*Year :* 2010

Metabolic effects of dietary proteins

BORTOLOTTI, Murielle

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Département de Physiologie

# *METABOLIC EFFECTS OF DIETARY PROTEINS*

**Thèse de doctorat ès Sciences de la Vie (PhD)**

Présentée à la Faculté de Biologie et de Médecine de l'Université de Lausanne par

**Murielle Bortolotti**

Diplômée en Biologie à l'Université de Lausanne

**Jury**

Prof. Peter Vollenweider, Président du Jury

Prof. Luc Tappy, Directeur de Thèse

Dr. Kevin Acheson, Expert

Prof. Yves Boirie, Expert

Lausanne 2010

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

**Ecole Doctorale**

**Doctorat ès sciences de la vie**

# Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

| | | | |
|---|---|---|---|
| ***Président*** | Monsieur | Prof. | Peter **Vollenweider** |
| ***Directeur de thèse*** | Monsieur | Prof. | Luc **Tappy** |
| ***Experts*** | Monsieur | Dr | Kevin **Acheson** |
| | Monsieur | Dr | Yves **Boirie** |

le Conseil de Faculté autorise l'impression de la thèse de

## Madame Murielle Bortolotti

Biologiste diplômée de l'Université de Lausanne

intitulée

**METABOLIC EFFECTS OF DIETARY PROTEINS**

Lausanne, le 25 juin 2010

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Peter Vollenweider

# *PREFACE*

***REMERCIEMENTS***

Une page qui se heurte elle aussi à la problématique d'une thèse, comment adresser le particulier de façon aussi ciblée et concise que possible, tout en sachant que par essence on s'écarte de l'exhaustif, de même comment dire MERCI à toutes ces personnes qui ont contribué à la réalisation de ce travail directement ou indirectement, tout un sachant que ce MERCI est tout aussi imparfait et que ces cinq lettres n'ont ni le poids ni la mesure de refléter ce qui m'a été donné; une problématique n'existe pas sans contexte, une thèse est à mon sens avant tout le fruit d'un travail aux multiples (res)-sources …

- à mon Directeur de Thèse, *Luc Tappy*, qui m'a encadrée, formée, toujours soutenue, et encouragée dont j'apprécie et estime non seulement son savoir-faire et ses compétences de recherche, mais surtout son savoir-être et ses qualités humaines…
- à mon Jury d'expertise, *Prof. P.Vollenweider, Dr. K.Acheson et Prof. Y.Boirie* qui ont accepté de lire ce travail, de l'expertiser et de l'étayer par leur connaissance…
- à mes relecteurs, *Virgile* et *Toto_London*…
- à tout l'*équipage Tappy* ; avec à son bord
  - les valeurs sûres *Valentine, Nathalie, Philippe*, *Eunika*, mais aussi *Christophe*, *Kaori*, et celles en devenir *Léonie & Fanny…*
  - les rescapés de la Clinique le temps d'une traversée ou deux, *Guillaume, Christel, Caroline, Frédérique, David &Rodrigue…*
  - les plus jeunes moussaillons dont j'ai essayé au mieux d'orienter la chaloupe, *Liliane, Julien, Elena, Mattia, Evelyne, Vanessa & Yannick*….
  - et les compagnons de « Bonne Fortune » *Andi*, *Raph*, *Kim-Anne*, *Mme Defago* et *Olivier…*
- à des « Drôles de Dames », infirmières de recherche entre autre, *Christiane, Christine & Françoise* avec qui j'ai pu partager mes *Passions* mais aussi en vivre d'autres…
- à ces volontaires que j'ai tentés de choyer au mieux dont les plus fidèles, *Christophe, Florent, Miguel &Raphaël...*

***MAIS AUSSI***

- à *KOEN*, my best friend, a beautiful dancer & dance master, such a person who is always here to support me …to ask me for a "quick drink"!...
- à *MA PRECIEUSE FAMILLE*, mes parents, et mes deux frères, *Laurent et Raphaël* qui sont à eux 4 une ressource intarissable à laquelle je peux puiser toute sorte d'essence
- à la *TROUPE UNE FOIS*, *Reb, Claire et Nelly*, avec qui j'ai revisité l'euphorie de l'AMOUR adolescent
- au *BLEU* avec son (ex)-team, *Julio*, *Verrrrrrrrronika* et *Victor*, ses bières, et ses habitués qui sont devenus de mes amis, *Giosué* et *Sonja*
- à *Pascale, Mariame, Delphine, Floriane, Thalia, Benoît, JP, Stéphane, Antoinette, Sarah…Nicolas…&…*

- à la *DANSE* une belle échappée, ma meilleure antidote….

*M.E.R.C.I*

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***GLOSSARY***

| | |
|---|---|
| ABCA-1 | ATP-binding cassette transporter-1 |
| ACC | acetyl-CoA carboxylase |
| Acyl-CoA | acyl-coenzyme A |
| ALAT | alanine aminotransferase |
| ASAT | aspartate aminotransferase |
| ATGL | adipose triglyceride lipase |
| BA | bile acid |
| BAT | brown adipose tissue |
| BCAA | branched chain amino-acids |
| BMI | body mass index |
| BMR | basal metabolic rate |
| BOHB | beta-hydroxybutyrate |
| C | control |
| CA | cholic acid |
| CCK | cholecystokinin |
| CD | control diet |
| CD36 | cluster of differenciation 36 |
| CDCA | chenodeoxycholic acid |
| CM | control meal |
| CPT-1 | carnitine palmitoyltransferase I |
| CVD | cardiovascular disease |
| D5 desaturase | D5 desaturase |
| D6 desaturase | D6 desaturase |
| DCA | deoxycholic acid |
| EAA | essential amino acids |
| ECF | extracellular fluid |
| EGP | endogenous glucose production |
| EGIR | European Group for the Study of Insulin Resistance |
| FFA | free fatty acid |
| FAS(N) | fatty acid synthase |
| FAO | Food and Agriculture Organization of United Nation |
| FXR | farnesoid X receptor |
| GC-MS | gas chromatography-mass spectroscopy |
| GC-IRMS | gas chromatography-isotope ratio mass spectroscopy |
| GFR | glomerular filtration rate |
| GLP-I | glucagon-like peptide-I |
| HDL | high density lipoprotein |
| HF | high-fat |

| | |
|---|---|
| HFHP | high-fat high-protein |
| $^{1}$H-MR spectroscopy | proton magnetic resonance spectroscopy |
| $^{1}$H-NMR spectroscopy | proton nuclear magnetic resonance spectroscopy |
| HOMA-IS | homeostasis model assessment of insulin sensitivity |
| HPD | high-protein diet |
| HPM | high-protein meal |
| HSL | hormone sensitive lipase |
| IDF | International Diabetes Federation |
| IE | isotopic enrichment |
| IHCL | intrahepatocelluar lipids |
| IKKβ | I-kappa-β kinase |
| IL-1 | interleukin 1 |
| IL-6 | interleukin 6 |
| LDL | low-density lipoprotein |
| LDLR | low density lipoprotein receptor |
| LPL | Lipoprotein lipase |
| LXRα | Liver X receptor-alpha |
| m/z | mass to charge ratio |
| N | nitrogen |
| NAFLD | non alcoholic fatty liver disease |
| NASH | non alcoholic steatohepatitis |
| NB | nitrogen balance |
| NCEP | USA National Cholesterol Education Program |
| NEFA | non-esterified fatty acid |
| NFKβ | nuclear factor kappa β |
| NS | non-significant |
| PDE-3b | phosphodiesterase 3b |
| PPARs | peroxisome proliferator-activated receptors |
| PPARG | peroxisome proliferator-activated receptor gamma |
| PLIN | perilipin |
| PYY | peptide tyrosine tyrosine |
| Ra | rate of appearance |
| Rd | rate of disappearance |
| ROI | region of interest |
| SA | specific activity |
| SCD | stearoyl-CoA desaturase |
| SEM | standard error of the mean |
| SIM | selective ion monitoring |
| SRE | sterol regulatory-element |
| SREBP-1 | sterol regulatory-element binding protein-1 |

