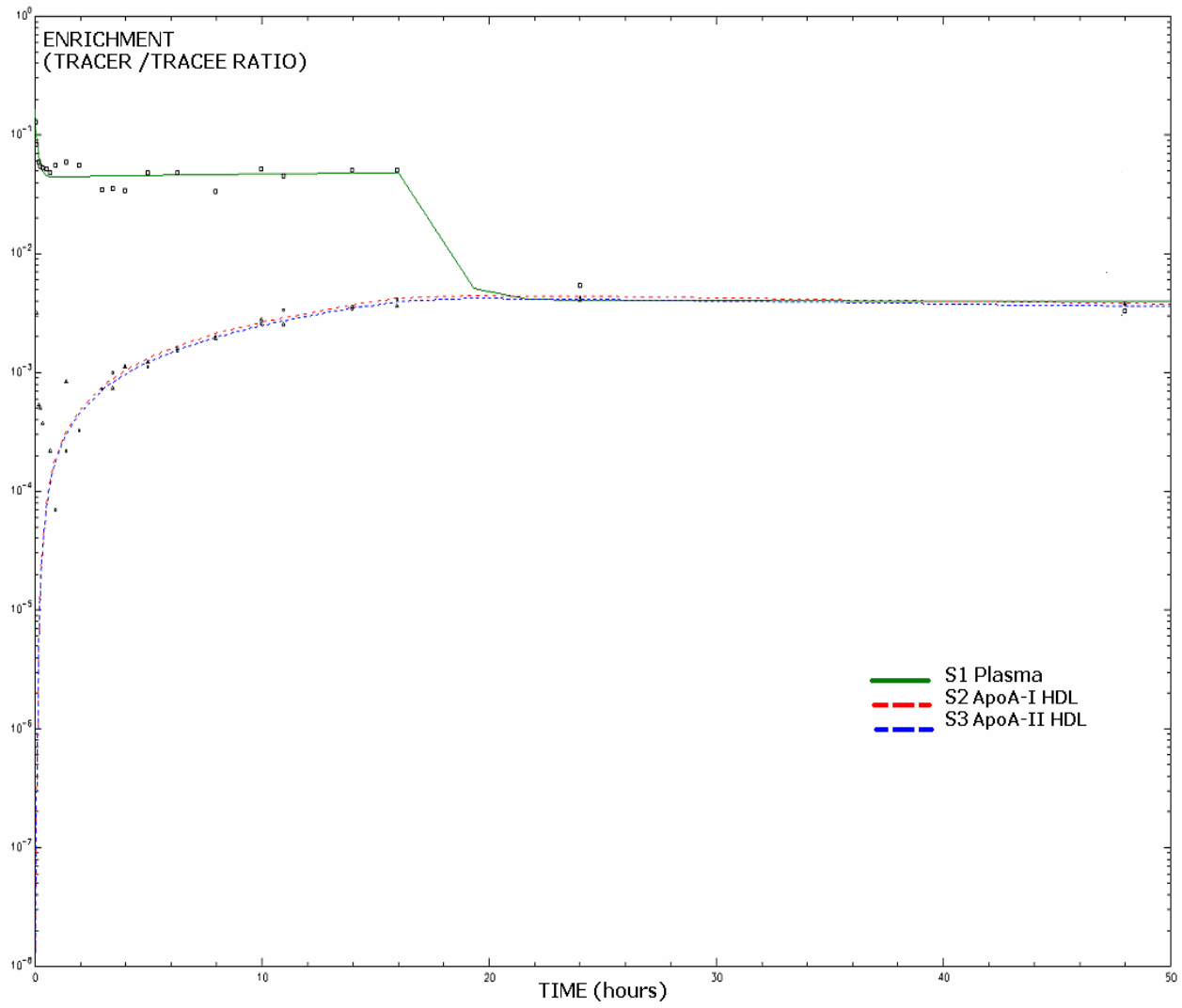
Figure 49. Apo A-I and A-II HDL enrichment curves for STEP II diet study.



N=1 volunteer.

° (curve points) observed tracer/tracee ratios.

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

s=site of determination.

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2.1.4.3.2.3 Lipoprotein kinetic parameters

The apo B-100-rich lipoproteins kinetic parameters calculated were: $VLDL_1$, $VLDL_2$, 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 $VLDL_1$, $VLDL_2$ 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:
  - $VLDL_1$ 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 $VLDL_1$ to $VLDL_2$ (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.
  - $VLDL_2$ 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 $VLDL_2$ to IDL (12.06 versus 6.62 pools/day; *P*=0.63) and decreased, albeit not significantly, $VLDL_2$ 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).

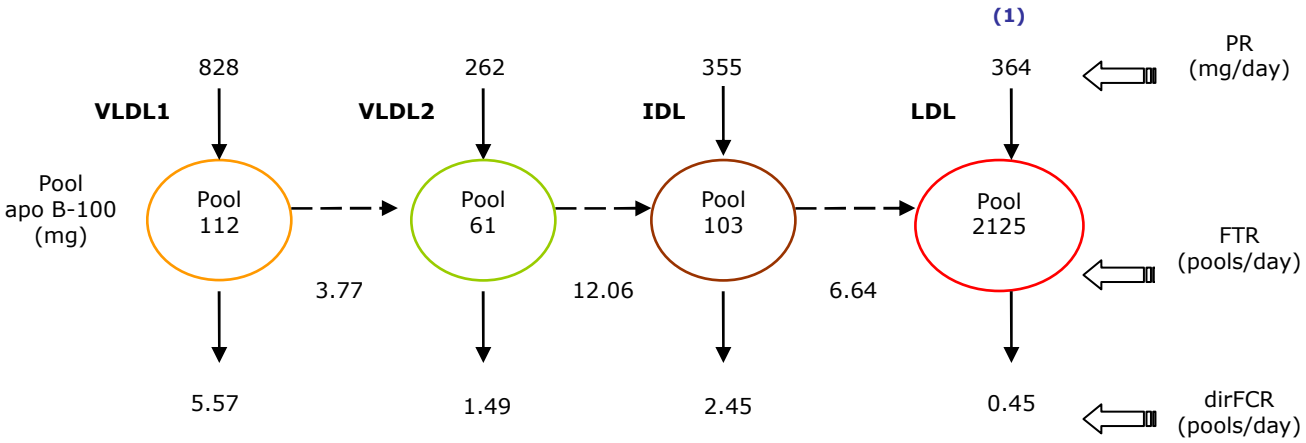
Table 50. Kinetic parameters describing apo B-100-rich lipoproteins metabolism.

	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

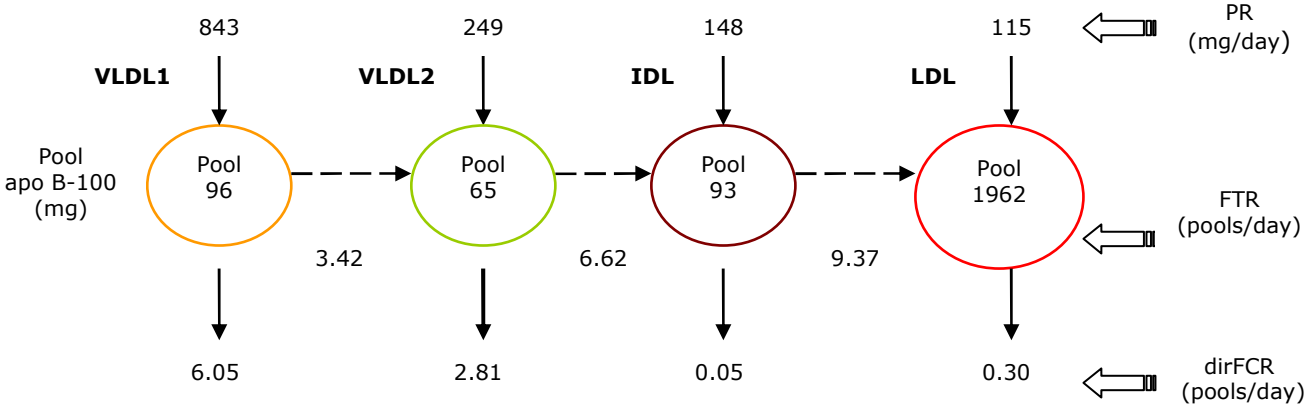
Results expressed as mean ± standard deviation (SD).
Significance *P*<0.05.
MED=Mediterranean; vs.=versus; PR=production rate;
dirFCR=direct fractional catabolic rate; FTR=fractional transfer rate.

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

Mediterranean diet



STEP II diet



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.

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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.

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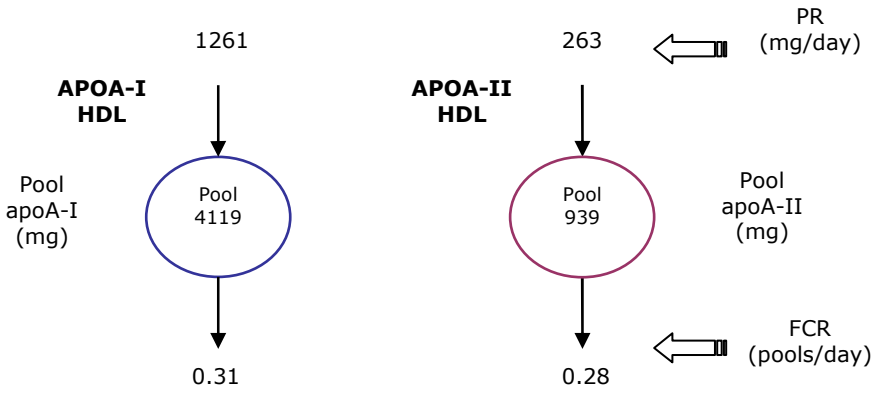
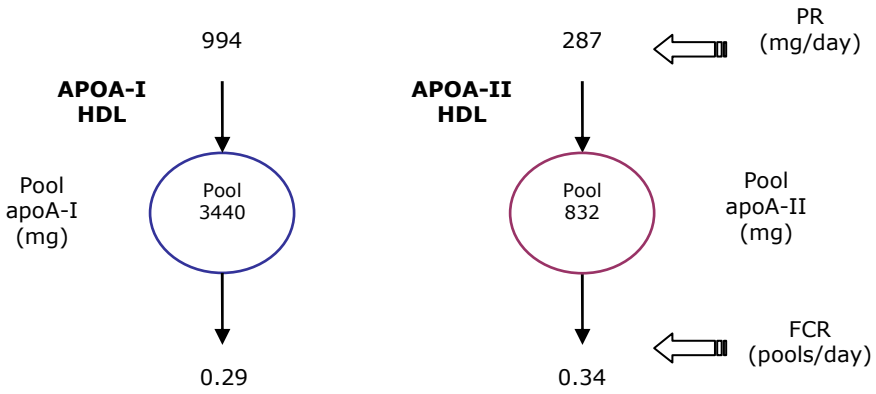
Figure 51. Flow diagrams of the kinetic parameters describing apoA-I and A-II HDL metabolism.

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.

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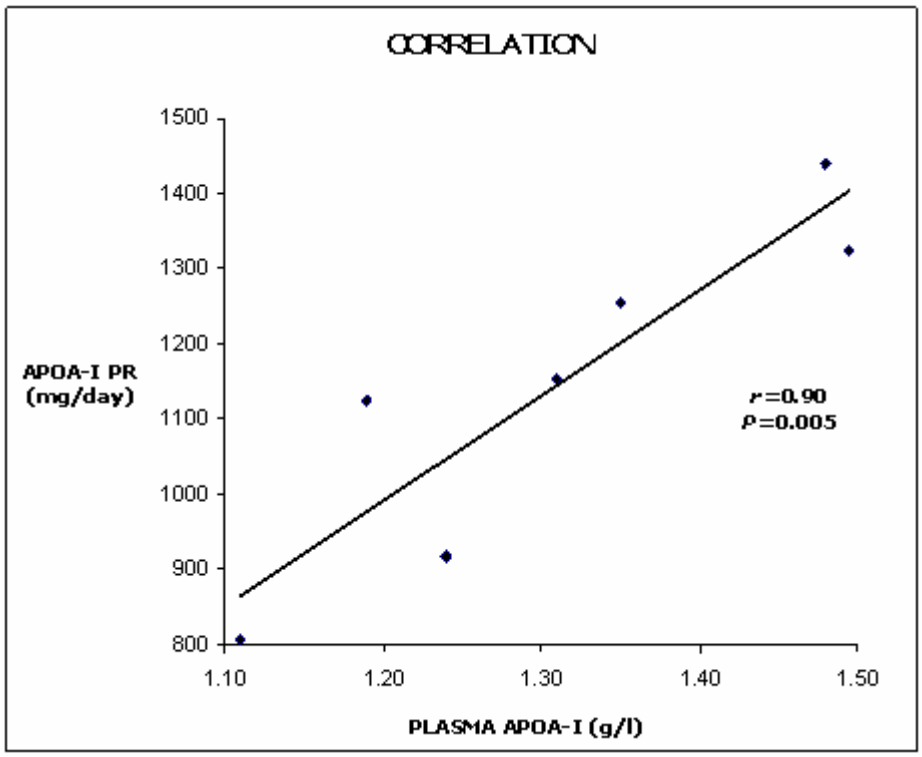
2.1.4.3.2.4 Parameter correlations

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 concentracions (g/l) and apoA-I PR (mg/day) (A); and between apoA-I plasma concentracions (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.

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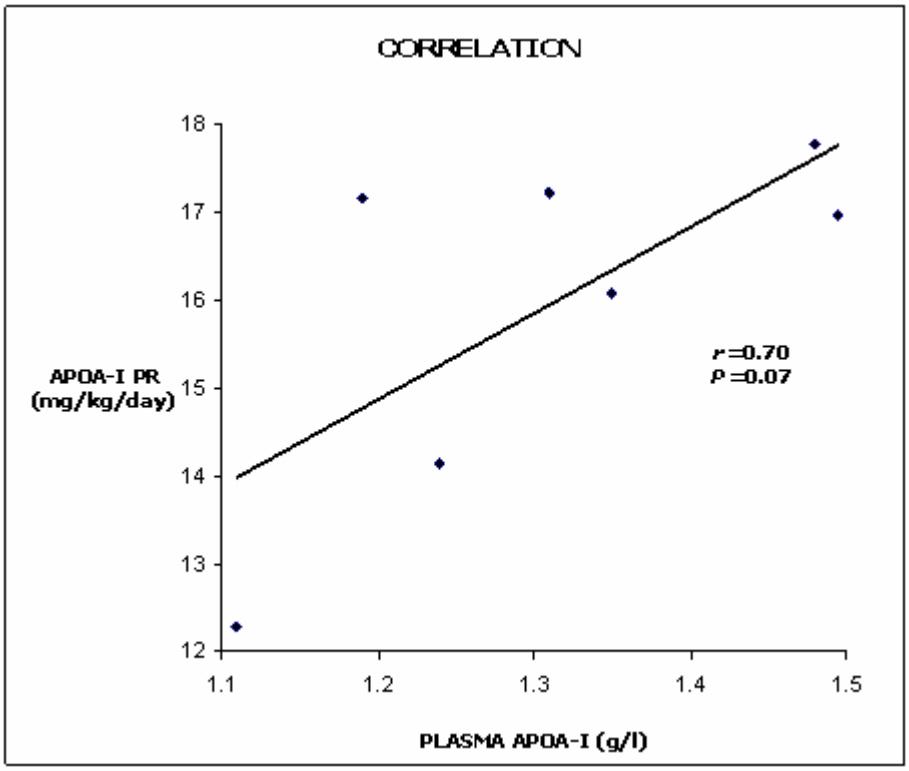
(B)



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.

	r	*P*
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.

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DISCUSSION

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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
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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 attractive [286-292].

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 studies [339-342]. 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 in administered [436]. 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.

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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 caracterisation 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 $^{2}H_3$-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 acurate GC-MS detection.

$^{2}H_3$-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 $^{2}H_3$-L-leucine is optimised. Further, leucine is an essential amino acid that is metabolised in muscle with a uniderectional pathway of catabolism to its keto acid [311]. We used a dose of 0.7 mg $^{2}H_3$-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 apos 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

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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, $^{2}H_3$-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 beginnig 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-synthetised 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 beeen 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 $^{2}H_3$-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,

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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, $^{2}H_3$-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].

$VLDL_1$ and $VLDL_2$ were separated from plasma using cumulative ultracentrifugation. IDL, LDL and HDL were separated using sequential preparative ultracentrifugation. Apo B-100 from $VLDL_1$, $VLDL_2$, 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

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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 apos [269].