| | |
|---|---|
| T2D | Type 2 Diabetes |
| TG | triacyglycerol |
| TNF-α | tumor necrosis factor-alpha |
| TO | turnover |
| tPAI-1 | tissue-plasminogen-activator-inhibitor-1 |
| TZDs | Thiazolidinedione |
| | |
| UNO | Unitated Nations Office |
| UCP | Uncoupling protein |
| | |
| VLDL | very-low density lipoprotein |
| $\dot{V}O_2$ | rate of oxygen consumption |
| $\dot{V}CO_2$ | rate of carbon dioxide production |
| | |
| w | body weight |
| WPS | whey protein supplementation |
| WHO | World Health Organisation |

***SUMMARY***

Non-alcoholic fatty liver disease (NAFLD) is characterized by an elevated intra-hepatocellular lipid (IHCL) concentration (> 5%). The incidence of NAFLD is frequently increased in obese patients, and is considered to be the hepatic component of the metabolic syndrome. The metabolic syndrome, also characterized by visceral obesity, altered glucose homeostasis, insulin resistance, dyslipidemia, and high blood pressure, represents actually a major public health burden.

Both dietary factors and low physical activity are involved in the development of the metabolic syndrome. In animals and healthy humans, high-fat or high-fructose diets lead to the development of several features of the metabolic syndrome including increased intrahepatic lipids and insulin resistance. In contrast the effects of dietary protein are less well known, but an increase in protein intake has been suggested to exert beneficial effects by promoting weight loss and improving glucose homeostasis in insulin-resistant patients. Increased postprandial thermogenesis and enhanced satiety after protein ingestion may be both involved. The effects of dietary protein on hepatic lipids have been poorly investigated in humans, but preliminary studies in rodents have shown a reduction of hepatic lipids in carbohydrate fed rats and in obese rats.

In this context this work aimed at investigating the metabolic effects of dietary protein intake on hepatic lipid metabolism and glucose homeostasis in humans. The modulation by dietary proteins of exogenous lipid oxidation, net lipid oxidation, hepatic beta-oxidation, triglycerides concentrations, whole-body energy expenditure and glucose tolerance was assessed in the fasting state and in postprandial states. Measurements of IHCL were performed to quantify the amount of triglycerides in the liver.

In an attempt to cover all these metabolic aspects under different point of views, these questions were addressed by three protocols involving various feeding conditions.

Study I addressed the effects of a 4-day hypercaloric high-fat high-protein diet on the accumulation of fat in the liver (IHCL) and on insulin sensitivity. Our findings indicated that a high protein intake significantly prevents intrahepatic fat deposition induced by a short-term hypercaloric high-fat diet, adverse effects of which are presumably modulated at the liver level.

These encouraging results led us to conduct the second study (Study II), as we were also interested in a more clinical approach to protein administration and especially if increased protein intakes might be of benefit for obese patients. Therefore the effects of one-month whey protein supplementation on IHCL, insulin sensitivity, lipid metabolism, glucose tolerance and renal function were assessed in obese women. Results showed that whey protein supplementation reduces hepatic steatosis and improves the plasma lipid profile in obese patients, without adverse effects on glucose tolerance or creatinine clearance. However since patients were fed *ad-libitum*, it remains possible that spontaneous carbohydrate and fat intakes were reduced due to the satiating effects of protein.

The third study (Study III) was designed in an attempt to deepen our comprehension about the mechanisms involved in the modulation of IHCL. We hypothesized that protein improved lipid metabolism and, therefore, we evaluated the effects of a high protein meal on postprandial lipid metabolism and glucose homeostasis after 4-day on a control or a high protein diet. Our results did not sustain the hypothesis of an increased postprandial net lipid oxidation, hepatic beta oxidation and exogenous lipid oxidation. Four days on a high-protein diet rather decreased exogenous fat oxidation and enhanced postprandial triglyceride concentrations, by impairing probably chylomicron-TG clearance.

Altogether the results of these three studies suggest a beneficial effect of protein intake on the reduction in IHCL, and clearly show that supplementation of proteins do not reduce IHCL by stimulating lipid metabolism, e.g. whole body fat oxidation, hepatic beta oxidation, or exogenous fat oxidation. The question of the effects of high-protein intakes on hepatic lipid metabolism is still open and will need further investigation to be elucidated.
The effects of protein on increased postprandial lipemia and lipoproteins kinetics have been little investigated so far and might therefore be an interesting research question, considering the tight relationship between an elevation of plasmatic TG concentrations and the increased incidence of cardiovascular diseases.

***RÉSUMÉ***

La stéatose hépatique non alcoolique se caractérise par un taux de lipides intra-hépatiques élevé, supérieur à 5%. L'incidence de la stéatose hépatique est fortement augmentée chez les personnes obèses, ce qui mène à la définir comme étant la composante hépatique du syndrome métabolique. Ce syndrome se définit aussi par d'autres critères tels qu'obésité viscérale, altération de l'homéostasie du glucose, résistance à l'insuline, dyslipidémie et pression artérielle élevée. Le syndrome métabolique est actuellement un problème de santé publique majeur.

Tant une alimentation trop riche et déséquilibrée, qu'une faible activité physique, semblent être des causes pouvant expliquer le développement de ce syndrome. Chez l'animal et le volontaire sain, des alimentations enrichies en graisses ou en sucres (fructose) favorisent le développement de facteurs associés au syndrome métabolique, notamment en augmentant le taux de lipides intra-hépatiques et en induisant le développement d'une résistance à l'insuline. Par ailleurs, les effets des protéines alimentaires sont nettement moins bien connus, mais il semblerait qu'une augmentation de l'apport en protéines soit bénéfique, favorisant la perte de poids et l'homéostasie du glucose chez des patients insulino-résistants. Une augmentation de la thermogenèse postprandiale ainsi que du sentiment de satiété pourraient en être à l'origine.

Les effets des protéines sur les lipides intra-hépatiques chez l'homme demeurent inconnus à ce jour, cependant des études préliminaires chez les rongeurs tendent à démontrer une diminution des lipides intra hépatiques chez des rats nourris avec une alimentation riche en sucres ou chez des rats obèses.

Dans un tel contexte de recherche, ce travail s'est intéressé à l'étude des effets métaboliques des protéines alimentaires sur le métabolisme lipidique du foie et sur l'homéostasie du glucose. Ce travail propose d'évaluer l'effet des protéines alimentaires sur différentes voies métaboliques impliquant graisses et sucres, en ciblant d'une part les voies de l'oxydation des graisses exogènes, de la beta-oxydation hépatique et de l'oxydation nette des lipides, et d'autre part la dépense énergétique globale et l'évolution des concentrations sanguines des triglycérides, à jeun et en régime postprandial. Des mesures des lipides intra-hépatiques ont aussi été effectuées pour permettre la quantification des graisses déposées dans le foie.

Dans le but de couvrir l'ensemble de ces aspects métaboliques sous différents angles de recherche, trois protocoles, impliquant des conditions alimentaires différentes, ont été entrepris pour tenter de répondre à ces questions.

La première étude (Etude I) s'est intéressée aux effets d'une suralimentation de 4 jours enrichie en graisses et protéines sur la sensibilité à l'insuline et sur l'accumulation de graisses intra-hépatiques. Les résultats ont démontré que l'apport en protéines prévient l'accumulation de graisses intra-hépatiques induite par une suralimentation riche en graisses de courte durée ainsi que ses effets délétères probablement par le biais de mécanismes agissant au niveau du foie.

Ces résultats encourageants nous ont conduits à entreprendre une seconde étude (Etude II) qui s'intéressait à l'implication clinique et aux bénéfices que pouvait avoir une supplémentation en protéines sur les graisses hépatiques de patients obèses. Ainsi nous avons évalué pendant un mois de supplémentation l'effet de protéines de lactosérum sur le taux de graisses intrahépatiques, la sensibilité à l'insuline, la tolérance au glucose, le métabolisme des graisses et la fonction rénale chez des femmes obèses. Les résultats ont été encourageants ; la supplémentation en lactosérum améliore la stéatose hépatique, le profil lipidique des patientes obèses sans pour autant altérer la tolérance au glucose ou la clairance de la créatinine. L'effet satiétogène des protéines pourrait aussi avoir contribué à renforcer ces effets.

La troisième étude s'est intéressée aux mécanismes qui sous-tendent les effets bénéfiques des protéines observés dans les 2 études précédentes. Nous avons supposé que les protéines devaient favoriser le métabolisme des graisses. Par conséquent, nous avons cherché à évaluer les effets d'un repas riche en protéines sur la lipémie postprandiale et l'homéostasie glucidique après 4 jours d'alimentation contrôlée soit isocalorique et équilibrée, soit hypercalorique enrichie en protéines. Les résultats obtenus n'ont pas vérifié l'hypothèse initiale ; ni une augmentation de l'oxydation nette des lipides, ni celle d'une augmentation de la béta-oxydation hépatique ou de l'oxydation d'un apport exogène de graisses n'a pu être observée. A contrario, il semblerait même plutôt que 4 jours d'alimentation hyperprotéinée inhibent le métabolisme des graisses et augmente les concentrations

sanguines de triglycérides, probablement par le biais d'une clairance de chylomicrons altérée.

Globalement, les résultats de ces trois études nous permettent d'attester que les protéines exercent un effet bénéfique en prévenant le dépôt de graisses intra-hépatiques et montrent que cet effet ne peut être attribué à une stimulation du métabolisme des lipides via l'augmentation des oxydations des graisses soit totales, hépatiques, ou exogènes. La question demeure en suspens à ce jour et nécessite de diriger la recherche vers d'autres voies d'exploration. Les effets des protéines sur la lipémie postprandiale et sur le cinétique des lipoprotéines n'a que peu été traitée à ce jour. Cette question me paraît néanmoins importante, sachant que des concentrations sanguines élevées de triglycérides sont étroitement corrélées à une incidence augmentée de facteurs de risque cardiovasculaire.

# *CHAPTER 1*
# *INTRODUCTION*

## *1.1 GENERAL CONTEXT: METABOLIC SYNDROME AND OBESITY*

### 1.1.1 Epidemiology of obesity

Overweight and obesity are defined as excessive fat accumulation that may impair health. Body Mass Index (BMI) is a simple index calculated as weight (kg)/height ($m^2$) that is commonly used for classifying overweight (BMI > 25 kg/$m^2$) and obesity (BMI > 30 kg/$m^2$) among individuals. Obesity is associated with a chronic imbalance between energy intakes (consumption of high energy dense food) and energy expenditure (low physical activity). Before the 20$^{th}$ century, obesity was rare. In 1997, obesity was formally recognized by the World Health Organisation (WHO) as a global epidemic. In 2005, WHO's latest projection indicated that globally 1.6 billion adults (> 15 years old) were overweight and 400 million at least were obese. WHO projections are approximately 2.3 billion overweight and 700 million obese individuals by 2015 (1).

Obesity, and more particularly visceral obesity, is often associated with Type 2 Diabetes (T2D), sleep apnea and other risk factors for cardiovascular diseases (CVD). Their concomitant occurrence has been found to reduce life expectancy, increasing mortality and morbidity and all together are now recognized to define the Metabolic Syndrome (2).

## 1.2 The Metabolic Syndrome

Different organisations such as WHO (3) and the European Group For the Study of Insulin Resistance (EGIR) in 1999 (4), the US National Cholesterol Education Program Adult Panel III (NCEP) in 2001 (5) and the International Diabetes Federation (IDF) in 2005 (6), proposed different criteria to define the "Metabolic Syndrome", but no consensus was then approved (7). There are currently two major definitions for the metabolic syndrome, also named Insulin Resistance Syndrome or Metabolic Syndrome X, one provided by IDF, and the other by the revised NCEP ATP III. Even if these two definitions contrast in the evaluation of accumulation of visceral fat and adiposity, the IDF and NECP ATP III definitions use the same threshold values for the other criteria. **Table 1** summarizes these criteria among the four definitions (8).

**Table 1** Features of the World Health Organization (WHO), European Group for the Study of Insulin Resistance (EGIR), National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP-III) and International Diabetes Federation (IDF) definitions of metabolic syndrome

| | **WHO (WHO consultation, 1999). Impaired glucose tolerance or diabetes and/or insulin resistance and two other factors** | **EGIR (Balkau and Charles, 1999[10]). Presence of fasting hyperinsulinaemia (the highest 25%) and two other factors** | **ATP-III (NCEP Expert Panel on Detection, 2001). Three or more of the following factors (TAGs and HDL counted separately)** | **IDF. Central obesity and two other factors (www.idf.org)** |
|---|---|---|---|---|
| Central obesity | WHR ⩾0.9 (men), 0.85 (women) and/or BMI >30 kg/m$^2$ | Waist ⩾94 cm (men), ⩾80 cm (women) | Waist ⩾102 cm (men), >88 cm (women) | Waist >94 cm (European men), >90 cm (Asian men), >80 cm (women) |
| Blood pressure (mm Hg) | ⩾140/90 | ⩾140/90 or treated for hypertension | >130/85 or treated for hypertension | ⩾130 SBP or ⩾85 DBP or treated for hypertension |
| Dyslipidaemia (mmol/l) | TAGs ⩾1.7, HDL <0.9 (men), <1.0 (women) | TAGs ⩾2.0 or HDL-cholesterol <1.0 or treated for dyslipidaemia | TAGs ⩾1.7, HDL-cholesterol: <1.0 (men), <1.3 (women) | TAGs ⩾1.7, HDL-cholesterol, <1.04 (men), <1.29 (women) |
| Dysglycaemia (mmol/l) | Fasting glucose ⩾6.1 and/or 2 h post-challenge glucose ⩾7.8 on diabetes | Fasting plasma glucose >6.1, but non-diabetic | Fasting plasma glucose ⩾6.1 | Fasting glucose ⩾5.6 or previous diagnosis of impaired glucose tolerance or diabetes |
| Insulin resistance | Glucose uptake during hyperinsulinaemic–euglycaemic clamp in lowest quartile for population | Presence of fasting hyperinsulinaemia (ie, among the highest 25% of the non-diabetic population) | Not applicable | Not applicable |
| Other factors | WHO (WHO consultation, 1999). Impaired glucose tolerance or diabetes and/or insulin resistance and two other factors) | EGIR (Balkau and Charles, 1999[10]). Presence of fasting hyperinsulinaemia (the highest 25%) and two other factors | Microalbuminuria (urinary albumin excretion rate >20 μg/min or albumin/creatinine ratio >30 mg/g) | None |

BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; SBP, systolic blood pressure; TAG, triacylglycerol; WHR, waist/hip ratio.