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 sofware used in the design and determination of lipoprotein kinetic parameters. It has several advantatges [337, 338, 483]:

* it developes 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 $^{2}H_3$-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 $VLDL_1$, $VLDL_2$, IDL and LDL compartments. The model also consideres 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 isotopes [457].

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 data [264]. The model is simpler than the apo B-100 model and includes a site for $^{2}H_3$-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 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.

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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; ^{125}I and ^{131}I=radioisotopes (iodine)

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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% ± 0.04. Its composition was: oleic acid of 73.01% ± 1.03, palmitic acid of 12.43% ± 1.07, linoleic acid of 9.76% ± 0.56, stearic acid of 2.10% ± 0.20, palmitoleic acid of 1.22% ± 0.20, arachidic acid of 0.56% ± 0.26 and linolenic acid of 0.46% ± 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 profiles [506]. 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

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(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].

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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 $VLDL_1$, $VLDL_2$, IDL and LDL increased in the Mediterranean diet, although only significantly in $VLDL_2$ (+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 $VLDL_1$, $VLDL_2$, IDL, LDL and HDL and the EC:TG ratio in $VLDL_1$, $VLDL_2$, IDL and LDL; albeit the decrease was only significant for $VLDL_1$ 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/m^2, respectively). The Mediterranean diet, compared to the STEP II diet, induced a not significant change in body weight by a mean of ≤1%. However, in the 4 volunteers subset, we observed that the Mediterrenean diet significantly decreased the body weight (-0.58 kg) and showed a trend, albeit not significant, towards a decreased BMI (-0.21 kg/m^2). 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 $VLDL_1$, $VLDL_2$, 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 (≤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 $VLDL_1$, $VLDL_2$ 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

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apo B-100 LDL PR and, possibly, an increased FCR as well. Apo B-100 VLDL PR and FCR did not change [569].

Abbott el 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 $VLDL_1$, $VLDL_2$, 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 LDL_3 and apo B-100. Plasma TG did not change significantly. $VLDL_1$ and $VLDL_2$ 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 PPARα which interacts with unsaturated FAs.

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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 risk [524]. 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 events [585]. 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 concentrations [586]. 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)	A high-CH, very low-fat diet 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)	A very low-fat, low-CH diet 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)	A STEP II diet 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 replacing SFAs with MUFAs 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)	A low-fat diet reduction in apoA-I production explained decrease of HDLC. A decrease in apoA-I FCR possibly attributable to body weight reduction) A high-MUFA diet 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)	A Mediterranean-type diet, compared with a STEP II diet, increased plasma apoA-I levels, showed a trend towards an increase in plasma HDL C levels and decreaseed apo B-100/apoA-I ratio. A Mediterranean-type diet, compared with a STEP II diet, 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.

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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 [559-566].

Reference	Design
Vélez-Carrasco et al Arterioscler Thromb Vasc Biol. 1999. 19: 918-924	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 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 All meals and drinks prepared and provided (metabolic unit) Body weight maintained constant
Frenais et al Atheros. 2001. 157: 131-135	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
Matthan et al Arterioscler Thromb Vasc Biol. 2004. 24: 1092-1097	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 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 Body weight maintained constant Analysis of the macronutrient of diets

wk=week; CH=carbohydrates; SFAs=saturated fatty acids;
MUFAs=monounsaturated fatty acid;
PUFAs=polyunsaturated fatty acids; EPA=eicosapentanoic acid,
DHA=docosahexanoic acid.

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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		Baseline diet	STEP II diet
		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		Before supplementation	After maxEPA® capsules
		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	
		Unsaturated fat	Hydrogenated fat / Saturated fat
		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.

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Reference	Design
Desroches et al J Lipid Res 2004. 45. 2331-2338	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 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 **(1)** *P*<0.01 All meals prepared and provided (metabolic unit) Diets consumed *ad libitum* 3-day food records at baseline
Chan et al Am J Clin Nutr. 2006. 84: 37-43	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) 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%)

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
$^2H_3$ -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 Monoexponential function	Low-fat/High-CH diet ApoA-I Before / After / % 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)** High-MUFA diet ApoA-I Before / After / % 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)** Low-fat/High-CH diet ApoA-II Before / After / % 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 High-MUFA diet ApoA-II Before / After / % 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
$^2H_3$-L-leucine Single bolus injection Blood samples collected until 96 hours Fasted condition Multicompartmental model	After intervention diets Placebo / Fish oils / Atorv / Fish oil +Atorv / % Main effect fish oils ApoA-I Baseline 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 Week 6 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 Baseline 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 Week 6 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.

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

Reference	Design
Our study	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 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 Composition of diets determined using nutrition data bases and software 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 **(1)** *P*=0.02 **(2)** *P*=0.01 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

wk=week; CH=carbohydrates; SFAs=saturated fatty acids;
MUFAs=monounsaturated fatty acids;
PUFAs=polyunsaturated fatty acids; EPA=eicosapentanoic acid;
DHA=docosahexanoic acid.

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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	
	After Mediterranean diet	After STEP II diet
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	
	After Mediterranean diet	After STEP II diet
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.

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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 whweras decreased FCR.
Cortese C et al Eur J Clin Invest. 1983. 13:79-85 (radioisotope)	8 hyperlipidemic subjects	A low-fat diet (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)	A low-fat, high-CH diet decreased VLDL to LDL convertion, 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.

UNIVERSITAT ROVIRA I VIRGILI
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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 replacing SFAs with MUFAs 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 low-fat diet and high-MUFA diets.
Our study	10 men (moderate hypercholesterolemic)	A Mediterranean diet, compared to the STEP II diet, 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 therapeutic low-fat diet (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.

UNIVERSITAT ROVIRA I VIRGILI
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Autora: Katia Uliaque Cugat
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Method	Kinetic parameters
Primed constant infusion ^{13}C –leucine during 9 hours Fasted conditions Algebraic functions (linear regression)	Minimal fat diet / Moderate fat diet / Controls 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) Before / After 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 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 Placebo / Fish oils / Atorv / Fish+Atorv / % Main effect fish oils 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 ± 20 / 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.

<table>
<tr><th>Reference</th><th>Design</th></tr>
<tr><td>Gill et al
Am J Clin Nutr. 2003.
78: 47-56</td><td>
Randomised, blinded, crossover study
3 dietary interventions for 6-wk
<table>
<tr><th></th><th>Low-MUFA diet</th><th>Moderate-MUFA diet</th><th>High-MUFA diet</th></tr>
<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>
</table>
Washout period between diet ≥ 8-wk

Composition of diets determined using nutrition data bases and software
<table>
<tr><th></th><th>Low-MUFA</th><th>Moderate-MUFA</th><th>High-MUFA</th><th></th></tr>
<tr><td>CH %</td><td>52.20 ± 0.60</td><td>52.50 ± 0.70</td><td>53.20 ± 0.80</td><td></td></tr>
<tr><td>Prot %</td><td>12.40 ± 0.40</td><td>12.20 ± 0.30</td><td>12.30 ± 0.40</td><td></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><td></td></tr>
<tr><td>SFA %</td><td>14.70 ± 0.30</td><td>11.20 ± 0.20</td><td>7.30 ± 0.10</td><td>(1)</td></tr>
<tr><td>MUFA %</td><td>7.80 ± 0.20</td><td>10.30 ± 0.20</td><td>13.70 ± 0.20</td><td>(1)</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><td>(1)</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><td>(1)</td></tr>
</table>
(1) P<0.01

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
</td></tr>
<tr><td>Chan et al
Am J Clin Nutr. 2003.
77: 300-307</td><td>
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)

Isocaloric diets
Checked by capsules count
</td></tr>
<tr><td>Matthan et al
Arterioscler
Thromb Vasc Biol. 2004.
24: 1092-1097</td><td>
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

Composition of diets determined by chemical analysis of food
<table>
<tr><th></th><th>Unsaturated fat</th><th>Hydrogenated fat</th><th>Saturated fat</th></tr>
<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>
</table>
Body weight maintained constant
Analysis of the macronutrient of diets
</td></tr>
</table>

wk=week; CH=carbohydrates; SFAs=saturated fatty acids;
MUFAs=monounsaturated fatty acids PUFAs=polyunsaturated fatty acids.

Method	Kinetic parameters

$^2H_3$-L-leucine Bolus infusion Blood samples collected until 48 hours Fasted condition Multicompart mental model

	Low-MUFA diet	High-MUFA diet	
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 $^2H_3$-L-leucine Blood samples collected until 96 hours Fasted condition Multicompart mental 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

$^2H_3$-L-leucine Primed constant infusion for 15 hours Blood samples collected until 15 hours 12 hours O/N fasted + fed hourly for 20 hours Multicompart mental model

	Experimental diets			
	Unsaturated fat	Hydrogenated fat	Saturated 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.

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<table>
<tr><th>Reference</th><th>Design</th></tr>
<tr><td>Desroches et al
J Lipid Res 2004.
45: 2331-2338</td><td>
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 graind and vegetables

Composition of diets determined using nutrition data bases and software

<table>
<tr><th></th><th>Low-fat/high-CH diet</th><th>High-MUFA diet</th><th></th></tr>
<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>
</table>

(1) P<0.01

All meals prepared and provided (metabolic unit)
Diets consumed ad libitum
3-day food records at baseline
</td></tr>
<tr><td>Our study</td><td>
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

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

Composition of diets determined using nutrition data bases and software

<table>
<tr><th></th><th>Stabilisation diet</th><th>Mediterranean diet</th><th>STEP II diet</th><th></th></tr>
<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>
</table>

(1) P=0.02
(2) P=0.01

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
</td></tr>
</table>

wk=week; CH=carbohydrates; SFAs=saturated fatty acids;
MUFAs=monounsaturated fatty acids; PUFAs=polyunsaturated fatty acids.

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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 Mono-exponential function		Low-fat/High-CH diet	
	Before	After	% change
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
		High-MUFA diet	
	Before	After	% change
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) $P<0.05$			
$^{2}H_3$-L-leucine Primed constant infusion for 16 hours Blood samples collected until 48 hours Fasted condition Multi-compartmental model	After Mediterranean diet	After STEP II diet	
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.

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CONCLUSIONS

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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

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the primary and secondary prevention of CVD in subjects who may be predisposed to the disease.

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1. Introduction's bibliography

1. http://www.fao.org (Food and Agriculture Organization of the United Nations).

2. http://www.americanheart.org (American Heart Association's).

3. www.who.int/whr/en/ (World Health Organization (WHO) world health report).

4. Villar Álvarez F. La prevención cardiovascular en España. Promoviendo el uso de las recomendaciones. Rev Esp Salud Publica. 2004. 78: 421-434.

5. Navab M, Fogelman AM et al. Pathogenesis of atherosclerosis. Am J Cardiol. 1995. 9: 18C-23C.

6. Ross R. Atherosclerosis: an inflammatory disease. New Engl J Med. 1999. 340: 115–126.

7. Tall AR, Wang N. Tangier disease as a test of the reverse cholesterol transport hypothesis. J Clin Invest. 2000. 106: 1205-1207.

8. Lusis AJ. Atherosclerosis. Nature. 2000. 407: 233-241.

9. Viles-Gonzalez JF, Fuster V, Badimon JJ. Atherothrombosis: A widespread disease with unpredictable and life-threatening consequences. Eur Heart J. 2004. 14: 1197-1207.

10. Fuster V, Moreno PR et al. Atherothrombosis and high-risk plaque: part I: evolving concepts. J Am Coll Cardiol. 2005. 46: 937-954.

11. Glass CK, Witztum JL. Atherosclerosis: the road ahead. Cell. 2001. 104: 503-516.

12. Hegele RA. Monogenic dyslipidemias: window on determinants of plasma lipoprotein metabolism. Am J Hum Genet. 2001. 69: 1161-1177.

13. Stocker R, Keaney JF Jr. Role of oxidative modifications in atherosclerosis. Physiol Rev. 2004. 84: 1381-1478.

14. Berliner JA, Navab M et al. Atherosclerosis: basic mechanisms. Oxidation, inflammation and genetics. Circulation. 1995. 91: 2488-2496.

15. Kiechl S, Willeit J. Bruneck Study Group. The natural course of atherosclerosis. Part I: Incidence and progression. Arterioscler Thromb Vasc Biol. 1999. 19: 1484-1490.

16. Kiechl S, Willeit J. Bruneck Study Group. The natural course of atherosclerosis. Part II: Vascular remodelling. Arterioscler Thromb Vasc Biol. 1999. 19: 1491-1498.

17. Libby P. Atherosclerosis: the new view. Sci Am. 2002. 286: 46-55.

18. Steinberg D. Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. Nat Med. 2002. 8: 1211-1217.

19. Navab M, Ananthramaiah GM et al. The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. J Lipid Res. 2004. 45: 993-1007.

20. Cullen P, Rauterberg J, Lorkowski S. The pathogenesis of atherosclerosis. Handb Exp Pharmacol. 2005. 170: 3-70.

21. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med. 2005. 352: 1685-1695.

22. Quehenberger O. Thematic review series: the immune system and atherogenesis. Molecular mechanisms regulating monocyte recruitment in atherosclerosis. J Lipid Res. 2005. 46: 1582-1590.

23. Reiss AB, Glass AD. Atherosclerosis: immune and inflammatory aspects. J Investig Med. 2006. 54: 123-131.

24. www.accessmedicine.com (online Harrison's Principles of Internal Medicine, 16th Edition, The McGraw-Hill companies).

25. Vane JR, Änggard EE, Botting RM. Regulatory functions of the vascular endothelium. New Engl J Med. 1990. 323: 27–36.

26. Schaefer EJ, Lamon-Fava S et al. Factors associated with low and elevated plasma high density lipoprotein cholesterol and apolipoprotein AI levels in the Framingham Offspring Study. J Lipid Res. 1994. 35: 871-882.

27. Stary HC, Chandler AB et al. A definition of advanced types of lesions and a histological classification of atherosclerosis. Aterioscler Thromb Vasc Biol. 1995. 15: 1512-1531.

28. Raines EW, Ross R. Is overamplification of the normal macrophage defensive role critical to lesion development? Ann N Y Acad Sci. 1997. 811: 76-85 (discussion 85-87).

29. Braun-Dullaeus RC, Sedding DG. Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. Nat Med. 2002. 8: 1249-1256.

30. Li AC, Glass CK. The macrophage foam cell as a target for therapeutic intervention. Nat Med. 2002. 8: 1235-1242.

31. Takahashi K, Takeya M, Sakashita N. Multifunctional roles of macrophages in the developmemt and prograssion of atherosclerosis in humans and experimental animals. Med Electron Microsc. 2002. 35: 179-203.

32. Ghazalpour A et al. Toward a biological network for atherosclerosis. J Lipid Res. 2004. 10: 1793-1805.

33. Braunersreuther V, Mach F. Leukocyte recruitment in atherosclerosis: potential targets for therapeutic approaches? Cell Mol Life Sci. 2006. 63: 2079-2088.

34. Badimon L, Martinez-Gonzalez J, Llorente-Cortes V, Rodriguez C, Padro T. Cell biology and lipoproteins in atherosclerosis. Curr Mol Med. 2006. 6: 439-456.

35. Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: beyond lipid uptake. Arterioscler Thromb Vasc Biol. 2006. 26: 1702-1711.

36. Libby P, Jiang Geng Y et al. Macrophages and atherosclerotic plaques stability. Curr Opin Lipidol. 1996. 7: 330-335.

37. Lee RT, Libby P. The unstable atheroma. Arteriscler Thromb Vasc Biol. 1997. 17: 1859-1867.

38. Zaman AG, Helt G et al. The role of plaque rupture and thrombosis in coronary artery disease. Atherosclerosis. 2000. 149: 251-266.

39. Tabas I. Apoptosis and plaque destabilization in atherosclerosis: the role of macrophage apoptosis induced by cholesterol. Cell Death Differ. 2004. 11 (Suppl 1): S12-S16.

40. Ballantyne CM, Hoogeveen RC. Role of lipid and lipoprotein profiles in risk assessment and therapy. Am Heart J. 2003. 14: 227-233.

41. Fruchart JC, Nierman MC et al. Risk Factors for Atherosclerosis and Patient Risk Assessment Circulation. 2004. 23 (Suppl 1): III15-III19.

42. Hawkins MA. Markers of increased cardiovascular risk: are we measuring the most appropriate parameters? Obes Res. 2004. 12 (Suppl 2): 107S-114S.

43. Weber C. Platelets and chemokines in atherosclerosis: partners in crime. Circ Res. 2005. 96: 612-616.

44. Smith SC Jr. Current and future directions of cardiovascular risk prediction. Am J Cardiol. 2006. 97: 28A-32A.

45. Pearson TA, Mensah GA, Alexander RW et al. Markers of inflammation and cardiovascular disease: application to clinical and public health practice. A statement for healthcare professionals from the Centers for Disease Control and Prevention and The American Heart Association. Circulation. 2003. 107: 499-511.

46. Fruchart JC, Nierman MC, Stroes ES, Kastelein JJ, Duriez P. New risk factors for atherosclerosis and patient risk assessment. Circulation. 2004. 109 (Suppl 1): III15-III19.

47. de Maat MP, Trion A. C-reactive protein as a risk factor versus risk marker. Curr Opin Lipidol. 2004. 15: 651-657.

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48. Koschinsky ML. Lipoprotein(a) and the link between atherosclerosis and thrombosis. Can J Cardiol. 2004. 20 (Suppl B): 37B-43B.

49. Kannel WB. Overview of hemostatic factors involved in atherosclerotic cardiovascular disease. Lipids. 2005. 40: 1215-1220.

50. Eaton CB. Traditional and emerging risk factors for cardiovascular disease. Prim Care. 2005. 32: 963-976.

51. Kaul S, Zadeh AA, Shah PK. Homocysteine hypothesis for atherothrombotic cardiovascular disease: not validated. J Am Coll Cardiol. 2006. 48: 914-923.

52. Barter PJ, Rye KA. Homocysteine and cardiovascular disease: is HDL the link? Circ Res. 2006. 99: 565-566.

53. Stamler J, Daviglus ML, Garside DB et al. Relationship of baseline serum cholesterol levels in 3 large cohorts of younger men to long-term coronary, cardiovascular, and all-cause mortality and to longevity. JAMA. 2000. 284: 311-318.

54. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20536 high-risk individuals: a randomised placebo-controlled trial. Lancet. 2002. 360: 7–22.

55. Ito MK, Cheung RJ, Gupta EK, Birtcher KK, Chong PH, Bianco TM, Bleske BE. Key articles, guidelines, and consensus papers relative to the treatment of dyslipidemias—2005. Pharmacotherapy. 2006. 26: 939-1010.

56. Erhardt LR, Gotto A Jr. The evolution of European guidelines: changing the management of cholesterol levels. Atherosclerosis. 2006. 185: 12-20.

57. Commission on Chronic Illness. Chronic Illness in a Large City: The Baltimore Study. Comission on Chronic Illness. Harvard University Press. Vol IV. 1957.

58. Commission on Chronic Illness. Chronic Illness in the United States. Comission on Chronic Illness. Harvard University Press. Vol I. 1957.

59. Kannel WB, McGee D, Gordon T. A general cardiovascular risk profile: the Framingham Study. Am J Cardiol. 1976. 38: 46-51.

60. The Expert Panel. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Final report. Circulation. 2002. 106: 3143-3421.

61. Grundy SM, Cleeman JI, Merz CN et al. Coordinating Committee of the National Cholesterol Education Program. Implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines. Arterioscler Thromb Vasc Biol. 2004. 24: 149-161.

62. Stone NJ, Bilek S, Rosenbaum S. Recent National Cholesterol Education Program Adult Treatment Panel III update: adjustments and options. Am J Cardiol. 2005. 96: 53E-59E.

63. Crouse JR 3rd. Thematic review series: patient-oriented research. Imaging atherosclerosis: state of the art. J Lipid Res. 2006. 47: 1677-1699.

64. Jaffer FA, Weissleder R. Molecular imaging in the clinical arena. JAMA. 2005. 293: 855-862.

65. Anderson KM, Wilson PW et al. An update coronary risk profile. A statement for health professionals. Circulation. 199. 83: 356-362.

66. Assmann G, Cullen P, Schulte H. Simple scoring scheme for calculating the risk of acute coronary events based on the 10-year follow-up of the Prospective Cardiovascular Münster (PROCAM) study. Circulation. 2002. 105: 310-315.

67. Conroy RM, Pyörälä K et al. SCORE project group. Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project. Eur Heart J. 2003. 11: 987-1003.

68. De Backer G, Ambrosioni E et al. European Society of Cardiology Committee for Practice Guidelines. European guidelines on cardiovascular disease prevention in clinical practice. Third Joint Task Force of European and other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of eight societies and by invited experts). Atherosclerosis. 2004. 2: 381-391.

69. Brotons C, Royo-Bordonada MA, Alvarez-Sala L et al. Comite Espanol Interdisciplinario para la Prevencion Cardiovascular (CEIPC). Spanish adaptation of the European Guide to Cardiovascular Prevention (I). Aten Primaria. 2004. 34: 427-432.

70. Marrugat J, D'Agostino R, Sullivan L et al. An adaptation of the Framingham coronary heart disease risk function to Euroepan Mediterranean areas. J Epidemiol Community Health. 2003. 57: 634-638.

71. Goldstein JL, Hobbs HH, Brown MS. Familial hypercholesterolemia. In The Metabolic & Molecular Bases of Inherited Disease. McGraw-Hill. 2001. Vol 2: 2863-2913.

72. Alberts B, Johnson A et al. Molecular Biology of the Cell. 4th ed. Garland Publishing. New York. 2002.

73. Pauciullo P. Lipoprotein transport and metabolism: a brief update. Nutr Metab Cardiovasc Dis . 2002. 12: 90-97.

74. Durrington P. Dyslipidaemia. Lancet. 2003. 362: 717-731.

75. Berglund L. Lipoprotein metabolism: a well-tried tool to characterize dyslipidemic mechanisms. Arterioscler Thromb Vasc Biol. 2006. 26: 1201-1203.

76. Merkel M, Eckel RH, Goldberg IJ. Lipoprotein lipase: genetics, lipid uptake, and regulation. J Lipid Res. 2002. 43: 1997-2006.

77. Redgrave TG. Chylomicron metabolism. Biochem Soc Trans. 2004. 32: 79-82.

78. Hussain MM, Fatma S et al. Intestinal lipoprotein assembly. Curr Opin Lipidol. 2005. 16: 281-285.

79. Lopez-Miranda J, Perez-Martinez P et al. Postprandial lipoprotein metabolism, genes and risk of cardiovascular disease. Curr Opin Lipidol. 2006. 17: 132-138.

80. Jin W, Marchadier D, Rader DJ. Lipases and HDL metabolism. Trends Endocrinol Metab. 2002. 13: 174-178.

81. Stein Y, Stein O. Lipoprotein lipase and atherosclerosis. Atherosclerosis. 2003. 170: 1-9.

82. Tacken PJ, Hofker MH et al. Living up to a name: the role of the VLDL receptor in lipid metabolism. Curr Opin Lipidol. 2001. 12: 275-259.

83. Talmud PJ, Stephens JW. Lipoprotein lipase gene variants and the effect of environmental factors on cardiovascular disease risk. Diabetes Obes Metab. 2004. 6: 1-7.

84. Takahashi S, Sakai J, Fujino T et al. The very low-density lipoprotein (VLDL) receptor: characterization and functions as a peripheral lipoprotein receptor. J Atheroscler Thromb. 2004. 11: 200-208.

85. Lusis AJ, Fogelman AM, Fonarow GC. Genetic basis of atherosclerosis: part I: new genes and pathways. Circulation. 2004. 110: 1868-1873.

86. Heeren J, Beisiegel U, Grewal T. Apolipoprotein E recycling: implications for dyslipidemia and atherosclerosis. Arterioscler Thromb Vasc Biol. 2006. 26: 442- 448.

87. Badellino KO, Rader DJ. The role of endothelial lipase in high-density lipoprotein metabolism. Curr Opin Cardiol. 2004. 19: 392-395.

88. Santamarina-Fojo S, Gonzalez-Navarro H et al. Hepatic lipase, lipoprotein metabolism, and atherogenesis. Arterioscler Thromb Vasc Biol. 2004. 24: 1750-1754.

89. Hussain MM, Strickland DK, Bakillah A. The mammalian low-density lipoprotein receptor family. Annu Rev Nutr. 1999. 19: 141-172.

90. Herz J, Hui DY. Lipoprotein receptors in the vascular wall. Curr Opin Lipidol. 2004. 15: 175-181.

91. Moore KJ, Freeman MW. Scavenger receptors in atherosclerosis: beyond lipid uptake. Arterioscler Thromb Vasc Biol. 2006. 26: 1702-1711.

92. Edge SB, Hoeg JM et al. Apolipoprotein B synthesis in humans: liver synthesizes only apolipoprotein B-100. Metabolism. 1985. 34: 726-730.

93. Chan DC, Barrett H, Watts G. Lipoprotein transport in the metabolic syndrome: methodological aspects of stable isotope kinetic studies. Clinical Science. 2004. 107: 221–232.

94. Jin W, Marchadier D, Rader DJ. Lipases and HDL metabolism. Trends Endocrinol Metab. 2002. 13: 174-178.

95. Stein O, Stein Y. Lipid transfer proteins (LTP) and atherosclerosis. Atherosclerosis. 2005. 178: 217-230.

96. Zannis VI, Chroni A, Krieger M. Role of apoA-I, ABCA1, LCAT, and SR-BI in the biogenesis of HDL. J Mol Med. 2006. 84: 276-294.

97. Inazu A, Koizumi J, Mabuchi H. Cholesteryl ester transfer protein and atherosclerosis. Curr Opin Lipidol. 2000. 11: 389-396.

98. Barter PJ. Brewer HB Jr, Chapman MJ. et al. Cholesteryl ester transfer protein: a novel target for raising HDL and inhibiting atherosclerosis. Arterioscler Thromb Vasc Biol. 2003. 2: 160-167.

99. van Berkel TJ, Van Eck M et al. Scavenger receptor classes A and B: their roles in atherogenesis and the metabolism of modified LDL and HDL. Ann N Y Acad Sci. 2000. 902: 113-126.

100. Rader DJ. High-density lipoproteins as an emerging therapeutic target for atherosclerosis. JAMA. 2003. 290: 2322-2324.

101. Connelly MA, Williams DL. Scavenger receptor BI: a scavenger receptor with a mission to transport high density lipoprotein lipids. Curr Opin Lipidol. 2004. 15: 287-295.

102. van Eck M, Pennings M et al. Scavenger receptor BI and ATP-binding cassette transporter A1 in reverse cholesterol transport and atherosclerosis. Curr Opin Lipidol. 2005. 3: 307-315.

103. van der Velde AE, Groen AK. Shifting gears: liver SR-BI drives reverse cholesterol transport in macrophages. J Clin Invest. 2005. 115: 2699-2701.

104. Huby T, Doucet C, Dachet C et al. Knockdown expression and hepatic deficiency reveal an atheroprotective role for SR-BI in liver and peripheral tissues. J Clin Invest. 2006. 116: 2767-2776.

105. Jessup W, Gelissen IC et al. Roles of ATP binding cassette transporters A1 and G1, scavenger receptor BI and membrane lipid domains in cholesterol export from macrophages. Curr Opin Lipidol. 2006. 17: 247-257.

106. van Tol A. Phospholipid transfer protein. Curr Opin Lipidol. 2002. 13: 135-159.

107. Stefkova J, Poledne R, Hubacek JA. ATP-binding cassette (ABC) transporters in human metabolism and diseases. Physiol Res. 2004. 53: 235-243.

108. Garcia-Calvo M, Lisnock J, Bull HG et al. The target of ezetimibe is Niemann-Pick C1-Like 1 (NPC1L1). Proc Natl Acad Sci U S A. 2005. 102: 8132-8137.

109. Hazard SE, Patel SB. Sterolins ABCG5 and ABCG8: regulators of whole body dietary sterols. Pflugers Arch. 2006. 27: 1-8.

110. Davies JP, Ioannou YA. The role of the Niemann-Pick C1-like 1 protein in the subcellular transport of multiple lipids and their homeostasis. Curr Opin Lipidol. 2006. 17: 221-226.

111. Boadu E, Choi HY, Lee DW et al. Correction of apolipoprotein A-I-mediated lipid efflux and HDL particle formation in human Niemann-Pick type C disease fibroblasts. J Biol Chem. 2006. 281: 37081-37090.

112. Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? Circulation. 2006. 113: 2548-2555.

113. Toth PP. High-density lipoprotein as a therapeutic target: clinical evidence and treatment strategies. Am J Cardiol. 2005. 96: 50K-58K; discussion 34K-35K.

114. Ballantyne C, Arroll B, Shepherd J. Lipids and CVD management: towards a global consensus. Eur Heart J. 2005. 26: 2224-2231.

115. Choi BG, Vilahur G, Yadegar D et al. The role of high-density lipoprotein cholesterol in the prevention and possible treatment of cardiovascular diseases. Curr Mol Med. 2006. 6: 571-587.

116. Balbisi EA. Management of hyperlipidemia: new LDL-C targets for persons at high-risk for cardiovascular events. Med Sci Monit. 2006. 12: RA34-RA39.

117. Choi BG, Vilahur G, Viles-Gonzalez JF, Badimon JJ. The role of high-density lipoprotein cholesterol in atherothrombosis. Mt Sinai J Med. 2006. 73: 690-701.

118. Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. Circ Res. 2005. 96: 1221-1232.

119. Gotto AM, Whitney Jr E et al. Relation between baseline and on-treatment lipid parameters and first acute major coronary events in the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS). Circulation. 2000. 101: 477–484.

120. Rader DJ. High-density lipoproteins as an emerging therapeutic target for atherosclerosis. JAMA. 2003. 17: 2322-2324.

121. Lada AT, Rudel LL. Associations of low density lipoprotein particle composition with atherogenicity. Curr Opin Lipidol. 2004. 15: 19-24.

122. Asztalos BF. High-density lipoprotein metabolism and progression of atherosclerosis: new insights from the HDL Atherosclerosis Treatment Study. HDL Atherosclerosis Treatment Study. Curr Opin Cardiol. 2004. 19: 385-391.

123. NIH Consensus Conference. Triglyceride, high-density lipoprotein, and coronary heart disease. NIH Consensus Development Panel on Triglyceride, High-Density Lipoprotein, and Coronary Heart Disease. JAMA. 1993. 269: 505–510.

124. Sharrett AR, Ballantyne CM et al. Coronary heart disease prediction from lipoprotein cholesterol levels, triglycerides, lipoprotein(a), apolipoproteins A-I and B, and HDL density subfractions: the Atherosclerosis Risk in Communities (ARIC) Study. Circulation. 2001. 104: 1108–1113.

125. Szapary PO, Rader DJ. The triglyceride-high-density lipoprotein axis: an important target of therapy? Am Heart J. 2004. 148: 211-221.

126. Packard CJ, Saito Y. Non-HDL cholesterol as a measure of atherosclerotic risk. J Atheroscler Thromb. 2004. 11: 6-14.

127. Kinsell LW, Partridge J, Boling L et al. Dietary modification of serum cholesterol and phospholipid levels. J Clin Endocrinol Metab. 1952. 12: 909-13.

128. Hu F, Stampfer M. et al. Dietary fat intake and the risk of coronary heart disease in women. New Engl J Med. 1997. 337: 1491–1499.

129. Moreno JJ, Mitjavila MT. The degree of unsaturation of dietary fatty acids and the development of atherosclerosis. J Nutr Biochem. 2003. 14: 182-195.

130. Jakobsen MU, Overvad K, Dyerberg J, Schroll M, Heitmann BL. Dietary fat and risk of coronary heart disease: possible effect modification by gender and age. Am J Epidemiol. 2004. 160: 141-149.

131. Lichtenstein AH. Thematic review series: patient-oriented research. Dietary fat, carbohydrate, and protein: effects on plasma lipoprotein patterns. J Lipid Res. 2006. 47: 1661-1667.

132. Shekelle RB, Shryock AM, Paul O et al. Diet, serum cholesterol, and death from coronary heart disease. The Western Electric Study. N Engl J Med. 1981. 304: 65-70.

133. Kushi LK, Lew RA, Stare FJ et al. Diet and 20-year mortality from coronary heart disease. The Ireland-Boston Diet-Heart Study. N Engl J Med. 1985. 312: 811-818.

134. Kromhout D, Menotti A et al. Dietary saturated and trans fatty acids and cholesterol and 25-year mortality from coronary heart disease: the Seven Countries study. Prev Med. 1995. 24: 308–315.

135. Skuladottir GV, Gudmundsdottir S et al. Plasma fatty acids and lipids in two separate, but genetically comparable, Icelandic populations. Lipids. 1995. 30: 649–655.

UNIVERSITAT ROVIRA I VIRGILI
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136. Kratz M. Dietary cholesterol, atherosclerosis and coronary heart disease. Handb Exp Pharmacol. 2005. 170: 195-213.

137. Simopoulos AP, Cleland LG. Omega-6/Omega-3 Essential Fatty Acid Ratio: The Scientific Evidence. World Rev Nutr Diet. 2003. Karger Ed. Vol 92.