An important parameter that is missing in these global definitions, dating back five years, is the relatively importance of ectopic fat deposition in the liver, also called intrahepatocellular lipids (IHCL)(8, 9).

Nowadays, studies in humans and rodents tend to link the accumulation of fat in the liver to features of the metabolic syndrome (10) independently of obesity (11), even though the prevalence of fat in the liver increases in parallel with obesity (9, 12). Fat accumulation in the liver seems to be a key player in the pathogenesis of insulin resistance and the metabolic syndrome (13). Indeed, hepatic lipid metabolism is a highly regulated process, in which many pathways involve nuclear receptors and transcription factors (14). It is tightly controlled by intracellular lipidic products, levels of non esterified fatty acid (NEFA), but also by other metabolites, like carbohydrates or proteins and hormones, such as insulin and glucagon. It appears that an overflow of any metabolite can disturb this delicate equilibrium and causes hepatic fat accumulation, which can in turn degenerate into liver function impairment and subsequent pathologies (14).

Non-alcoholic fatty liver disease (NAFLD) is defined by fatty infiltration of the liver, exceeding 5% to 10 % by weight (15), but not due to excessive alcohol abuse. In most cases patients have no or few symptoms and are diagnosed by abnormal liver function tests (elevated plasma ASAT, ALAT concentrations) (16). Steatosis is associated with insulin resistance and components of the metabolic syndrome, such as elevated fasting glycemia (increased endogenous glucose production (EGP)) and hyperlipidemia (increased VLDL-triglycerides secretion and decreased HDL-cholesterol concentrations) (9, 17). In some cases, low grade inflammation occurs, which can progress and degenerate into non-alcoholic steatohepatitis (NASH), the most extreme form of NAFLD (18), which can then lead to the development of cirrhosis.

The gold standard method for diagnosis of NAFLD is the liver biopsy, with a histological scoring system uniformly used to assess the severity of the disease (*NAFLD activity score* or *the fibrosis score*) (19). The major limitation of the liver biopsy is sampling variability (20). Moreover it is not a practical screening tool given the cost, time-intensive nature and potential adverse effects of the procedure (21). Liver imaging, using tools such as ultrasound, computed tomography or magnetic resonance spectroscopy (MRS), is a current area of research attempting to quantify the degree of steatohepatitis. However, sensitivity might be

low and the degree of fibrosis cannot be determined. Current research is also attempting to define diagnostic panels relying on several biomarkers to predict fibrosis score and degree of steatohepatitis, such as the ELF-test (22, 23). Recently a proteomic analysis revealed key changes in serum protein expression levels between control subjects and patients with different stages of NAFLD (24). However future validation of these potential biomarkers is still required before such tests can replace liver biopsy.

Several pharmaceutical agents have been proposed for the treatment of NAFLD including insulin sensitizers, such as thiazolidinediones (rosiglitazone/pioglitazone) or metformin, antioxidants, or antihyperlipidemic agents, such as statins. The effects of these drugs on the pathological features of NAFLD are controversial and they may even be associated with significant levels of hepatotoxicity (23).

Among treatments, weight loss appears to be the most successful (23). Indeed many studies have focused on improvements in the liver biochemistry profile in response to weight loss, and documented that accumulation of fat in the liver was decreased (25).

Even though surgical options for weight loss are being increasingly used in the USA for the treatment of morbid obesity (26), less irreversible and invasive propositions might also be efficient, such as lifestyle intervention.

An abundance of epidemiological and interventional studies demonstrate the beneficial effects of changes in lifestyle habits, such as dieting and/or increased physical activity (27), on obesity, metabolic syndrome and NAFLD. Combining dieting (decrease in energy intakes) and physical activity (increase in energy expenditure) is indeed highly efficient, provided that compliance is good. In this context, much attention has been paid to dietary proteins, as they have intrinsic properties which might tend to favour and potentiate weight loss in comparison to dietary carbohydrates or lipids.

Before going into more details regarding the research on protein diets, it might be useful first to go back to some historical considerations regarding our knowledge on protein metabolism, and second to do a quick review of their properties without paying too much attention to the details and complexity of their metabolism.

### *1.2 PROTEIN OVER HISTORY*

This section is mainly inspired by the work of Kenneth Carpenter, who published in four parts " A Short History of Nutritional Science"; which covers the period from 1785 up to 1985, in the Journal of Nutrition (28-31). Just a few major points are summarized here in an attempt to give some insights into the prominent role of protein in Nutritional Sciences.

Before 1885, nearly all of the nutritional studies had been carried out in Western Europe and most were concerned with the need for either protein or energy. Early Nutritional Science started in France in the eighteenth century with the "Chemical Revolution", providing the necessary tools to address ideas about food and body composition in a quantitative and scientific way.

An important finding was reported to the Academy of Science in 1785 by Claude Berthollet, who discovered that decomposition of animal matter produced ammonia. The discovery of nitrogen not only in animal matter, but also in some fractions of vegetable products, such as gluten in wheat flour, and its absence from sugars, starch and fats, led scientists to question about the origin of animal nitrogen rich-tissues.

The question of whether animals could use atmospheric nitrogen to "animalize" ingested food of low nitrogen content was addressed in 1816 by François Magendie, a French surgeon who had converted to physiology. The experimental part of his demonstration consisted of feeding dogs with one selected nutrient, sugar, which did not contain nitrogen. Since the dog died after one month, he repeated the experiment with other foods like olive oil, gum or butter, which all led to similar results. These successive experiments allowed him to conclude that "none of this food was "pre-eminently nutritive" and that at least "the majority of the nitrogen in a dog's tissue must come from the food he consumed".

Fifteen years later, Jean-Baptiste Boussingault, a French chemist, further addressed this question. He demonstrated that leguminous plants, contrary to cereal grains, use atmospheric nitrogen for their growth. He also performed nutritional trials in cows and horses by controlling and recording all their food intakes. He estimated the amount of nitrogen intake and losses, and was then able to conclude that the animal's food provided sufficient nitrogen to meet their needs and that there was no need to hypothesize that they had to obtain nitrogen from the atmosphere.

These experiments led Jean-Baptiste Boussingault to propose that the relative nutritional values of plant food could be assessed from their nitrogen contents. Vitamins, minerals and trace elements had not been discovered at this time.

At that time if the origin of nitrogenous compounds in animal tissue was commonly recognized, and attributed to the vegetal kingdom, the question of their function, use and process was of much more debate.

Justus Liebig, a German organic chemist, advanced that "protein was the only true nutrient", providing both the machinery of the body and the fuel for its work and that an explosive breakdown of the protein molecules led to the production and excretion of urea.