138. Lands WEM. Biochemistry and physiology of n-3 fatty acids. FAEB J. 1992. 6: 2530-2536.

139. He K, Rimm EB, Merchant A et al. Fish Consumption and Risk of Stroke in Men. JAMA. 2002. 288: 3130-3136.

140. www.fda.gov/ohrms/dockets/dockets/03q0401/03q-0401-c000005-01-vol7.pdf (US Food and Drug Administration Announces Qualified Health Claims for Omega-3 Fatty Acids).

141. Wijendran V, Hayes KC. Dietary n-6 and n-3 fatty acid balance and cardiovascular health. Annu Rev Nutr. 2004. 24: 597-615.

142. Schmidt EB, Arnesen H et al. Marine n-3 polyunsaturated fatty acids and coronary heart disease. Part I. Background, epidemiology, animal data, effects on risk factors and safety. Thromb Res. 2005. 115: 163-170.

143. Kris-Etherton PM, Pearson TA et al. High-monounsaturated fatty acid diets lower both plasma cholesterol and triacyglycerol concentrations. Am J Clin Nutr. 1999. 70: 1009-1015.

144. Perez-Jimenez F, Lopez-Miranda J, Mata P. Protective effect of dietary monounsaturated fat on arteriosclerosis: beyond cholesterol. Atherosclerosis. 2002. 163: 385-398.

145. Montoya MT, Porres A, Serrano S et al. Fatty acid saturation of the diet and plasma lipid concentrations, lipoprotein particle concentrations, and cholesterol efflux capacity. Am J Clin Nutr. 2002. 75: 484-491.

146. Ros E. Dietary cis-monounsaturated fatty acids and metabolic control in type 2 diabetes. Am J Clin Nutr. 2003. 78 (3 Suppl): 617S-625S.

147. Martinez-Gonzalez MA, Bes-Rastrollo M. The cardioprotective benefits of monounsaturated fatty acid. Altern Ther Health Med. 2006. 12: 24-30.

148. Rassart E, Bedirian A, Do Carmo S et al. Apolipoprotein D. Biochim. Biophys. Acta. 2000. 1482: 185-198.

149. Duchateau PN, Movsesyan I et al. Plasma apo L concentrations correlate with plasma triglycerides and cholesterol levels in normolipidemic, hyperlipidemic and diabetic subjects. J Lipid Res. 2000. 41: 1231-1236.

150. Pennacchio LA, Olivier M et al. An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. Science. 2001. 5540: 169-173.

151. Gautier T, Masson D et al. Apo CI deficiency markedly augments plasma lipoprotein changes mediated by human cholesteryl ester transfer protein (CETP) in CETP transgenic/Apo CI-knocked out mice. J Biol Chem. 2002. 277: 31354-31363.

152. Paromov VM, Morton RE. Lipid transfer inhibitor protein defines the participation of high density lipoprotein subfractions in lipid transfer reactions mediated by cholesterol ester transfer protein (CETP). J Biol Chem. 2003. 278: 40859-40866.

153. McIlhargey TL, Yang Y et al. Identification of a lipoprotein lipase cofactor-binding site by chemical cross-linking and transfer of apo C-II-responsive lipolysis from lipoprotein lipase to hepatic lipase. J Biol Chem. 2003. 25: 23027-23035.

154. Ribalta J, Vallve JC, Girona J, Masana L. Apolipoprotein and apolipoprotein receptor genes, blood lipids and disease. Curr Opin Clin Nutr Metab Care. 2003. 6: 177-187.

155. Olivieri O, Bassi A et al. Apo C-III, metabolic syndrome, and risk of coronary artery disease. J Lipid Res. 2003. 12: 2374-2381.

156. Sniderman AD, Fuberg CD, Keech et al. Apolipoproteins versus lipids as indices of coronary risk and as target for statin treatment. Lancet 2003. 361: 777-780.

157. Sniderman AD. Applying apo B to the diagnosis and therapy of the atherogenic dyslipoproteinemias: a clinical diagnostic algorithm. Curr Opin Lipidol. 2004. 15: 433-438.

158. Yusuf S, Hawken S, Ounpuu S et al. INTERHEART Study Investigators. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. Lancet. 2004. 364: 937-952.

159. Song Y, Stampfer MJ, Liu S. Meta-analysis: apolipoprotein E genotypes and risk for coronary heart disease. Ann Intern Med. 2004. 141: 137-147.

160. Hummasti S, Laffitte BA, Watson MA et al. Liver X receptors are regulators of adipocyte gene expression but not differentiation: identification of apoD as a direct target. J Lipid Res. 2004. 45: 616-625.

161. Wolfrum C, Poy MN, Stoffel M. Apo M is required for the preβ-HDL formation ad cholesterol efflux to HDL and protects against atherosclerosis. Nat Med. 2005. 11: 418-422.

162. Lai CQ, Parnell LD, Ordovas JM. The APOA1/C3/A4/A5 gene cluster, lipid metabolism and cardiovascular disease risk. Curr Opin Lipidol. 2005. 16: 153-166.

163. Navab M, Anantharamaiah GM, Reddy ST et al. An oral apoJ peptide renders HDL antiinflammatory in mice and monkeys and dramatically reduces atherosclerosis in apolipoprotein E-null mice. Arterioscler Thromb Vasc Biol. 2005. 25: 1932-1937.

164. Albert TS, Duchateau PN, Deeb SS et al. Apolipoprotein L-I is positively associated with hyperglycemia and plasma triglycerides in CAD patients with low HDL. J Lipid Res. 2005. 46: 469-474.

165. Matsuura E, Kobayashi K et al. Oxidized LDL/beta2-glycoprotein I complexes: new aspects in atherosclerosis. Lupus. 2005. 14: 736-741

166. Orlandi A, Pucci S, Ciucci A et al. Modulation of clusterin isoforms is associated with all-trans retinoic acid-induced proliferative arrest and apoptosis of intimal smooth muscle cells. Arterioscler Thromb Vasc Biol. 2005. 25: 348-353.

167. Grosskopf I, Baroukh N, Lee SJ et L. Apolipoprotein A-V deficiency results in marked hypertriglyceridemia attributable to decreased lipolysis of triglyceride-rich lipoproteins and removal of their remnants. Arterioscler Thromb Vasc Biol. 2005. 25: 2573-2579.

168. Dahlback B, Nielsen LB. Apolipoprotein M--a novel player in high-density lipoprotein metabolism and atherosclerosis. Curr Opin Lipidol. 2006. 17: 291-295.

169. Vanhollebeke B, Pays E. The function of apolipoproteins L. Cell Mol Life Sci. 2006. 63: 1937-1944.

170. Reilly M, Rader DJ. Apolipoprotein E and Coronary Disease: A Puzzling Paradox. PLoS Med. 2006. 3: e258.

171. Walldius G, Jungner I. The apoB/apoA-I ratio: a strong, new risk factor for cardiovascular disease and a target for lipid-lowering therapy; a review of the evidence. J Intern Med. 2006. 259: 493-519.

172. Whitfield AJ, Barrett PH, van Bockxmeer FM, Burnett JR. Lipid disorders and mutations in the APOB gene. Clin Chem. 2004. 50: 1725-1732.

173. Davidson NO, Shelness GS. APOLIPOPROTEIN B: mRNA editing, lipoprotein assembly, and presecretory degradation. Annu Rev Nutr. 2000. 20: 169-193.

174. Lamarche B, Tchernof A et al. Fasting insulin and apo B levels and low-density lipoprotein particle size as risk factors for ischemic heart disease. JAMA. 1998. 279: 1955-1961.

175. Packard CJ. Understanding coronary heart disease as a consequence of defective regulation of apo B metabolism. Curr Opin Lipidol. 1999. 10: 237-244.

176. Asztalos BF, Roheim PS, Milani RL et al. Distribution of ApoA-I-containing HDL subpopulations in patients with coronary heart disease. Arterioscler Thromb Vasc Biol. 2000. 20: 2670-2676.

177. de Beer MC, Durbin DM, Cai L et al. Apolipoprotein A-I conformation markedly influences HDL interaction with scavenger receptor BI. J Lipid Res. 2001. 42: 309-313.

178. Scott BR, McManus DC, Franklin V et al. The N-terminal globular domain and the first class A amphipathic helix of apolipoprotein A-I are important for lecithin:cholesterol acyltransferase

activation and the maturation of high density lipoprotein in vivo. J Biol Chem. 2001. 276: 48716-48724.

179. Cho KH, Durbin DM, Jonas A. Role of individual amino acids of apolipoprotein A-I in the activation of lecithin:cholesterol acyltransferase and in HDL rearrangements. J Lipid Res. 2001. 42: 379-389.

180. Temel RE, Walzem RL et al. Apolipoprotein A-I is necessary for the in vivo formation of high density lipoprotein competent for scavenger receptor BI-mediated cholesteryl ester-selective uptake. J Biol Chem. 2002. 277: 26565-26572.

181. Rye KA, Barter PJ. Formation and metabolism of prebeta-migrating, lipid-poor apolipoprotein A-I. Arterioscler Thromb Vasc Biol. 2004. 24: 421-428.

182. Navab M, Anantharamaiah GM, Reddy ST et al. Apolipoprotein A-I mimetic peptides. Arterioscler Thromb Vasc Biol. 2005. 25: 1325-1331.

183. Pownall HJ, Ehnholm C. The unique role of apolipoprotein A-I in HDL remodeling and metabolism. Curr Opin Lipidol. 2006. 17: 209-213.

184. Curtiss LK, Valenta DT et al. What is so special about apolipoprotein AI in reverse cholesterol transport? Arterioscler Thromb Vasc Biol. 2006. 26: 12-19.

185. Stein O, Stein Y. Atheroprotective mechanisms of HDL. Atherosclerosis. 1999. 144: 285-301.

186. Segrest JP, Li L, Anantharamaiah GM et al. Structure and function of apo A-I and high-density lipoprotein. Curr Opin Lipidol. 2000. 11: 105-115.

187. Mackness MI, Durrington PN, Mackness B. How high-density lipoprotein protects against the effects of lipid peroxidation. Curr Opin Lipidol. 2000. 11: 383-388.

188. Barter P, Kastelein J et al. Future Forum Editorial Board. High density lipoproteins (HDL) and atherosclerosis; the unanswered questions. Atherosclerosis. 2003. 168: 195-211.

189. Rye KA, Wee K et al. Apo A-II inhibits high density lipoprotein remodeling and lipid-poor apo A-I formation. J Biol Chem. 2003. 25: 22530-22536.

190. Boucher J, Ramsamy TA et al. Apo A-II regulates HDL stability and affects hepatic lipase association and activity. J Lipid Res. 2004. 5: 849-858.

191. Martin-Campos JM, Escola-Gil JC et al. Apolipoprotein A-II, genetic variation on chromosome 1q21-q24, and disease susceptibility. Curr Opin Lipidol. 2004. 15: 247-253.

192. Blanco-Vaca F, Escola-Gil JC et al. Role of apoA-II in lipid metabolism and atherosclerosis: advances in the study of an enigmatic protein. J Lipid Res. 2001. 42: 1727-1739.

193. Tailleux A, Duriez P, Fruchart JC. ApoA-II, HDL metabolism and atherosclerosis. Atherosclerosis. 2002. 164: 1-13.

194. Allayee H, Castellani LW et al. Biochemical and Genetic association pf plasma apoA-II levels with familial combined hyperlipidemia. 2003. Circ Res. 92: 1262-1267.

195. Assmann G, Nofer JR. Atheroprotective effects of high-density lipoproteins. Annu Rev Med. 2003. 54: 321-341.

196. Kalopissis AD, Pastier D, Chambaz J. Apolipoprotein A-II: beyond genetic associations with lipid disorders and insulin resistance. Curr Opin Lipidol. 2003. 14: 165-172.

197. Ribas V, Sanchez-Quesada JL, Anton R et al. Human apolipoprotein A-II enrichment displaces paraoxonase from HDL and impairs its antioxidant properties: a new mechanism linking HDL protein composition and antiatherogenic potential. Circ Res. 2004. 95: 789-797.

198. de Beer MC, Castellani LW, Cai L et al. ApoA-II modulates the association of HDL with class B scavenger receptors SR-BI and CD36. J Lipid Res. 2004. 45: 706-715.

199. Rotllan N, Ribas V, Calpe-Berdiel L et al. Overexpression of human apolipoprotein A-II in transgenic mice does not impair macrophage-specific reverse cholesterol transport in vivo. Arterioscler Thromb Vasc Biol. 2005. 25: e128-e132.

200. de Beer MC, van der Westhuyzen DR, Whitaker NL et al. SR-BI-mediated selective lipid uptake segregates apoA-I and apoA-II catabolism. J Lipid Res. 2005. 46: 2143-2150.

201. Dugue-Pujol S, Rousset X, Pastier D et al. Human apolipoprotein A-II induces hypertriglyceridemia in transgenic mice by associating with triglyceride-rich lipoproteins in plasma and impairing their catabolism. J Lipid Res. 2006. 47: 2631-2639.

202. Rocco AG, Mollica L, Gianazza E et al. A model structure for the heterodimer apoA-I Milano-apoA-II supports its peculiar susceptibility to proteolysis. Biophys J. 2006. 91: 3043-3049.

203. Broedl UC, Jin W, Fuki IV et al. Endothelial lipase is less effective at influencing HDL metabolism in vivo in mice expressing apoA-II. J Lipid Res. 2006. 47: 2191-2197.

204. Hu FB, Willett WC. Optimal diets for prevention of coronary heart disease. JAMA. 2002. 288: 2569-2578.

205. Bravata DM, Sanders L, Huang J et al. Efficacy and safety of low-carbohydrate diets: a systematic review. JAMA. 2003. 289: 1837-1850.

206. Esposito K, Marfella R, Ciotola M et al. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. JAMA. 2004. 292: 1440-1446.

207. Griel AE, Kris-Etherton PM. Beyond saturated fat: the importance of the dietary fatty acid profile on cardiovascular disease. Nutr Rev. 2006. 64: 257-262.

208. Miller Iii ER, Erlinger TP, Appel LJ. The Effects of Macronutrients on Blood Pressure and Lipids: An Overview of the DASH and OmniHeart Trials. Curr Atheroscler Rep. 2006. 8: 460-465.

209. Zarraga IG, Schwarz ER. Impact of dietary patterns and interventions on cardiovascular health. Circulation. 2006. 114: 961-973.

210. Basu A, Devaraj S, Jialal I. Dietary factors that promote or retard inflammation. Arterioscler Thromb Vasc Biol. 2006. 26: 995-1001.

211. Hooper L, Thompson RL, Harrison RA et al. Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. BMJ. 2006. 332: 752-760.

212. Krauss RM, Eckel RH, Howard B et al. AHA Dietary Guidelines: revision 2000: A statement for healthcare professionals from the Nutrition Committee of the American Heart Association. Circulation. 2000. 102: 2284-2299.

213. Lichtenstein AH, Appel LJ, Brands M et al. American Heart Association Nutrition Committee.Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee. Circulation. 2006. 114: 82-96.

214. Lada AT, Rudel LL. Dietary monounsaturated versus polyunsaturated fatty acids: which is really better for protection from coronary heart disease? Curr Opin Lipidol. 2003. 14: 41-46.

215. Howard BV, Van Horn L, Hsia J et al. Low-fat dietary pattern and risk of cardiovascular disease: the Women's Health Initiative Randomized Controlled Dietary Modification Trial. JAMA. 2006. 295: 655-666.

216. Ross E. Intestinal absorption of triglyceride and cholesterol. Dietary and pharmacological inhibition to reduce cardiovascular risk. Atherosclerosis. 2000. 151: 357–380.

217. Han SN, Leka LS, Lichtenstein AH et al. Effect of a therapeutic lifestyle change diet on immune functions of moderately hypercholesterolemic humans. J Lipid Res. 2003. 44: 2304-2310.

218. German JB, Dillard CJ. Saturated fats: what dietary intake? Am J Clin Nutr. 2004. 80: 550-559.

219. Varady KA, Jones PJ. Combination diet and exercise interventions for the treatment of dyslipidemia: an effective preliminary strategy to lower cholesterol levels? J Nutr. 2005. 135: 1829-1835.

220. Helsing E. Traditional diets and disease patterns of the Mediterranean, circa 1960. Am J Clin Nutr. 1995. 61 (6 Suppl): 1329S-1337S.

221. Trichopoulou A, Lagiou P. Healthy traditional Mediterranean diet: an expression of culture, history, and lifestyle. Nutr Rev. 1997. 55: 383-339.

222. Simopoulos AP. The Mediterranean diets: What is so special about the diet of Greece? The scientific evidence. J Nutr. 2001. 131 (11 Suppl): 3065S-3073S.

223. Trichopoulou A. Traditional Mediterranean diet and longevity in the elderly: a review. Public Health Nutr. 2004. 7: 943-947.

224. Keys A et al. Coronary heart disease in seven countries. XVI. The electrocardiogram in prediction of five-year coronary heart disease incidence among men aged forty through fifty-nine. Circulation. 1970. 41: 1-198.

225. Willett W, Sacks F et al. Mediterranean diet pyramid: a cultural model for healthy eating. Am J Clin Nutr. 1995. 61: 1402–1406.

226. Willett WC. The Mediterranean diet: science and practice. Public Health Nutr. 2006. 9: 105-110.

227. http://www.nal.usda.gov/fnic/foodcomp/Data/HG72/hg72_2002.pdf (Nutritive Value of Foods, United States Department of Agriculture).

228. Ramirez-Tortosa MC, Urbano G, Lopez-Jurado M et al. Extra-virgin olive oil increases the resistance of LDL to oxidation more than refined olive oil in free-living men with peripheral vascular disease. J Nutr. 1999. 129: 2177-2183.

229. Kris-Etherton PN. Monounsaturated fatty acids and risk of cardiovascular disease. Circulation. 1999. 100: 1253–1258.

230. Jansen S, Lopez-Miranda J, Castro P et al. Low-fat and high-monounsaturated fatty acid diets decrease plasma cholesterol ester transfer protein concentrations in young, healthy, normolipemic men. Am J Clin Nutr. 2000. 72: 36-41.

231. Hu FB, Stampfer MJ et al. Trends in the incidence of coronary heart disease and changes in diet and lifestyle in women. New Engl J Med. 2000. 343: 530–537.

232. Hargrove RL, Etherton TD, Pearson TA et al. Low fat and high monounsaturated fat diets decrease human low density lipoprotein oxidative susceptibility in vitro. J Nutr. 2001. 131: 1758-1763.

233. Mertens A, Holvoet P. Oxidized LDL and HDL: antagonists in atherothrombosis. FASEB J. 2001. 15: 2073-2084.

234. Fuentes F, López-Miranda J et al. A Mediterranean and NCEP-I diet improve endothelial function in hypercholesterolemic males. Ann Intern Med. 2001. 134: 1115–1119.

235. Tuck KL, Hayball PJ. Major phenolic compounds in olive oil: metabolism and health effects. Nutr Biochem. 2002. 13: 636-644.

236. Martinez-Gonzalez MA, Alonso A et al. What is protective in the Mediterranean diet? Atherosclerosis. 2003. 166: 405-457.

237. Togna GI, Togna AR et al. Olive oil isochromans inhibit human platelet reactivity. J Nutr. 2003. 133: 2532-2526.

238. Carluccio MA, Siculella L et al. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: antiatherogenic properties of Mediterranean diet phytochemicals. Arterioscler Thromb Vasc Biol. 2003. 23:622-629.

239. Esposito K, Marfella R, Ciotola M et al. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. JAMA. 2004. 292: 1440-1446.

240. Hashim YZ, Eng M et al. Components of olive oil and chemoprevention of colorectal cancer. Nutr Rev. 2005. 63: 374-386.

241. Pitsavos C, Panagiotakos DB, Tzima N et al. Adherence to the Mediterranean diet is associated with total antioxidant capacity in healthy adults: the ATTICA study. Am J Clin Nutr. 2005. 82: 694-699.

242. Appel LJ, Sacks FM, Carey VJ et al. OmniHeart Collaborative Research Group. Effects of protein, monounsaturated fat, and carbohydrate intake on blood pressure and serum lipids: results of the OmniHeart randomized trial. JAMA. 2005. 294: 2455-2464.

243. Perez-Jimenez F, Alvarez de Cienfuegos G, Badimon L et al. International conference on the healthy effect of virgin olive oil. Eur J Clin Invest. 2005. 35: 421-424.

244. Hamdi HK, Castellon R. Oleuropein, a non-toxic olive iridoid, is an anti-tumor agent and cytoskeleton disruptor. Biochem Biophys Res Commun. 2005. 334: 769-778.

245. Serra-Majem L, Roman B, Estruch R. Scientific evidence of interventions using the Mediterranean diet: a systematic review. Nutr Rev. 2006. 64: S27-S47

246. Alonso A, Ruiz-Gutierrez V, Martinez-Gonzalez MA. Monounsaturated fatty acids, olive oil and blood pressure: epidemiological, clinical and experimental evidence. Public Health Nutr. 2006. 9: 251-257.

247. Perona JS, Cabello-Moruno R, Ruiz-Gutierrez V. The role of virgin olive oil components in the modulation of endothelial function. J Nutr Biochem. 2006. 17: 429-445.

248. Bogani P, Galli C et al. Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. Atherosclerosis. 2006. [Epub ahead of print].

249. Ramírez-Tortosa MC, Urbano G et al. Extra-virgin olive oil more than refined olive oil increases the resistence of low density lipoprotein to oxidation in free-living male patients with peripheral vascular disease. J Nutr. 1999. 129: 2177-2183.

250. Visioli F, Galli C. The effects of minor constituents of olive oil on cardiovascular disease: new findings. Nutr Rev. 1998. 59: 142-147.

251. Visioli F, Caruso D, Grande S et al. Virgin Olive Oil Study (VOLOS): vasoprotective potential of extra virgin olive oil in mildly dyslipidemic patients. Eur J Nutr. 2005. 44: 121-127.

252. Covas MI, de la Torre K, Farre-Albaladejo M et al. Postprandial LDL phenolic content and LDL oxidation are modulated by olive oil phenolic compounds in humans. Free Radic Biol Med. 2006. 40: 608-616.

253. http://biophysics.bumc.bu.edu/research/atherolipo/athlip.html (Techniques).

254. www.aacb.asn.au/educ/exam/bofe_syllabus.pdf (Analitical Biochemistry).

255. http://bioprotocols.endlex.com/ (Biological Research Protocol Books).

256. Burnett J R, Barrett PHR. Apo B metabolism: tracer kinetics, models, and metabolic studies. Crit Rev Clin Lab Sci. 2002. 39: 89–137.

257. Pont F, Duvillard L et al. Development of compartmental models in stable-isotope experiments: application to lipid metabolism. Arterioscler Thromb Vasc Biol. 1998. 18: 853-860.

258. Millar JS, Packard CJ. Heterogeneity of apo B-100-containing lipoproteins: what we have learnt from kinetic studies. Curr Opin Lipidol. 1998. 9: 197-202.

259. Ramakrishnan R. Studying apolipoprotein turnover with stable isotope tracers - correct analysis is by modeling enrichments. J Lipid Res. 2006. 2006. 47: 2738-2753.

260. Barrett PH, Chan DC, Watts GF. Thematic review series: patient-oriented research. Design and analysis of lipoprotein tracer kinetics studies in humans. J Lipid Res. 2006. 47: 1607-1619.

261. Parhofer KG, Barrett PHR et al. Determination of kinetic parameters of apo B metabolism using amino acids labeled with stable isotopes. J Lipid Res. 1991. 32: 1311–1323.

262. Schaefer JR, Rader DJ, Brewer Jr HB. Investigations of lipoprotein kinetics using endogenous labeling with stable isotopes. Curr Opin Lipidol. 1992. 3: 227–232.

263. Watson TD. New in-vivo techniques for measuring lipoprotein metabolism. Curr Opin Lipidol. 1995. 6: 182–186.

264. Demant T, Packard CJ et al. Sensitive methods to study human apo B metabolism using isotope-labeled amino acids. Am J Physiol. 1996. 270: E1022–E1036.

265. Patterson BW. Use of stable isotopeically labeled tracers for studies of metabolic kinetics: an overview. Metab Clin Exp. 1997. 46: 322–329.

266. Pietzsch J, Lattke P, Julius U. Oxidation of apolipoprotein B-100 in circulating LDL is related to LDL residence time. In vivo insights from stable-isotope studies. Arterioscler Thromb Vasc Biol. 2000. 20: E63-E67.

267. Marsh JB, Welty FK, Schaefer EJ. Stable isotope turn over of apolipoproteins of high-density lipoproteins in humans. Curr Opin Lipidol. 2000. 11: 261–266.

268. Duvillard L, Pont F, Florentin E et al. Significant improvement of apolipoprotein B-containing lipoprotein metabolism by insulin treatment in patients with non-insulin-dependent diabetes mellitus. Diabetologia. 2000. 43: 27-35.

269. Marsh JB, Welty FK et al. Apo B metabolism in humans: studies with stable isotope-labeled amino acid precursors. Atherosclerosis. 2002. 162: 227–244.

270. Nierman MC, Prinsen BH, Rip J et al. Enhanced conversion of triglyceride-rich lipoproteins and increased low-density lipoprotein removal in LPLS447X carriers. Arterioscler Thromb Vasc Biol. 2005. 25: 2410-2415.

271. Ikewaki K, Schaefer JR, Frischmann ME et al. Delayed in vivo catabolism of intermediate-density lipoprotein and low-density lipoprotein in hemodialysis patients as potential cause of premature atherosclerosis. Arterioscler Thromb Vasc Biol. 2005. 25: 2615-2622.

272. Ooi EM, Watts GF, Farvid MS et al. High-density lipoprotein apolipoprotein A-I kinetics in obesity. Obes Res. 2005. 13: 1008-1016.

273. Parhofer KG, Barrett PH. Thematic review series: patient-oriented research. What we have learned about VLDL and LDL metabolism from human kinetics studies. J Lipid Res. 2006. 47: 1620-1630.

274. Rashid S, Patterson BW, Lewis GF. Thematic review series: patient-oriented research. What have we learned about HDL metabolism from kinetics studies in humans? J Lipid Res. 2006. 47: 1631-1642.

275. Chan DC, Barrett PH, Watts GF. Recent studies of lipoprotein kinetics in the metabolic syndrome and related disorders. Curr Opin Lipidol. 2006. 17: 28-36.

276. Tremblay AJ, Lamarche B, Cohn JS et al. Effect of ezetimibe on the in vivo kinetics of apoB-48 and apoB-100 in men with primary hypercholesterolemia. Arterioscler Thromb Vasc Biol. 2006. 26: 1101-1106.

277. Chetiveaux M, Lalanne F, Lambert G et al. Kinetics of prebeta1 HDL and alphaHDL in type II diabetic patients. Eur J Clin Invest. 2006. 36: 29-34.

278. Steiner G, Poapst ME et al. Metabolism of the apo B-containing lipoproteins. Methods Enzymol. 1986. 129: 395–420.

279. Grundy SM, Mok HY et al. Transport of very low density lipoprotein triglycerides in varying degrees of obesity and hypertriglyceridemia. J Clin Invest. 1979. 63: 1274-1283.

280. Ramakrishnan R, Arad Y, Wong S, Ginsberg HN. Nonuniform radiolabeling of VLDL apo B: implications for the analysis of studies of the kinetics of the metabolism of lipoproteins containing apo B. J Lipid Res. 1990. 31: 1031-1042.

281. Fisher WR, Zech LA, Kilgore LL, Stacpoole PW. Metabolic pathways of apo B in heterozygous familial hypercholesterolemia: studies with a [3H]leucine tracer. J Lipid Res. 1991. 32: 1823-1836.

282. Gaw A, Packard CJ, Shepherd J. VLDL and LDL turnover: methods and clinical application. Curr Opin Lipidol. 1992. 3: 384-388.

283. Halliday D, Venkatesan S, Pacy P. Apolipoprotein metabolism: a stable-isotope approach.Am J Clin Nutr. 1993. 57 (Suppl 5): 726-730 (discussion 730-731).

284. Demant T, Packard CJ. Studies of apo B-100 metabolism using radiotracers and stable isotopes. Eur J Pediatr. 1997. 156 Suppl 1: 75-77.

285. Young VR, Ajami A. The Rudolf Schoenheimer Centenary Lecture. Isotopes in nutrition research. Proc Nutr Soc. 1999. 58: 15-32.

286. Schoenheimer R. The dynamic state of body constituents. 1942. Harvard University Press.

287. Matthews CM. The theory of tracer experiments with 131I-labelled plasma proteins. Phys Med Biol. 1957. 2: 36-53.

288. Schoenheimer R, Rittenberg D. Deuterium as an indicator in the study of intermediary metabolism. J Biol Chem. 1935. 11: 163-192.

289. Schoenheimer R, Rutner S, Rittenberg D. Studies in protein metabolism: metabolic activity of body proteins investigated with L-leucine containing two isotopes. J C Chem. 1939. 130: 730-732.

290. Bier DM. Stable isotopes in biosciences, their measurement and models for amino acid metabolism. Eur J Pediatr. 1997. 156 (Suppl 1): 2-8.

291. Vogt J. Availability of stable isotope tracers for human use. Eur J Pediatr. 1997. 156 (Suppl 1): 9-11.

292. Koletzko B, Sauerwald T, Demmelmair H. Safety of stable isotope use. Eur J Pediatr. 1997. 156 (Suppl 1): 12-17.

293. Ikewaki K, Zech LA et al. Apolipoprotein A-II production rate is a major factor regulating the distribution of apolipoprotein A-I among HDL subclasses LpA-I and LpA-I:A-II in normolipidemic humans. Arterioscler Thromb Vasc Biol. 1995. 15: 306-312.

294. Ikewaki K, Zech LA et al. ApoA-II kinetics in humans using endogenous labeling with stable isotopes: slower turn over of apoA-II compared with the exogenous radiotracer method. J Lipid Res. 1996. 37: 399-407.

295. Cobelli C, Toffolo G, Foster DM. Tracer-to-tracee ratio for analysis of stable isotope tracer data: link with radioactive kinetic formalism. Am J Physiol. 1992. 262: E968–E975.

296. Ostlund RE Jr, Matthews DE. [^{13}C]Cholesterol as a tracer for studies of cholesterol metabolism in humans. J Lipid Res. 1993. 34: 1825–1831.

297. Ikewaki K, Rader DJ et al. Evaluation of apo A-I kinetics in humans using simultaneous endogenous stable isotope and exogenous radiotracer methods. J Lipid Res. 1993. 34: 2207–2215.

298. Verges B, Rader D et al. In vivo metabolism of apo A-IV in severe hypertriglyceridemia: a combined radiotracer and stable isotope kinetic study. J Lipid Res. 1994. 35: 2280–2291.

299. http://www.uga.edu/~sisbl/stable.html (Stable Isotope).

300. Klein PD, Klein ER. Stable isotopes: origins and safety. J Clin Pharmacol. 1986. 26: 378-382.

301. Jones PJ, Leatherdale ST. Stable isotopes in clinical research: safety reaffirmed. Clin Sci (Lond). 1991. 80: 277-280.

302. Gregg CT, Hutson JY et al. Substantial replacement of mammalian body carbon with carbon-13. Life Sci. 1973. 13: 775-782.

303. Thompson GN, Pacy PJ, Ford GC, Halliday D. Practical considerations in the use of stable isotope labelled compounds as tracers in clinical studies. Biomed Environ Mass Spectrom. 1989. 18: 321-317.

304. Wolfe RR. Radioactive and stable isotope tracers in biomedicine-principles and practice of kinetic analysis. John Wiley Ed. 1992.

305. Urey HC, Brickwedge FG, Murphy GM. A hydrogen isotopere of mass 2. Physical Reviews. 1932. 40: 1-15.

306. Lewis GN. The biochemistry of water containing heavy hidrogen. 1933. J Am Chem Soc. 4: 3503-3504.

307. http://www.fact-index.com/d/de/deuterium.html (Deuterium).

308. Blake M, Crespi HL, Katz JJ. Studies with deuterated drugs. J Pharm Sci. 1975. 64: 367-391.

309. Jacques JA. Compartmental Analysis in Biology and Medicine. University of Michigan Press, Ann Arbor, MI. 1985.

310. Lichtenstein AH, Cohn JS et al. Comparision of deuterated leucine, valine, and lysine in the measurement of human A-I and B-100 kinetics. J Lipid Res. 1990. 31: 1693- 1701.

311. Fisher WR, Venkatakrishnan V et al. The 3H-leucine tracer: its use in kinetic studies of plasma lipoproteins. Metabolism. 1997. 46: 333-342.

312. Cohn JS, Wagner DA et al. Measurement of VLDL and LDL apo B-100 and HDL apoA-I in human subjects using deuterated leucine. Effect of FAting and feading. J Clin Invest. 1990. 85: 804-811.