First Edward Smith, a British physician and physiologist, and then two Swiss scientists, Adolf Fick and Johannes Wislicenus, were able to refute this statement by proving that protein breakdown and urea production were not proportionate to the amount of work load. This led Edward Franklin to assess a structural function to proteins and to draw the analogy of a muscle to a steam engine in which the engine did not consume itself when working, but remained intact while using an entirely different fuel.

The term "protein", from Greek *prôtos*, meaning "of primary quality, first, essential" was suggested in 1839 by Gerrit Mulder, a Dutch worker, who assessed that the so-called "animal substance", recognized to contained ca 16% nitrogen, had a common radical combined with different proportions of phosphorus, sulphur or both.

Since 1885 research on protein and energy metabolism increased throughout the world, which allowed to greatly broaden our understanding about protein needs and nutritional requirements. At this time the important question was to determine normal protein requirements.

Standards of high protein intakes were recommended in Germany, by Voit, but also in USA by Atwater, based on observational studies of protein intake in middle working individuals' class. It was believed that a high protein intake (ca 120 g/day) insured good physical health, and even though vegetarians with a lower protein intake could remain in nitrogen balance, they exposed themselves to disadvantages. Russel Chittenden, Professor of Physiological Chemistry at Yale, demonstrated among three groups of young men, with various physical activity levels, that ~ 60 g protein intake/day was sufficient to ensure athletic performance, good physical and mental activity and that it represented even more some kind of

"physiological economy". Other scientists were reluctant to accept this recommendation, but later studies would only confirm this finding.

Nutrition scientists then believed that proteins ingested with food were absorbed almost intact and were only slightly modified. They were reluctant to the discoveries by workers in the digestive physiology, stipulating that proteins were cleaved into more soluble derivatives, the "amino-acids" under the action of digestive enzymes (such as pepsin or trypsin) for their assimilation.

In 1902 it was still believed that "such a profound decomposition would be a waste of chemical potential energy, and that a reunion of such products is highly improbable". Therefore studies on supplementation of mixtures of amino acids, as substitutes for dietary proteins, were conducted in animals to test this hypothesis. This led to the discovery of new and essential amino acids, such as tryptophan by Hopkins and Coyle in 1902, methionine in the 1920s at Harward, isoleucine and threonine in the 1930s by Womack and Rose. By the 1930's, William Rose was able to supply all the essential amino acids needed for the growth of rats, and to definitely support the "amino-acid" concept.

Starting in 1942, Rose set out to extend the study to humans adults to find the correct pattern of amino acids which was needed to maintain nitrogen balance. Fifteen years later, he was still not able to demonstrate that mixture of amino acids could completely replace protein intake as nitrogen balance was achieved for low levels of amino acids but only with energy intakes higher than what was required with equivalent quantities of intact protein. He concluded however that as the nitrogen balance had been reached, the list of essential amino acids required by human adults had to be complete.

Meanwhile, the availability of isotopes, allowed a new approach for the study of the fate and distribution of nutrients in the body. In 1939, Rudolf Schoenheimer a pioneer in that domain (*see chapter 2: what is a tracer?*) was able to quantify protein breakdown, recycling and excretion.

In 1960, the FAO (Food and Agriculture organisation of the United Nation) wrote that "deficiency of protein in the diet is the most serious and widespread problem in the world". This statement relied on the discovery that "kwashiorkor", an acute form of childhood

protein energy malnutrition, highly prevalent in third-world countries, might be treated by high protein nutritional supplement. Much work was carried on to develop substitutes, but it appeared later that this globalised worldwide interest was moreover a pretext for food industries to make profit and to develop insights into food engineering. Treatment for Kwashiorkor has since switched to providing more concentrated food and by correcting for electrolyte deficiencies.

Nowadays, even if scientific reports suggest that there is no need for higher protein supplementation in well-nourished populations, it is still widely believed amongst individuals that a high protein intake may be beneficial by improving health, well-being and physical condition. Food industry reinforces this opinion and makes profit by selling a variety of expensive protein or amino acid powders.

## *1.3 PROTEIN METABOLISM (32-34)*

### 1.3.1 Generalities: protein and amino acids

Protein are major components of the body not only quantitatively (they account for more than half of the dry weight of a cell) but also qualitatively when considering the diversification of their biological functions.
They are the "work-horses" involved in multiple functional roles as biological catalyst (enzymes), regulators of gene expression, transporters of substances in plasma or through membranes, defenders of the organism (antibodies, interleukins), but also as components of cellular organelles and extracellular matrices.
This infinite diversity is allowed by the specificity of their single components; the amino acids. A protein is a linear sequence of amino acids, varying in length from tens to thousands of blocks. There are over twenty different amino acids, which allow a huge diversity of possible combinations, theoretically $20^n$, n being the number of amino acids present in the protein. This diversity is even more important when it is considered that some proteins, named heteroproteins, contain other components such as ions, carbohydrates or lipids. Among the 20 usual amino acids, some amino acids cannot be or are only partially synthesized by the organism and must be obtained from the food. Amino acids are divided in three categories; the indispensable amino acids, such as valine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine, trypthophan and histidine; the conditionally indispensable amino acids, such as glycine, arginine, glutamine, proline, cystine, tyrosine, and the dispensable amino acids, such as glutamic acids, alanine, serine, aspartic acids, and asparagine.

In most cells and tissues, proteins are continuously synthesized and degraded. The whole process of synthesis (anabolism) and degradation (catabolism) is referred to as protein turnover. To maintain protein homeostasis and nitrogen balance, but also to control lean mass gain (in growth) or loss (in dieting), these processes must be tightly regulated by the ambient intracellular end extracellular milieu (pool of amino acids, nutritional status, hormones insulin and glucagon).

### 1.3.2 Nitrogen balance

The principal metabolic systems responsible for the maintenance of body protein and amino acid homeostasis include protein synthesis and degradation, amino acid interconversions, and amino acid synthesis. In adults, the maintenance of a constant body composition implies that nitrogen intake = nitrogen excretion and that protein synthesis = protein breakdown.

Due to the lack of a more specific test, nitrogen balance (NB), being the resultant of whole body protein metabolism, has been accepted as a common criterion to evaluate adequate intakes and, though imprecise, is broadly investigated in clinics. Nitrogen balance (NB) determination requires a careful estimate of the intake (I) and all routes of nitrogen loss, namely urines (U), faces (F) and dermal losses (S); then

$$NB = I- (U+F+S).$$

In clinical practice, and even clinical trials, accurate evaluation of nitrogen balance is impossible since estimation of nitrogen intake (dietary records/absorption) and loss in urine or faeces are often partial and incomplete. Moreover other routes of nitrogen losses, like sweat or dermal losses, are very difficult to evaluate. Nitrogen balance evaluation should also be corrected for changes in the total body urea pool if blood urea concentrations do not remain constant.

Another important point to take in consideration is that nitrogen balance is not only affected by dietary protein intake but also by the level of dietary energy intake (35, 36). Consequently the estimation of protein requirements, based on nitrogen balance can be affected by the amount of energy available (36).

*Effect of varying protein intake on nitrogen balance*

Since body protein content in non-growing humans is maintained within narrow limits nitrogen balance is regulated around zero. When nitrogen intake varies, this regulation is achieved by compensatory changes in nitrogen excretion. When protein intake is below requirements, body proteins tended to be conserved, and nitrogen excretion is diminished over time. When a protein-free diet is consumed, a small amount of nitrogen excretion occurs, which represents the obligatory oxidation of protein for basal protein turnover. On the other hand, when protein is fed in excess, nitrogen excretion is increased to compensate for the excess intake and nitrogen balance is conserved around zero. Compensatory excretion is however limited and cannot account for extremely high levels of protein intake (upper limit estimated to be ca 225 g protein/day). When dietary protein intake is altered, nitrogen balance needs 4 to 7 days to achieve equilibration. Consequently, transient net accumulations or loss of nitrogen can occur.

*Effect of varying caloric intake on nitrogen balance*

The relationship between the sensitivity of nitrogen balance to the addition or removal of energy from the diet is characterized by a linear improvement in nitrogen balance with increasing in energy intake in a normal range of protein or energy intake. It was estimated that, with adequate dietary protein, nitrogen balance improved by 0.1 to 0.3 g/day per 100 excess kcal added to the diet (37). In rodents, when the diet provided adequate amounts of protein, increments in energy intake, produced by adding either carbohydrate or fat in sub-maintenance diets, caused a linear improvement in nitrogen balance, whereas when the diet contained no protein, addition of fat or carbohydrate failed to influence nitrogen balance. (38). Also in humans, increments in energy consumption result in better sparing and utilization of dietary nitrogen with higher amount of dietary protein (60g) compared to lower (40g) (39).

*Interaction of nitrogen intake and caloric intake on nitrogen balance*

The effects of nitrogen and energy intake on energy balance are interrelated and complex. The following figure (**Figure 1**), reproduced from "Basics in Clinical Nutrition"(34) illustrates to what extent changes in nitrogen and energy intake may modulate nitrogen balance.

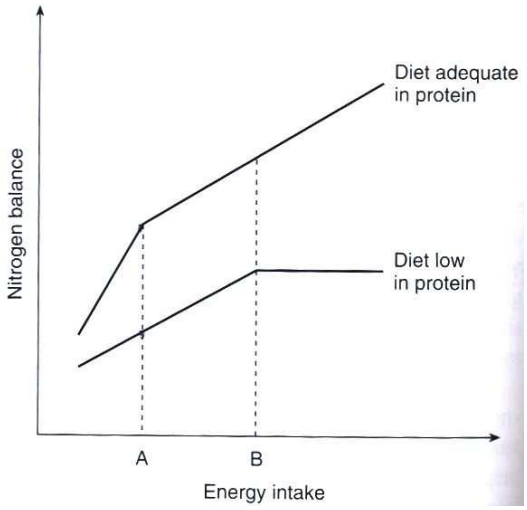


**Fig1. Relationship between nitrogen balance and energy intake with diets of different protein levels**. Between energy intake (A) low and (B) high the two lines are parallel (Reproduced from Munro and Allison, 1964, Vol. 1, p 381)

It has been observed that the level of energy intake determines the degree of change in nitrogen balance that occurs in response to a change in nitrogen intake. Conversely, the level of protein intake determines the quantitative effect of energy intake on energy balance. Therefore optimum body protein nutrition is achieved when protein and energy intakes are sufficient to meet or balance the needs for amino acids, nitrogen and daily energy expenditure.

The protein requirement of an individual is defined as the lowest level of dietary protein intake that will balance the losses from the body in an individual with adequate energy intake and moderate physical activity. Actual safe levels of protein requirements were issued by an International Commission (convened by FAO, WHO and UNO (United Nations Office)) in 1985, presupposing the use of first class proteins high in essential amino

acids and with an adequate energy supply (40). Protein needs differ with age; requirements for young adults range between 0.75-0.80 g/kg body weight, but proteins needs are considerably higher in newborns, children, and pregnant women. Protein requirements are also considerably increased in early lactation (+ 17.5g protein/day) and in catabolic states such as in disease or physical activity. The minimum protein requirement is considered to be 0.45 g/kg body weight.

### 1.3.3 Disposal of dietary amino acids and roles of specific organs (33)

*Events in the intestine*

Dietary proteins amounts to ca 100g/day for an adult and the charge of exogenic protein is degraded and absorbed by the gastrointestinal tract. Approximately an additional 70g arises in the intestine as a result of intestinal secretions and mucosal cell degranulation. Since faecal nitrogen is about 10g protein/day, an equivalent of 160g protein are absorbed per day (**see Figure 2**).

The process of protein digestion begins in the stomach, with the action of pepsin. In the duodenum and small intestine, proteases secreted by the pancreas (exocrine function), such as trypsin, chymotrypsin and carboxypeptidase, cleave proteins into small peptides and free amino acids. The brush border of the small intestine contains peptidases, which reduces peptides to dipeptides and amino acids, which are be then transported across the intestinal mucosa.

The small intestine is an important site of amino acid catabolism; glutamate, aspartate and glutamine are 90% catabolised in the first pass. Some of the indispensable amino acids, such as valine, leucine, isoleucine, lysine, methionine, phenylalanine and threonine, are also significantly catabolised. For the above mentioned amino acids, as much as 30% to 50% does not enter the hepatic portal circulation. As such oxidation is often partial, these amino acids are converted into other amino acids such as (alanine/proline/citrulline) which can then be delivered by the portal vein.

*Role of the liver*

The liver is recognized as the only organ with the ability to metabolize almost all amino acids. However amino acid metabolization is often partial, with the carbon skeleton being converted into glucose or ketone bodies. Hepatic cells contain two enzymatic pathways which allow detoxification of ammonium arising from the catabolism of amino acids in the peripheral organs and tissues; urea and glutamine synthesis. The capacity of the liver to synthesize urea is very high, so that it can rapidly respond to increased protein ingestion. Changes in concentrations of *N*-acetylglutamate tightly regulated Carbamoyl-phosphate-synthetase-I, the first enzyme involved in urea synthesis, however the mechanisms involved in N-acetylglutamate increases are still unclear but seem probably to be mediated by glucagon and glucocorticoïds.

The liver removes about 2/3 of the absorbed amino acids, while the other third, enriched in branched chain amino acid (BCAA) reaches the systemic circulation and the peripheral tissues. In the liver, one third of amino acids is used for protein synthesis, the remainder being metabolised (**see Figure 2**).

*Skeletal muscle and kidney*

Skeletal muscle is the major site for BCAA catabolism, as the liver has only very low activities of the first two enzymes involved in their catabolism; the branched-chain amino transferase and the branched-chain keto acid deshydrogenase. The nitrogen end-products are glutamine and alanine, but the bulk of these ketoacids is metabolized within the muscle in humans.

The kidneys are also involved in amino acids metabolism in an attempt to maintain acid-base status. The two sulphur-containing amino acids, methionine and cysteine, generate sulphuric acid on oxidation. The hydrogen ions are neutralized by the bicarbonate buffer system. Glutamine, and to a lesser extent, glycine are the predominant substrates metabolized by the kidney which one involved in the repletion of the bicarbonate pool.

Amino acid metabolism contrasts in two important ways with that of carbohydrate and lipid, which confers its properties and particularities.

The first point is that the body has no specific store for amino acids. Proteins are primarily synthesized for their specific physiological functions, which implies that all amino acids must be available to tissues simultaneously to fulfil protein synthesis requirements. Any excess of amino acids should be rapidly degraded to prevent electrolytic disturbances, as amino acids are charged molecules.