313. Rennie MJ. An introduction to the use of tracers in nutrition and metabolism. Proc Nutr Soc. 1999. 58: 935-944.

314. Searle GL, Strisower EH, Chaikoff IL. Glucose pool and glucose space in the normal and diabetic dog. Am J Physiol. 1954. 176: 190-194.

315. Fisher WR, Zech LA et al. The metabolism of apolipoprotein B in subjects with hypertriglyceridemia and polydisperse LDL. J Lipid Res. 1980. 21: 760-774.

316. Foster DM et al. Estimating the fractional synthetic rate of plasma apolipoproteins and lipids from stable isotope data. J Lipid Res. 1993. 34: 2193-2205.

317. Welty FK, Lichtenstein AH, Barrett PH et al. Human apolipoprotein (Apo) B-48 and Apo B-100 kinetics with stable isotopes. Arterioscler Thromb Vasc Biol. 1999. 19: 2966-2974.

318. Packard CJ. The role of stable isotopes in the investigation of plasma lipoprotein metabolism. Baillieres Clin Endocrinol Metab. 1995. 9: 755-772

319. Packard CJ, Gaw A, Demant T, Shepherd J. Development and application of a multicompartmental model to study very low density lipoprotein subfraction metabolism. J Lipid Res. 1995. 36: 172-187.

320. Foster DM, Barrett PHR. Considerations in designing and analysing data from apo B turn over studies. In Hypercholesterolemia, Hypocholesterolemia, Hypertriglyceridemia. Malmendier CL Ed. Plenum Press. 1999. pp: 201–211.

321. Barrett PHR, Foster DM. Design and analysis of lipid tracer kinetic studies. Curr Opin Lipidol. 1996. 7: 143–148.

322. Wagenmakers AJ. Tracers to investigate protein and amino acid metabolism in human subjects. Proc Nutr Soc. 1999. 58: 987-1000.

323. Cobelli C, Toffolo G, Bier DM, Nosadini R. Models to interpret kinetic data in stable isotope tracer studies. Am J Physiol. 1987. 253: 551-564.

324. Cobelli C, Saccomani MP. Domain of validity of classical models of leucine metabolism assessed by compartmental modeling. Math Biosci. 1991. 107: 3-20.

325. Patterson BW, Hachey DL et al. Incorporation of a stable isotopeically labeled amino acid into multiple human apolipoproteins. J Lipid Res. 1991. 32: 1063-1072.

326. Cobelli C, Saccomani MP et al. Compartmental model of leucine kinetics in humans. Am J Physiol. 1991. 261: 539-550.

327. Cobelli C, Caumo A. Using what is accessible to measure that which is not: necessity of model of system. Metabolism. 1998. 47: 1009-1035.

328. Clifford AA, Müller HG. Mathematical modeling in experimental nutrition. Plenum Press. 1998.

329. Wilson PD, Dainty JR. Modelling in nutrition: an introduction. Proc Nutr Soc. 1999. 58: 133-138.

330. Packard CJ, Demant T et al. Apo B metabolism and the distribution of VLDL and LDL subfractions. J Lipid Res. 2000. 41: 305-317.

331. Berman M. Kinetic analysis and modeling: theory and application to lipoproteins. In Lipoprotein Kinetics and Modeling. Berman M, Grundy SM, Howard B. V Ed. Academic Press, New York. 1982. pp: 3–26.

332. Vélez-Carrasco W, Lichtenstein AH et al. Human apoA-I kinetics within TRL and HDL. J Lipid Res. 1999. 40: 1695-1700.

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333. Ouguerram K, Krempf M et al. A new labeling approach using stable isotopes to study in vivo plasma cholesterol metabolism in humans. Metabolism. 2002. 51: 5-11.

334. Hugh P, Barrett R, Foster DM. Design and analysis of lipid tracer kinetic studies. Curr Opin Lipidol. 1996. 7: 143-1488.

335. Cryer DR, Matsushima T, Marsh JB et al. Direct measurement of apo B synthesis in human VLDL using stable isotopes and mass spectrometry. J Lipid Res. 1986. 27: 508-516.

336. http://depts.washington.edu/saam2/ (SAAM Institute Inc, University of Washington).

337. Barrett PH, Bell BM et al. SAAM II: simulation, analysis, and modeling software for tracer and pharmacokinetic studies. Metab Clin Exp. 1998. 47: 484–492.

338. Cobelli C, Foster DM. Compartmental models: theory and practice using the SAAM II software system. Adv Exp Med Biol. 1998. 445: 79–101.

2. Methodology's bibliography

339. Lewis B, Turner PR et al. Metabolic epidemiology of plasma cholesterol: a study of the mechanisms of variation of cholesterol levels within and between populations. Lancet. 1986, 2: 991-996.

340. Joven J, Villabona C et al. Abnormalities of lipoprotein metabolism in patients with the nephrotic syndrome. New Engl J Med. 1990. 323: 579-584.

341. Solà R, Masana L, Sardà P et al. Determinantes metabólicas de las concentraciones de colesterol en el hombre (I). Receptores y cinética de LDL. Med Clin (Barc). 1987. 89: 807-810.

342. Solà R, Masana L, Sardà P et al. Determinantes metabólicas de las concentraciones de colesterol en el hombre. (II). Factores dietéticos. Med Clin (Barc). 1987. 89: 811-814.

343. http://ww.mtas.es/insht/ntp/ntp 508.htm (Ministerio de Trabajo y Asuntos Sociales, Instituto Nacional de Seguridad e Higiene en el Trabajo, INSHT).

344. http:// www.iso.org (International Standards Organization).

345. James PW, Martin B et al. Apo B metabolism in homozygous familial hypercholesterolemia. J Lipid Res. 1989. 30: 159-169.

346. Lindgren FT et al. The isolation and quantitation analysis of serum lipoproteins. In Blood lipids and lipoproteins: Quantitation, Composition and Metabolism. Nelson GJ, Editor, Wiley-Interscience, New York. 1972. pp: 181-274.

347. Chapman MJ, Laplaud PM et al. Further resolution of the low density lipoprotein spectrum in normal human plasma: physicochemical characteristics of discrete subspecies separated by density gradient ultracentrifugation. J Lipid Res. 1988. 29: 442-458.

348. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest. 1955. 9: 1345-1353.

349. Segrest, JP, Albers JJ. Methods in Enzymology. Academic Pess, Inc. 1986. Vol 128.

350. Redgrave TG, Roberts DC, West CE. Separation of plasma lipoproteins by density-gradient ultracentrifugation. Anal Biochem. 1975. 65: 42- 49.

351. Egusa G, Brady DW et al. Isopropanol precipitation method for the determination of apo B specific activity and plasma concentrations during metabolic studies of the VLDL and LDL apo B. Lipid Res. 1983. 24: 1261-1267.

352. Kane JP. A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. Anal. Biochem. 1973. 53: 350-364.

353. Kane JP, Sata T et al. Apoprotein composition of VDLD of human serum. J. Clin. Invest. 1975. 56: 1622-1634.

354. Bloor WR. A method for the determination of fat in small amounts of blood. J Biol Chem. 1914. 17: 377.

355. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957. 226: 497-509.

356. http://www.chem.tamu.edu/rgroup/macfarlane/delipidation.htm (Delipidation).

357. http://www.cyberlipid.org/extract/extr0002.htm# (Lipid extraction).

358. http://www.chromatography.amershambiosciences.com (General Electric Company).

359. Ausubel FM et al. Current Protocols in Molecular Biology. Wiley, Inc. 1994. Vol.2.

360. Bradford, MM. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 1976. 72: 248-254.

361. Stoscheck, CM. Quantitation of Protein. Methods in Enzymology. 1990. 182: 50-69.

362. http://www.bio-rad.com (Bio-Rad Laboratories, Inc).

363. http://www.rpi.edu/dept/chem-eng/Biotech-Environ/LYO/ (Lyophilisation).

364. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970. 227: 680-685.

365. http://www.mcb.uct.ac.za/ sdspage.html (SDS-PAGE).

366. http://www.invitrogen.com (Invitrogen, Corp).

367. http://www.chemguide.co.uk/organicprops/aminoacids/proteinhydrolysis.html (The Hydrolysis of Proteins).

368. Segrest JP, Albers JJ. Methods in Enzymology. Academic Pess, Inc. 1986. Vol 182.

369. http://www.rit.edu/~pac8612/webionex/website/html/contents.html (Ion Exchange Chromatography).

370. http://sosnick.uchicago.edu/precpintro.html (Protein Precipitation).

371. http://www.protocol−online.org/prot/Molecular_Biology/Protein/Extraction___Purification/Protein_Precipitation/index.html (Protein Precipitation Protocols).

372. http://www.thermo.com (Thermo Electron Corp).

373. Chaves Das Neves HJ, Vasconcelos AM. Capillary gas chromatography of amino acids, including asparagine and glutamine: sensitive gas chromatographic-mass spectrometric and selected ion monitoring gas chromatographic-mass spectrometric detection of the N,O(S)-tert.-butyldimethylsilyl derivatives. J Chromatogr. 1987. 392: 249-58.

374. Calder AG, Smith A. Stable isotope ratio analysis of leucine and ketoisocaproic acid in blood plasma by Gas Chromatography/Mass Spectrometry. Use of tertiary butyldimethylsilyl derivatives. Rapid Commun Mass Spectrom. 1988. 2: 14-16.

375. Schwenk WF, Berg PJ et al. Use of t-butyldimethylsilylation in the Gas Chromatographic/Mass Spectrometric analysis of physiologic compounds found in plasma using electron-impact ionization. Analytical Biochemistry. 1984. 141: 101-109.

376. Woo KL, Chang, DK. Determination of 22 protein amino acids as N(O)-tert-butyldimethylsilyl derivatives by gas chromatography. J Chromatogr. 1993. 638: 93-107.

377. http://www.spectroscopynow.com (Spectroscopy, John Wiley & Sons, Ltd).

378. http://www.anc.univie.ac.at/scripts/Gas_Chromatography_in_Capillaries.pdf (Gas Chromatography).

379. http://www.unsolvedmysteries.oregonstate.edu/GCM_05.shtml (GC-MS, how does it work).

380. Demant T et al. Sensitive mass spectrometry techniques for measuring metabolism of human apo B in vivo. Clin Chem. 1994. 9: 1825-1827.

381. Lentner C. Geigy Scientific Tables. Physical Chemistry, Composition of Blood, Hematology, Somatometric Data. West Cadwell, NJ. 1981. Vol 3. pp: 92.

382. Brewer HB Jr, Fairwell T et al. The amino acid sequence of human APOA-I, an apolipoprotein isolated from high density lipoproteins. Biochem Biophys Res Commun. 1978. 80: 623-630.

383. Kane JP, Hardman DA, Paulus HE. Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons. Proc Natl Acad Sci USA. 1980. 77: 2465-2469.

384. Schaefer JR, Rader DJ et al. In vivo metabolism of apolipoprotein A-I in a patient with homozygous familial hypercholesterolemia. Arterioscler Thromb. 1992. 12: 843-848.

385. Foster DM, Boston RC. Using Computer Simulation Models of Physiological and Metabolic Processes in Laboratory Animals. ILAR J. 1997. 2: 58-68.

386. Foster DM. Developing and testing integrated multicompartment models to describe a single-input multiple-output study using the SAAM II software system. Adv Exp Med Biol. 1998. 445: 59-78.

387. Bilz S, Wagner S et al. Effects of atorvastatin versus fenofibrate on apo B-100 and apoA-I kinetics in mixed hyperlipidemia. J Lipid Res. 2001. 45: 174-185.

388. Cobelli C, Lepschy A, Jacur GR. Identifiability in tracer experiments. Fed Proc. 1980. 39: 91-96.

389. Cobelli C, DiStefano JJ 3rd. Parameter and structural identifiability concepts and ambiguities: a critical review and analysis. Am J Physiol. 1980. 239: R7-24.

390. Parhofer KG, Barrett PH et al. Effect of pravastatin on metabolic parameters of apolipoprotein B in patients with mixed hyperlipoproteinemia. Clin Investig. 1993. 71: 939-946.

391. Parhofer KG, Barrett PH, Schwandt P. Low density lipoprotein apolipoprotein B metabolism: comparison of two methods to establish kinetic parameters. Atherosclerosis. 1999. 144: 159-166.

392. Batista MC, Welty FK et al. Apolipoprotein A-I, B-100, and B-48 metabolism in subjects with chronic kidney disease, obesity, and the metabolic syndrome. Metabolism. 2004. 53: 255-261.

393. Chan DC, Watts GF et al. ATP-binding cassette transporter G8 gene as a determinant of apolipoprotein B-100 kinetics in overweight men. Arterioscler Thromb Vasc Biol. 2004. 24: 2188-2191.

394. Matthan NR, Jalbert SM et al. TRL, IDL, and LDL apolipoprotein B-100 and HDL apolipoprotein A-I kinetics as a function of age and menopausal status. Arterioscler Thromb Vasc Biol. 2005. 25: 1691-1696.

395. Demant T, Seeberg K et al. The metabolism of lipoprotein(a) and other apolipoprotein B-containing lipoproteins: a kinetic study in humans. Atherosclerosis. 2001. 157. 325-339.

396. Gill JMR, Brown JC et al. Effects of dietary MUFA on lipoprotein concentrations, compositios and subfraction distributions and on VLDL apo B kinetics: dose-dependent effects on LDL. Am J Clin Nutr. 2003. 78: 47-56.

397. Gaw A, Packard CJ et al. Overproduction of small VLDL (Sf 20-60) in moderate hypercholesterolemia relationships between apo b kinetics and plasma lipoproteins. J Lipid Res. 1995. 36: 158-171.

398. Gregersen MI, Rawson RA. Blood volume. Physiol Rev. 1959. 39: 307-42.

399. Dagher FJ, Lyons JH et al. Blood volume measurement: a critical study prediction of normal values: controlled measurement of sequential changes: choice of a bedside method. Adv Surg. 1965. 1: 69-109.

400. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951. 1: 265-275.

401. Hartree EF. Determination of protein: a modification of the Lowry method that gives a linear photometric response. Anal Biochem. 1972. 48: 422-427.

402. Dunn MJ. Protein determination of total protein concentration. Protein Purification Methods, Oxford. IRL Press. Harris. 1992.

403. Cummings MH, Watts GF et al. Comparison of immunoturbidimetric and Lowry methods for measuring concentration of very low density lipoprotein apolipoprotein B-100 in plasma. J Clin Pathol. 1994. 47: 176-178.

404. Price, NC. Proteins. Labfax, Oxford. Academic Press. 1996.

405. http://www-class.unl.edu/biochem/protein_assay/lowry_assay.htm (The Lowry Assay).

406. http://www.roche-ic.com/content/about_us/our_history/cobas_mira/ (Cobas Mira).

407. http://www.wakochemicals.de/ (Wako Chemicals GmbH).

408. http://www.beckmancoulter.com (Beckman Coulter, Inc.).

409. Schaefer EJ, Zech LA et al. Human apolipoprotein A-I and A-II metabolism. J Lipid Res. 1982. 23: 850-862.

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410. http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=hstat6.section.50534 (NIH Consensus Development Program. Outdated Consensus Development Reports. Chapter 47. Lowering Blood Cholesterol To Prevent Heart Disease).

411. Harris J, Benedict F. A biometric study of basal metabolism in man. Washington DC. Carnegie Institute of Washington. 1919.

412. http://www.gencat.net/salut/depsan/units/sanitat/pdf/catnutricio2003.pdf (Els Catalans i el Consum de dieta Mediterrània).

413. http://www.gencat.net/salut/depsan/units/sanitat/html/ca/dir210/index.html (Alimentació i nutrició).

414. http://www.searteriosclerosis.org (Sociedad Española de Arteriosclerosis).

415. Répertoire général des aliments. Feinberg M, Favier JC, Ireland-Ripert J. Fondation Française pour la Nutrition (FFN) and Centre Informatique sur la Qualité des Aliments (CIQUAL). 1987. pp: 147; 231.

416. Norman A, Bellocco R et al. Validity and reproducibility of self-reported total physical activity differences by relative weight. Int J Obes Relat Metab Disord. 2001. 5: 682-688.

417. http://www-users.med.cornell.edu/~spon/picu/calc/beecalc.htm (Basal Energy Expenditure: Harris-Benedict Equation).

418. http://wzar.unizar.es/curso/nutricion/d38.html (Ingesta alimentaria: Encuestas).

419. http://www.sigmaaldrich.com/catalog/search/ProductDetail/ALDRICH/486825 (L-Leucine-5,5,5-d_3).

420. www.boe.es (Boletín Oficial del Estado y Ministerio de Sanidad y Consumo).