The second point is the nature of amino acids end-products. Amino acid catabolism not only produces water and carbon dioxide, but also nitrogen containing end-products, such as urea and ammonium, and sulphur-containing end-products (principally sulfate). Ammonia is a potent neurotoxin, which implies that blood concentrations must be kept low. Its imminent detoxification via urea synthesis in confined to the liver. Sulphur-containing end-products involve the production of a strong metabolic acid, which have to be cleared by the kidney.

Taken together, the functionality and structure of proteins are maintained by a constant and tightly regulated protein turnover, which is an important energy requiring process and may be responsible for about 20% of basal metabolic rate (BMR). Similarly, dietary protein intake requires immediate metabolism, which is energy consuming and causes a subsequent increase in energy expenditure, which contribute substantially to postprandial thermogenesis.

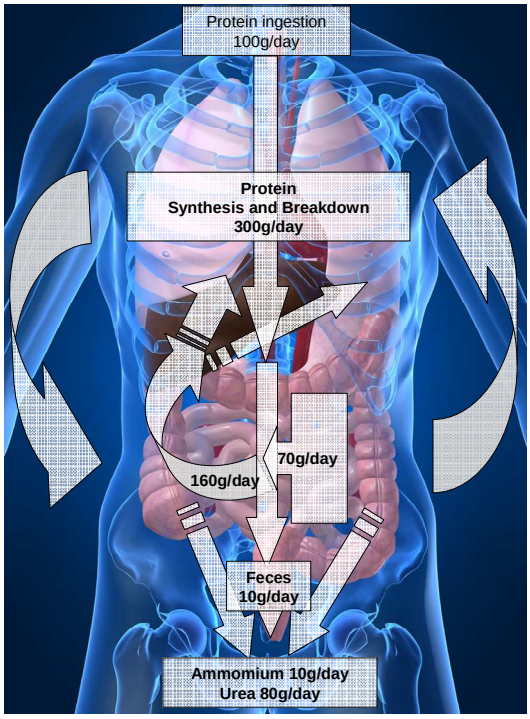


**Fig.2 Components of Adult Nitrogen Balance per day (in reference to Fürst P, 2004)(34)**
In nitrogen balance, for an adult per day; total protein absorption is about 160g, with dietary protein ingestion of 100g and intestinal secretion of 70g. Protein excretion amounts to 10g in faeces and catabolised protein are 90% excreted as urea and 10% as ammonium. 2/3 of the absorbed amino acids are metabolized in the liver, and the other third in peripheral tissues. The turnover of protein is estimated to be 300g/day.

### 1.3.4 Hormonal Regulation of protein metabolism

Insulin and glucagon are the principal hormones involved in protein and amino acids metabolism, and both are stimulated by amino acid and protein intake (41) .
Insulin has anabolic functions; it promotes protein synthesis by stimulating transcription and translation at the muscle level, and prevents proteolysis. Insulin also increases glucose utilization. An anabolic function has also been demonstrated for growth hormone and IGF-I either by promoting protein synthesis or inhibiting proteolysis (42). In opposition glucagon plays a major role in the catabolism of amino acids and counteracts hypoglycaemia by increasing hepatic glucose production. Glucocorticoïds and thyroid hormones also have catabolic functions by stimulating proteolysis and/or reducing protein synthesis.

The magnitude of these responses seems to be influenced by the quality and the quantity of protein in the diet. The nature of protein (animal *vs* vegetal) plays a major role as not only their amino acid composition but also the presence of biological compounds, such as isoflavones present in soya proteins, affects insulin secretion. Protein quantity has also been demonstrated to affect the magnitude of these responses, since diets with a low-protein composition are associated with reduced insulin secretion, whereas high-protein diets cause hypersecretion of insulin (41).

### *1.4 COMPLEXITY OF THE STUDY OF PROTEIN*

Due to the complexity of protein metabolism, science yields divergent conclusions regarding the physiological effects of dietary proteins. This could be partially explained by some of the parameters known to affect the time course of their metabolization and their subsequent effects on metabolism.

Indeed studies have shown that for the same amount of protein of comparable digestibility and with identical gastric emptying times, protein metabolism is affected by the consistency of the meal (liquid formula *vs* solid meal) (43), by the consistency of protein administration (intact protein *vs* hydrolysate) (44) or by the physical characteristics and types of other macronutrients present in the meal, such as carbohydrates, lipids or fibres (45),(46). Obviously differences in the kinetics of digestibility and absorption also affect postprandial responses (47), such as the elevation of glycemia, concentrations of the main regulatory hormones, or lipoprotein and cholesterol profiles (48). Studies also demonstrate differences in the anabolic properties of dietary proteins, when associated with physical exercise, pre or post exercise sessions (49),(50), or when taken as a single meal or as small portions spread throughout the day (51) .
Human and rodents studies have also investigated the specificity of each amino acid by assessing their own properties individually, usually during amino acid infusions (52). Effects of branched chains amino acids (BCAA) or essential amino acids (EAA), such as leucine, on regulation of satiety, energy expenditure, postprandial thermogenesis or insulin sensitivity have been extensively studied, but conclusions remain discrepant. Moreover, oral protein intake or parenteral feeding, infusions, provide different routes for amino acid administration which have to be taken in consideration.

Keeping these considerations in mind, I will discuss in more detail the actual knowledge about the main topics raised by this work.

### *1.5 NUTRITION AND PROTEINS*

Nutrition and proteins is a hot topic on its own, highly popular and mediatic. However dietary protein requirements and dietary recommendations should be acknowledged as problematic and may be very different depending upon specific needs and different populations. Four different major population categories can be distinguished based on their needs:

- Those who are underweight and undernourished and in whom muscle mass is insufficient, such as individuals affected by famine or the elderly. In this context nutritional studies focus particularly on the benefits of protein intake on whole body function and on muscle mass recovery when combined with sufficient energy intakes. The amounts, types and timing of protein supply should respond as efficiently as possible to the fundamental and human basic needs (53) (54).
- Those with kidney dysfunction, in which pathological state studies have to deal with how to provide sufficient amounts of protein while preventing the simultaneous overload of nitrogen or urea, which one known to be deleterious by increasing glomerular blood pressure and glomerular filtration rate (55).
- Those who are highly sportive and athletic, who aim to optimize performance by increasing muscle mass, strength and power and decreasing muscle breakdown. Studies focus on the best way to administer protein to induce positive nitrogen balance and anabolism. Physical activity and sufficient energy intake, instead of high protein intake, appear to be the major factors that promote muscle mass.
- Those who are obese and overweight with associated comorbidities defined in the metabolic syndrome. In such conditions, studies emphasize the effects of proteins effects on whole body metabolism, regulation of satiety and energy expenditure and subsequent weight and fat loss.

The major emphasis of this work was to gain a better understanding of the effects of proteins on features of the metabolic syndrome, by addressing especially the question of fat mass and lipid metabolism.

Our hypothesis relied on the present knowledge of protein properties which could more or less impact on improving certain features of the metabolic syndrome.

## *1.6 PROTEINS AND FEATURES OF THE METABOLIC SYNDROME*

### 1.6.1. Dietary proteins: satiety, energy expenditure, weight loss and weight maintenance

Even though common sense may have favoured higher-protein intakes for a long time to the detriment of carbohydrates, for weight reducing diets, such as the Atkins diet (1970) or "low-cab" diets, research on this topic only extensively began in the 1990s.

As reviewed by Westerep-Plantenga in Annual Nutrition Reviews (56), it is informative to define more accurately what a high-protein diet means. Normal- and high- protein diets need to be defined in relative and absolute terms in relation to energy intake.