421. Real Farmacopea Española. 2ª edición. Ministerio de Sanidad y Consumo. España. 2003.

422. Levin J. The Limulus Amebocyte Lysate Test: Perspectives and Problems. Watson SW, Levin J, Novitzky TJ, eds. Detection of Bacterial Endotoxin with the limulus Amebocyte Lysate Test. New York. Alan R. Liss. 1987. pp: 1-23.

423. Iwanaga S. The Limulus clotting reaction. Curr Opin Immunol. 1993. 5: 74-82.

424. http://www.victusinc.com/MedicalProd/Sets/FILTRACION%20EN%20LINEA.html (Filtración en línea de los fluidos intravenosos)

425. http://www.pall.com/datasheet_medical_6445.asp (Pall Posidyne® NEO Intravenous Filter).

426. http://www.omron-healthcare.com (Blood Pressure Monitor).

427. Lohman TG, Roche AF, Martorell R. Anthropometric standardization reference manual. Champaign, IL: Human Kinetics Books. 1988.

428. http://www.bendermedsystems.com (Bender MedSystems).

429. http://www.imtec-berlin.de/ (IMTEC Immundiagnostika GmbH).

430. http://www.mercodia.se/global/mainpage.asp?page_id=79 (Mercodia Oxidized LDL ELISA).

431. Fisher RA. Statistical Methods for Research Workers. 10th ed. Edinburgh: Oliver and Boyd. 1948.

432. http://www.statsoft.com/textbook/glosp.html (Pearson Correlation).

433. www.spss.com/ (SPSS Inc).

3. Discussion's bibliography

434. Chan DC, Barrett PH, Watts GF. Lipoprotein transport in the metabolic syndrome: methodological aspects of stable isotope kinetic studies. Clin Sci (Lond). 2004. 107: 221-232.

435. Barrett PH, Chan DC, Watts GF. Design and analysis of lipoprotein tracer kinetic studies in humans. J Lipid Res. 2006. 47: 1607-1619.

436. Recalde D, Velez-Carrasco W, Civeira F et al. Enhanced fractional catabolic rate of apo A-I and apo A-II in heterozygous subjects for apo A-I(Zaragoza) (L144R). Atherosclerosis. 2001. 154: 613-623.

437. Gitlin D, Cornwell DG et al. Studies on the metabolism of plasma proteins in the nephrotic syndrome. II. The lipoproteins. J Clin Invest. 1958. 37: 172-184.

438. Mcfarlane AS. Efficient trace-labelling of proteins with iodine. Nature. 1958. 182: 53.

439. Eaton RP, Berman M, Steinberg D. Kinetic studies of plasma free fatty acid and triglyceride metabolism in man. J Clin Invest. 1969. 48: 1560-1579.

440. Bilheimer DW, Eisenberg S, Levy RI. The metabolism of very low density lipoprotein proteins. Preliminary in vitro and in vivo observations. Biochim Biophys Acta. 1972. 260: 212-221.

441. Berman M, Hall M 3rd et al. Metabolism of apoB and apoC lipoproteins in man: kinetic studies in normal and hyperlipoproteininemic subjects. J Lipid Res. 1978. 19: 38-56.

442. Caslake MJ, Farish E, Shepherd J. Metabolism of apolipoprotein A-I in healthy young adults. Metabolism. 1978. 27: 437- 447.

443. Zech LA, Schaefer EJ et al. Metabolism of human apolipoproteins A-I and A-II: compartmental models. J Lipid Res. 1983. 24: 60-71.

444. Packard CJ, Boag DE et al. Effects of 1,2-cyclohexanedione modification on the metabolism of very low density lipoprotein apolipoprotein B: potential role of receptors in intermediate density lipoprotein catabolism. J Lipid Res. 1985. 26: 1058-1067.

445. Beltz WF, Kesaniemi YA et al. Development of an integrated model for analysis of the kinetics of apolipoprotein B in plasma very low density lipoproteins, intermediate density lipoproteins, and low density lipoproteins. J Clin Invest. 1985. 76: 575-585.

446. Patterson BW, Lee AM. Self-association and phospholipid binding properties of iodinated polipoprotein A-I. Biochemistry. 1986. 25: 4953-4957.

447. Reeds PJ, Hachey DL et al. VLDL apolipoprotein B-100, a potential indicator of the isotopic labeling of the hepatic protein synthetic precursor pool in humans: studies with multiple stable isotopically labeled amino acids. J Nutr. 1992. 122: 457-466.

448. Horowitz BS, Goldberg IJ et al. Increased plasma and renal clearance of an exchangeable pool of apolipoprotein A-I in subjects with low levels of high density lipoprotein cholesterol. J Clin Invest. 1993. 91: 1743-1752.

449. Foster DM, Barrett PH et al. Estimating the fractional synthetic rate of plasma apolipoproteins and lipids from stable isotope data. J Lipid Res. 1993. 34: 2193-2205.

450. Millar JS, Lichtenstein AH et al. Impact of age on the metabolism of VLDL, IDL, and LDL apolipoprotein B-100 in men. J Lipid Res. 1995. 36: 1155-1167.

451. Lamarche B, Uffelman KD et al. Triglyceride enrichment of HDL enhances in vivo metabolic clearance of HDL apo A-I in healthy men. J Clin Invest. 1999. 103: 1191-1199.

452. Berman M. Kinetic analysis of turn over data. Prog Biochem Pharmacol. 1979. 15: 67-108.

453. Matsushima T, Cryer DR et al. Measurement of apolipoprotein B synthesis in perfused rat liver using stable isotopes: [15N]hippurate as a measure of the intracellular [15N]glycine precursor enrichment. J Lipid Res. 1989. 30: 841-846.

454. Jacques JA. Compartmental Analysis in Biology and Medicine. 3rd edition. University of Michigan Press, Ann Arbor, MI. 1996.

455. Patterson BW, Zhao G, Klein S. Improved accuracy and precision of gas chromatography/mass spectrometry measurements for metabolic tracers. Metabolism. 1998. 47: 706-712.

456. Brewer HB Jr, Fairwell T, Kay L et al. Human plasma proapoA-I: isolation and amino-terminal sequence. Biochem Biophys Res Commun. 1983. 113: 626-632.

457. Rader DJ, Schaefer JR et al. Increased production of apolipoprotein A-I associated with elevated plasma levels of high-density lipoproteins, apolipoprotein A-I, and lipoprotein A-I in a patient with familial hyperalphalipoproteinemia. Metabolism. 1993. 42: 1429-1434.

458. Fisher WR, Venkatakrishnan V, Zech LA et al. Kinetic evidence for both a fast and a slow secretory pathway for apolipoprotein A-I in humans. J Lipid Res. 1995. 36: 1618-1628.

459. Frenais R, Ouguerram K et al. High density lipoprotein apolipoprotein AI kinetics in NIDDM: a stable isotope study. Diabetologia. 1997. 40: 578-583.

460. Duvillard L, Pont F et al. Inefficiency of insulin therapy to correct apolipoprotein A-I metabolic abnormalities in non-insulin-dependent diabetes mellitus. Atherosclerosis. 2000. 152: 229-237.

461. Maugeais C, Mahot P et al. Effect of dietary omega-3 fatty acids on high-density lipoprotein apolipoprotein AI kinetics in type II diabetes mellitus. Atherosclerosis. 2001. 157: 131-135.

462. Cohn JS, Batal R et al. Plasma turn over of HDL apoC-I, apoC-III, and apoE in humans: in vivo evidence for a link between HDL apoC-III and apoA-I metabolism. J Lipid Res. 2003. 44: 1976-1983.

463. Chetiveaux M, Ouguerram K et al. New model for kinetic studies of HDL metabolism in humans. Eur Clin Invest. 2004. 34: 262-267.

464. Okubo K, Ikewaki K et al. Abnormal HDL apolipoprotein A-I and A-II kinetics in hemodialysis patients: a stable isotope study. J Am Soc Nephrol. 2004. 15: 1008-1015.

465. Welty FK, Lichtenstein AH et al. Interrelationships between human apolipoprotein A-I and apolipoproteins B-48 and B-100 kinetics using stable isotopes. Arterioscler Thromb Vasc Biol. 2004. 24: 1703-1707.

466. Eggerman TL, Hoeg JM, Meng MS et al. Differential tissue-specific expression of human apoA-I and apoA-II. J Lipid Res. 1991. 32: 821-828.

467. Bjorkegren J, Packard CJ et al. Accumulation of large very low density lipoprotein in plasma during intravenous infusion of a chylomicron-like triglyceride emulsion reflects competition for a common lipolytic pathway. J Lipid Res. 1996. 37: 76-86.

468. Aguilar-Salinas CA, Hugh P et al. A familial combined hyperlipidemic kindred with impaired apolipoprotein B catabolism. Kinetics of apolipoprotein B during placebo and pravastatin therapy. Arterioscler Thromb Vasc Biol. 1997. 17: 72-82.

469. Dwyer KP, Barrett PH, Chan D et al. Oxazolinone derivative of leucine for GC-MS: a sensitive and robust method for stable isotope kinetic studies of lipoproteins. J Lipid Res. 2002. 43: 344-349.

470. Kinoshita M, Okazaki M et al. Simple method for analysis of apolipoproteins in high density lipoproteins by high performance liquid chromatography. J Biochem (Tokyo). 1983. 94: 615-617.

471. Young PM, Boehm TM, Brown JE. Resolution and quantitation of apolipoproteins A-I and A-II from human high-density lipoprotein by size exclusion high-performance liquid chromatography. J Chromatogr. 1984. 311: 79-92.

472. Mezdour H, Clavey V, Kora I et al. Anion-exchange fast protein liquid chromatographic characterization and purification of apolipoproteins A-I, A-II, C-I, C-II, C-III0, C-III1, C-III2 and E from human plasma. J Chromatogr. 1987. 414: 35-45.

473. Cohn JS, McNamara JR, Cohn SD et al. Plasma apolipoprotein changes in the triglyceride-rich lipoprotein fraction of human subjects fed a fat-rich meal. J Lipid Res. 1988. 29: 925-936.

474. Caslake M, Packard CJ et al. Plama triglyceride and low density lipoprotein metabolism. Eur J Clin Invest. 1992. 22: 96-104.

475. Foster DM, Barrett PHR et al. Estimating the fractional synthetic rate of plasma apolipoproteins and lipids from stable isotope data. J Lipid Res. 1993. 34: 2193-2205.

476. Demant TC, Packard CJ et al. A sensitive mass spectrometry technique for the measurement of human apo B metabolism in vivo. Clin Chem. 1994. 40: 1825-1827.

477. Packard CJ, Gaw A et al. Development and application of a multicompartmental model to study very low density lipoprotein subfraction metabolism. J Lipid Res. 1995. 36: 172-187.

478. Xiong Z. Simple one-STEP procedure for the separation of apolipoproteins A1 and A2 by high-performance liquid chromatography. J Chromatogr B Biomed Sci Appl. 1997. 702: 216-220.

479. Packard CJ, Shepherd J. Lipoprotein heterogeneity and apo B metabolism. Arterioscler Thromb Vasc Biol. 1997. 17: 3542-3556.

480. Nazeem Nanjee M, Brinton EA. Very Small Apolipoprotein A-I-containing Particles from Human Plasma: Isolation and Quantification by High-Performance Size-Exclusion Chromatography Clin Chem. 2000. 46: 207-223.

481. Chan DC, Watts GF et al. Apolipoproteins C-III and A-V as predictors of very-low-density lipoprotein triglyceride and apolipoprotein B-100 kinetics. Arterioscler Thromb Vasc Biol. 2006. 26: 590-596.

482. Fisher WR, Zech LA et al. The metabolism of apolipoprotein B in subjects with hypertriglyceridemia and polydisperse LDL. J Lipid Res. 1980. 21: 760-774.

483. Le NA, Melish JS, Roach BC et al. Direct measurement of apoprotein B specific activity in 125I-labeled lipoproteins. J Lipid Res. 1978. 19: 578-584

484. Nestel PJ, Billington T, Fidge NH. Slower removal of intestinal apolipoprotein B-48 than of apolipoprotein B-100 in severely hypertriglyceridemic subjects. Biochim Biophys Acta. 1983. 751: 422-427.

485. Stalenhoef AF, Malloy MJ et al. Metabolism of apolipoproteins B-48 and B-100 of triglyceride-rich lipoproteins in normal and lipoprotein lipase-deficient humans. Proc Natl Acad Sci USA. 1984. 81: 1839-1843.

486. Lichtenstein AH, Hachey DL et al. Measurement of human apolipoprotein B-48 and B-100 kinetics in triglyceride-rich lipoproteins using [5,5,5-2H3]leucine. J Lipid Res. 1992. 33: 907-914.

487. Adiels M, Packard C et al. A new combined multicompartmental model for apolipoprotein B-100 and triglyceride metabolism in VLDL subfractions. J Lipid Res. 2005. 46: 58-67.

488. Krauss RM, Burke DJ. Identification of multiple subclasses of plasma low density lipoproteins in normal humans. J Lipid Res. 1982. 23: 97-104.

489. Adiels M, Taskinen MR et al. Overproduction of large VLDL particles is driven by increased liver fat content in man. Diabetologia. 2006. 49: 755-765.

490. Pease RJ, Leiper JM. Regulation of hepatic apolipoprotein-B-containing lipoprotein secretion. Curr Opin Lipidol. 1996. 7: 132-138.

491. Boren J, Veniant MM, Young SG. Apo B-100-containing lipoproteins are secreted by the heart. J Clin Invest. 1998. 101: 1197-1202.

492. Ardern HA, Benson GM et al. Apolipoprotein B overproduction by the perfused liver of the St. Thomas' mixed hyperlipidemic (SMHL) rabbit. J Lipid Res. 1999. 40: 2234-2243.

493. Shepherd J, Patsch JR et al. Dynamic properties of human high density lipoprotein apoproteins. J Lipid Res. 1978. 19: 383-389.

494. Kunitake ST, Kane JP. Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. J Lipid Res. 1982. 23: 936-940

495. Grow TE. Factors affecting the exchange of apoproteins between human high density lipoprotein subclasses in vitro. Biochem Med. 1983. 29: 248-258.

496. Ponsin G, Pownall HJ. Equilibrium of apoproteins between high density lipoprotein and the aqueous phase: modelling of in vivo metabolism. J Theor Biol. 1985. 112: 183-192.

497. Blum CB, Levy RI et al. High density lipoprotein metabolism in man. J Clin Invest. 1977. 60: 795-807.

498. Schaefer EJ, Blum CB et al. Metabolism of high-density lipoprotein apolipoproteins in Tangier disease. N Engl J Med. 1978. 299: 905-910.

499. Fidge N, Nestel P, Ishikawa T et al. Turn over of apoproteins A-I and A-II of high density lipoprotein and the relationship to other lipoproteins in normal and hyperlipidemic individuals. Metabolism. 1980. 29: 643-653.

500. Frenais R, Ouguerram K et al. Apolipoprotein A-I kinetics in heterozygous familial hypercholesterolemia: a stable isotope study. J Lipid Res. 1999. 40: 1506-1511.

501. Gottrand F, Beghin L et al. Moderate red wine consumption in healthy volunteers reduced plasma clearance of apolipoprotein A-II. Eur J Clin Invest. 1999. 29: 387-394.

502. Velez-Carrasco W, Lichtenstein AH et al. Apolipoprotein A-I and A-II kinetic parameters as assessed by endogenous labeling with [(2)H(3)]leucine in middle-aged and elderly men and women. Arterioscler Thromb Vasc Biol. 2000. 20: 801-806.

503. Cooper GR, Smith SJ, Myers GL, Sampson EJ, Magid E. Estimating and minimizing effects of biologic sources of variation by relative range when measuring the mean of serum lipids and lipoproteins. Clin Chem. 1994. 40: 227-232.

504. Schaefer EJ, Lichtenstein AH et al. Effects of National Cholesterol Education Program STEP 2 diets relatively high or relatively low in fish-derived fatty acids on plasma lipoproteins in middle-aged and elderly subjects. Am J Clin Nutr. 1996. 63: 234-241.

505. http://www.etsea.udl.es/dept/tecal/cat/localitzacio/ (Departament de Tecnologia d'Aliments, DTA).

506. Chan DC, Watts GF et al. Regulatory effects of HMG CoA reductase inhibitor and fish oils on apolipoprotein B-100 kinetics in insulin-resistant obese male subjects with dyslipidemia. Diabetes. 2002. 51: 2377-2386.

507. Mattson FH, Grundy SM. Comparison of effects of dietary saturated, monounsaturated, and polyunsaturated fatty acids on plasma lipids and lipoproteins in man. J Lipid Res. 1985. 26: 194-202.

508. Keys A, Menotti A, Karvonen MJ et al. The diet and 15-year death rate in the seven countries study. Am J Epidemiol. 1986. 124: 903-915.

509. Grundy SM. Comparison of monounsaturated fatty acids and carbohydrates for lowering plasma cholesterol. N Engl J Med. 1986. 314: 745-748.

510. Mensink RP, Katan MB. Effect of monounsaturated fatty acids versus complex carbohydrates on high-density lipoproteins in healthy men and women. Lancet. 1987. 1: 122-125.

511. Garg A, Bonanome A et al. Comparison of a high-carbohydrate diet with a high-monounsaturated-fat diet in patients with non-insulin-dependent diabetes mellitus. N Engl J Med. 1988. 319: 829-834.

512. Ginsberg HN, Barr SL, Gilbert A et al. Reduction of plasma cholesterol levels in normal men on an American Heart Association STEP 1 diet or a STEP 1 diet with added monounsaturated fat. N Engl J Med. 1990. 322: 574-579.

513. Masana L, Camprubi M et al. The Mediterranean-type diet: is there a need for further modification? Am J Clin Nutr. 1991. 53: 886-889.

514. Mensink RP, Katan MB. Effect of dietary fatty acids on serum lipids and lipoproteins. A meta-analysis of 27 trials. Arterioscler Thromb. 1992. 12: 911-919.

515. Artaud-Wild SM, Connor SL et al. Differences in coronary mortality can be explained by differences in cholesterol and saturated fat intakes in 40 countries but not in France and Finland. A paradox. Circulation. 1993. 88: 2771-2779.

516. Yu S, Derr J et al. Plasma cholesterol-predictive equations demonstrate that stearic acid is neutral and monounsaturated fatty acids are hypocholesterolemic. Am J Clin Nutr. 1995. 61: 1129-1139.

517. Oliver MF. It is more important to increase the intake of unsaturated fats than to decrease the intake of saturated fats: evidence from clinical trials relating to ischemic heart disease. Am J Clin Nutr. 1997. 66(4 Suppl): 980S-986S.

518. Garg A. High-monounsaturated-fat diets for patients with diabetes mellitus: a meta-analysis. Am J Clin Nutr. 1998. 67 (3 Suppl): 577S-582S.

519. Hu FB, Manson JE, Willett WC. Types of dietary fat and risk of coronary heart disease: a critical review. J Am Coll Nutr. 2001. 20: 5-19.

520. Trichopoulou A, Costacou T et al. Adherence to a Mediterranean diet and survival in a Greek population. N Engl J Med. 2003. 348: 2599-2608.

521. Knoops KT, de Groot LC, Kromhout D et al. Mediterranean diet, lifestyle factors, and 10-year mortality in elderly European men and women: the HALE project. JAMA. 2004. 292: 1433-1439.

522. Pelkman CL, Fishell VK, Maddox DH et al. Effects of moderate-fat (from monounsaturated fat) and low-fat weight-loss diets on the serum lipid profile in overweight and obese men and women. Am J Clin Nutr. 2004. 79: 204-212.

523. Estruch R, Martinez-Gonzalez MA, Corella D et al. Effects of a Mediterranean-Style Diet on Cardiovascular Risk Factors: A Randomized Trial. Ann Intern Med. 2006. 145: 1-11.

524. Duffy D, Rader DJ. Emerging therapies targeting high-density lipoprotein metabolism and reverse cholesterol transport. Circulation. 2006. 113: 1140-1150.

525. Covas MI, Nyyssonen K, Poulsen HE et al. EUROLIVE Study Group. The effect of polyphenols in olive oil on heart disease risk factors: a randomized trial. Ann Intern Med. 2006. 145: 333-341.

526. Moreno JA, Lopez-Miranda J, Gomez P et al. Effect of phenolic compounds of virgin olive oil on LDL oxidation resistance. Med Clin (Barc). 2003. 120: 128-131.

527. Masella R, Vari R, D'Archivio M et al. Extra virgin olive oil biophenols inhibit cell-mediated oxidation of LDL by increasing the mRNA transcription of glutathione-related enzymes. J Nutr. 2004. 134. :785-791.

528. http://www.cfsan.fda.gov/~dms/qhcolive.html (Qualified Health Claims: Monounsaturated Fatty Acids from Olive Oil and Coronary Heart Disease November 1, 2004).

529. Stampfer MJ, Sacks FM et al. A prospective study of cholesterol, apolipoproteins, and the risk of myocardial infarction. N Engl J Med. 1991. 325: 373-381.

530. von Eckardstein A, Nofer JR, Assmann G. High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. Arterioscler Thromb Vasc Biol. 2001. 21: 13-27.

531. Olofsson SO, Boren J. Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis. J Intern Med. 2005. 258: 395-410.

532. Sacks FM, Svetkey LP, Vollmer WM et al. DASH-Sodium Collaborative Research Group. Effects on blood pressure of reduced dietary sodium and the Dietary Approaches to Stop Hypertension (DASH) diet. DASH-Sodium Collaborative Research Group. N Engl J Med. 2001. 344: 3-10.

533. Psaltopoulou T, Naska A et al. Olive oil, the Mediterranean diet, and arterial blood pressure: the Greek European Prospective Investigation into Cancer and Nutrition (EPIC) study. Am J Clin Nutr. 2004. 80: 1012-1018.

534. Lapointe A, Couillard C, Lemieux S. Effects of dietary factors on oxidation of low-density lipoprotein particles. J Nutr Biochem. 2006. 17: 645-658.

535. Mensink RP, Katan MB. Effect of a diet enriched with monounsaturated or polyunsaturated fatty acids on levels of low-density and high-density lipoprotein cholesterol in healthy women and men. N Engl J Med. 1989. 321: 436-441.

536. Chang NW, Huang PC. Effects of dietary monounsaturated fatty acids on plasma lipids in humans. J Lipid Res. 1990. 31: 2141-2147.

537. Cheung MC, Lichtenstein AH, Schaefer EJ. Effects of a diet restricted in saturated fatty acids and cholesterol on the composition of apolipoprotein A-I-containing lipoprotein particles in the fasting and fed states. Am J Clin Nutr. 1994. 60: 911-918.

538. Schaefer EJ, Lichtenstein AH, Lamon-Fava S et al. Efficacy of a National Cholesterol Education Program Step 2 diet in normolipidemic and hypercholesterolemic middle-aged and elderly men and women. Arterioscler Thromb Vasc Biol. 1995. 15: 1079-1085.

539. Katan MB. Effect of low-fat diets on plasma high-density lipoprotein concentrations. Am J Clin Nutr. 1998. 67 (3 Suppl): 573S-576S.

540. Walden CE, Retzlaff BM, Buck BL et al Differential effect of National Cholesterol Education Program (NCEP) Step II diet on HDL cholesterol, its subfractions, and apoprotein A-I levels in hypercholesterolemic women and men after 1 year: the beFIT Study. Arterioscler Thromb Vasc Biol. 2000. 20: 1580-1587.

541. Lichtenstein AH, Ausman LM, Jalbert SM et al. Efficacy of a Therapeutic Lifestyle Change/Step 2 diet in moderately hypercholesterolemic middle-aged and elderly female and male subjects. J Lipid Res. 2002. 43: 264-273.

542. Mensink RP, Zock PL et al. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. Am J Clin Nutr. 2003. 77: 1146-1155.

543. Bonanome A, Pagnan A et al. Effect of dietary monounsaturated and polyunsaturated fatty acids on the susceptibility of plasma low density lipoproteins to oxidative modification. Arterioscler Thromb. 1992. 12: 529-533.

544. Mata P, Alonso R, Lopez-Farre A et al. Effect of dietary fat saturation on LDL oxidation and monocyte adhesion to human endothelial cells in vitro. Arterioscler Thromb Vasc Biol. 1996. 16: 1347-1355.

545. Mata P, Varela O, Alonso R et al. Monounsaturated and polyunsaturated n-6 fatty acid-enriched diets modify LDL oxidation and decrease human coronary smooth muscle cell DNA synthesis. Arterioscler Thromb Vasc Biol. 1997. 17: 2088-2095.

546. Sola R, La Ville AE et al. Oleic acid rich diet protects against the oxidative modification of high density lipoprotein. Free Radic Biol Med. 1997. 22: 1037-1045.

547. Holvoet P, Vanhaecke J et al. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. Circulation. 1998. 98: 1487-1494.

548. Visioli F, Galli C. Olive oil: more than just oleic acid. Am J Clin Nutr. 2000. 72: 853.

549. Masella R, Giovannini C et al. Effects of dietary virgin olive oil phenols on low density lipoprotein oxidation in hyperlipidemic patients. Lipids. 2001. 36: 1195-1202.

550. Fung TT, Rimm EB et al. Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk. Am J Clin Nutr. 2001. 73: 61-67.

551. Patrick L, Uzick M. Cardiovascular disease: C-reactive protein and the inflammatory disease paradigm: HMG-CoA reductase inhibitors, alpha-tocopherol, red yeast rice, and olive oil polyphenols. A review of the literature. Altern Med Rev. 2001. 6: 248-271.

552. Hargrove RL, Etherton TD et al. Low fat and high monounsaturated fat diets decrease human low density lipoprotein oxidative susceptibility in vitro. J Nutr. 2001. 131: 1758-1763.

553. Steinberg D, Witztum JL. Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis? Circulation. 2002. 105: 2107-2111.

554. Chrysohoou C, Panagiotakos DB et al. Adherence to the Mediterranean diet attenuates inflammation and coagulation process in healthy adults: The ATTICA Study. J Am Coll Cardiol. 2004 Jul. 44: 152-158.

555. Esposito K, Marfella R et al. Effect of a mediterranean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome: a randomized trial. JAMA. 2004. 292: 1440-1446.

556. Silaste ML, Rantala M, Alfthan G et al. Changes in dietary fat intake alter plasma levels of oxidized low-density lipoprotein and lipoprotein(a). Arterioscler Thromb Vasc Biol. 2004. 24: 498-503.

557. Wahle KW, Caruso D et al. Olive oil and modulation of cell signaling in disease prevention. Lipids. 2004. 39: 1223-1231.

558. Michalsen A, Lehmann N, Pithan C et al. Mediterranean diet has no effect on markers of inflammation and metabolic risk factors in patients with coronary artery disease. Eur J Clin Nutr. 2006. 60: 478-485.

559. Shepherd J, Packard CJ et al. Effects of dietary polyunsaturated and saturated fat on the properties of high density lipoproteins and the metabolism of apolipoprotein A-I. J Clin Invest. 1978. 61: 1582-1592.

560. Brinton EA, Eisenberg S, Breslow JL. A low-fat diet decreases high density lipoprotein (HDL) cholesterol levels by decreasing HDL apolipoprotein transport rates. J Clin Invest. 1990. 85: 144-151.

561. Velez-Carrasco W, Lichtenstein AH et al. Dietary restriction of saturated fat and cholesterol decreases HDL ApoA-I secretion. Arterioscler Thromb Vasc Biol. 1999. 19: 918-924.

562. Frenais R, Ouguerram K et al. Effect of dietary omega-3 fatty acids on high-density lipoprotein apolipoprotein AI kinetics in type II diabetes mellitus. Atherosclerosis. 2001. 157: 131-135.

563. Gill JM, Brown JC, Caslake MJ et al. Effects of dietary monounsaturated fatty acids on lipoprotein concentrations, compositions, and subfraction distributions and on VLDL apolipoprotein B kinetics: dose-dependent effects on LDL. Am J Clin Nutr. 2003. 78: 47-56.

564. Matthan NR, Welty FK et al. Dietary hydrogenated fat increases high-density lipoprotein apoA-I catabolism and decreases low-density lipoprotein apo B-100 catabolism in hypercholesterolemic women. Arterioscler Thromb Vasc Biol. 2004. 24: 1092-1097.

565. Desroches S, Paradis ME et al. Apolipoprotein A-I, A-II, and VLDL-B-100 metabolism in men: comparison of a low-fat diet and a high-monounsaturated fatty acid diet. J Lipid Res. 2004. 45: 2331-2338.

566. Chan DC, Watts GF et al. Factorial study of the effect of n-3 fatty acid supplementation and atorvastatin on the kinetics of HDL apolipoproteins A-I and A-II in men with abdominal obesity. Am J Clin Nutr. 2006. 84: 37-43.

567. Shepherd J, Packard CJ, Gotto AM Jr, Taunton OD. Effects of dietary saturated and polyunsaturated fat on the metabolism of apolipoproteins A-I and B. Study of a patient with type IIb hyperlipoproteinaemia. Clin Chim Acta. 1978. 87: 395-404.

568. Huff MW, Nestel PJ. Metabolism of apolipoproteins CII, CIII1, CIII2 and VLDL-B in human subjects consuming high carbohydrate diets. Metabolism. 1982. 31: 493-498.

569. Cortese C, Levy Y et al. Modes of action of lipid-lowering diets in man: studies of apolipoprotein B kinetics in relation to fat consumption and dietary fatty acid composition. Eur J Clin Invest. 1983. 13: 79-85.

570. Nestel PJ, Connor WE et al. Suppression by diets rich in fish oil of very low density lipoprotein production in man. J Clin Invest. 1984. 74: 82-89.

571. Abbott WG, Swinburn B, Ruotolo G et al. Effect of a high-carbohydrate, low-saturated-fat diet on apolipoprotein B and triglyceride metabolism in Pima Indians. J Clin Invest. 1990. 86: 642-650.

572. Stacpoole PW, von Bergmann K et al. Nutritional regulation of cholesterol synthesis and apolipoprotein B kinetics: studies in patients with familial hypercholesterolemia and normal subjects treated with a high carbohydrate, low fat diet. J Lipid Res. 1991. 32: 1837-1848.

573. Pacy PJ, Mitropoulos KA et al. Metabolism of apolipoprotein B-100 and of triglyceride-rich lipoprotein particles in the absence of functional lipoprotein lipase. Atherosclerosis. 1993. 103: 231-243.

574. Gylling H, Miettinen TA. J Lipid Res. Effects of inhibiting cholesterol absorption and synthesis on cholesterol and lipoprotein metabolism in hypercholesterolemic non-insulin-dependent diabetic men. J Lipid Res. 1996. 37: 1776-1785.

575. Fisher WR, Zech LA, Stacpoole PW. Apolipoprotein B metabolism in hypertriglyceridemic diabetic patients administered either a fish oil- or vegetable oil-enriched diet. J Lipid Res. 1998. 39: 388-401.

576. Bordin P, Bodamer OA et al. Effects of fish oil supplementation on apolipoprotein B100 production and lipoprotein metabolism in normolipidaemic males. Eur J Clin Nutr. 1998. 52: 104-109.

577. Chan DC, Watts GF, Barrett PH et al. Regulatory effects of HMG CoA reductase inhibitor and fish oils on apolipoprotein B-100 kinetics in insulin-resistant obese male subjects with dyslipidemia. Diabetes. 2002. 51: 2377-2386.

578. Marsh JB, Welty FK et al. Apo B metabolism in humans: studies with stable isotope-labeled amino acid precursors. Atherosclerosis. 2002. 162: 227–244.

579. Daumerie CM, Woollett LA, Dietschy JM. Fatty acids regulate hepatic low density lipoprotein receptor activity through redistribution of intracellular cholesterol pools. Proc Natl Acad Sci USA. 1992. 89: 10797-10801

580. Calleja L, Trallero MC, Carrizosa C et al. Effects of dietary fat amount and saturation on the regulation of hepatic mRNA and plasma apolipoprotein A-I in rats. Atherosclerosis. 2000. 152: 69-78.

581. Sauvaget D, Chauffeton V, Dugue-Pujol S et al. In vitro transcriptional induction of the human apolipoprotein A-II gene by glucose. Diabetes. 2004. 53: 672-678.

582. Viguerie N, Vidal H, Arner P et al. Nutrient-Gene Interactions in Human Obesity--Implications for Dietary Guideline (NUGENOB) project. Adipose tissue gene expression in obese subjects during low-fat and high-fat hypocaloric diets. Diabetologia. 2005. 48: 123-131.

583. Fernandez ML, West KL. Mechanisms by which dietary fatty acids modulate plasma lipids. J Nutr. 2005. 135: 2075-2078.

584. Ordovas JM. Genetic interactions with diet influence the risk of cardiovascular disease. Am J Clin Nutr. 2006. 83: 443S-446S.

585. Mukherjee S. Genetics and molecular biology: rationale for targeting high-density lipoprotein cholesterol for therapeutic intervention. Curr Opin Lipidol. 2006. 17: 602-605.

586. Ashen MD, Blumenthal RS. Low HDL Cholesterol Levels. N Engl J Med. 2005 353: 1252-1260.

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