In relative terms, a protein diet is qualified as "normal" when protein intake accounts for 10%-15% total energy of the diet, whereas it is qualified as "high" when protein intake accounts for at least 18% to 30%, or even 47% total energy. However, in absolute terms the amount of protein intake can be the same or even greater in a "normal" protein diet. For example, as illustrated by Westertep-Plantenga a ~ 2800 kcal balanced diet, with a normal protein intake of 15 % represents 100g protein/day whereas a very-low-energy diet providing ~ 500 kcal, a high-protein intake of 47 % is equivalent to 52g protein/day.

Halton *et al.* indicate that the results of most studies converge to indicate that higher protein intakes are associated with increased satiety, energy expenditure, thermogenesis and weight loss (57).

The question of satiety is probably the most difficult to assess among these four parameters, since it does not rely on an objective *in vivo* variable. Up until now evaluating satiety has been limited to subjective observation. The two most widely methodologies to evaluate satiety are the use of visual analogue scale satiety ratings, or the quantification of energy intake during a subsequent meal. Moreover, satiety appears to be influenced not only by a wide variety of physico-chemical properties of food such as palatability, food mass, energy density, fibres and glycemic index but also by psychological and physical state of the subject, such as mood, motivation, stress, anxiety or tiredness. In other words, evaluating satiety is limited to subjective appreciation.

Even if the regulations of satiety and energy expenditure are far from being completely elucidated, some potential mechanisms have been suggested. A preliminary theory was proposed by Mellinkof in 1956 (58) which he termed the "amino-static" theory. Since high amino acid concentrations are correlated with a reduction in appetite it was believed that there was a satiety centre in the brain sensitive to amino acid concentrations. A recent paper from Koren *et al.* contradicted this hypothesis as they found an increase in satiety after high-carbohydrate and high-protein diets, a decrease in body weight, but no changes in plasma amino acid concentrations (59). Interestingly, *Nefti et al.* showed that protein-induced satiety was related to vagal feedback to the nucleus tracts solitarius in the brainstem and to the hypothalamus, where it suppresses feelings of hunger (60). Even if this hypothesis is engaging and makes sense as levels of amino acids are of primary importance regarding tissue growth, there is still a lack of evidence to support this hypothesis.

Among the mechanisms that might contribute to protein-induced satiety, three other hypotheses have been proposed by Veldhorst in a recent review, such as changes in the concentrations of satiety hormones, increases in energy expenditure or effects on the process of gluconeogenesis (61).

*Change in concentration of satiety hormones*

Taken together, studies have shown some evidence that a high-protein meal, when co-ingested with carbohydrates, stimulates the secretion of anorexigenic hormones such as Glucagon-like peptide-1 (GLP-I) (62) or PYY (63) whereas Ghrelin does not seem to be affected. Insufficient information is available on CCK to see any tendency.
Another hypothesis developed by Westerterp-Plantenga, relied on the observation that there is a relationship between energy expenditure and satiety (64). A possible explanation sustained by Veldhorst could be that increased energy expenditure at rest is concomitant with an elevation in oxygen consumption and body temperature that may lead to sensing oxygen deprivation, which in turn seems to promote satiety. This phenomenon has already been observed in situations of oxygen deprivation, such as high altitude or chronic obstructive pulmonary disease.

*Increase in energy expenditure & Gluconeogenesis*

The relatively strong thermic effect of protein intake may be mediated by the high ATP cost of postprandial protein synthesis. Amino acid oxidation may also play a major role, more particularly when they are administrated in excess, since they can not be stored and therefore have to be degraded. The ATP cost of protein metabolism depends on their nature, digestibility, and composition in amino acids. The longer the carbon chain of an amino acid, the more ATP is needed for its complete oxidation and urea synthesis.
Ingestion of rapidly digested protein, such as whey, results in a stronger increase in postprandial protein synthesis and amino acid oxidation than slowly digested protein, such as casein.
Gluconeogenesis, induced by protein intake, has also been suggested to contribute to satiety or to better food intake regulation. But so far this has only been demonstrated in an animal model up to now. It could be related to the improvement of glucose homeostasis, regulated by modulation of hepatic gluconeogenesis and subsequent glucose metabolism.

Protein induced satiety has been shown acutely, with single meals containing up to 81% protein, but also with high protein *ad-libitum* diets, lasting from 1 to six months, with a significant reduction in body weight attesting even to the satiety effect of protein and/or to the increase in energy expenditure.
Veldhorst et al (61) concluded that mechanisms explaining protein-induced satiety are primarily nutrient-specific, meaning dependant on the nature of protein, and are essentially related to elevated plasma amino acid concentrations.
Protein-induced satiety seems to be related to protein-induced energy expenditure and is believed to be of prime importance for protein induced weight loss and weight maintenance by Westerterp-Plantenga (56). This aspect of satiety was confirmed by Clifton in an editorial about high protein diets and weight control (65). Indeed *ad libitum* or energy controlled high-protein diets are helpful in increasing weight loss and in weight maintenance, by preventing weight regain.

### 1.6.2 Dietary proteins: Type II Diabetes

Insulin resistance and Type II Diabetes are major metabolic disorders associated with obesity and the metabolic syndrome. Consequently, the question of the metabolic effects of high protein diets on insulin resistance is of major importance.
When taken together, animals, humans and epidemiological studies show very discrepant results and underlie different outcomes on glucose homeostasis with high protein or amino acids intakes.

An extensive and interesting article by Tremblay *et al*. (66) reviewed in detail the role of dietary proteins and amino acids in the pathogenesis of insulin resistance. He reviewed experimental studies with amino acid infusion which demonstrate that protein and amino acids *per se* can have deleterious effects on glucose homeostasis by promoting insulin resistance and increasing gluconeogenesis. These effects are linked to the intrinsic properties of protein and amino acids. Increased postprandial amino acid availability, might have direct (substrate-mediated) and indirect (hormone-mediated) effects on glucose metabolism (67). Endogenous secretion of insulin and glucagon, both hormones that are involved in the regulation of glucose homeostasis and hepatic glucose metabolism by altering portal insulin/glucagon ratio is stimulated.

The direct effect of amino acid elevation can be shown when the endogenous release of glucoregulatory hormones is inhibited, such as during a continuous infusion of somatostatin. The direct effect of amino acid on hepatic glucose metabolism is an increase in gluconeogenesis and endogenous glucose production without any effect on glycogenolysis (68). In the case of insulin resistance, such as in Type II Diabetes, insulin secretion is insufficient to compensate for the hyperglycemia. The direct effect of amino acid infusion is a reduction in insulin-stimulated whole-body glucose disposal. Amino acids cause a reduction in the rate of skeletal muscle glycogen synthesis, which is linked to a direct inhibition of muscle glucose transport and/or phosphorylation.
Among the indirect effects of protein on stimulation of the endogenous release of glucoregulatory hormones in Type II Diabetes, it seems probable that β-cells, refractory to glucose, retain the ability to respond to non-glucose stimuli such as amino acids, allowing to adjust the increase in insulin concentration.

A long the same thoughts, when dietary habits are assessed by food intake questionnaires in a large sample of individuals, a high meat intake is linked to adverse effects on glucose metabolism, insulin resistance, and glucose intolerance (69).
Taken together, experimental studies with amino acid infusions and epidemiological data demonstrate direct induction of insulin resistance and stimulation of endogenous glucose production.

However, intervention studies with high-protein diets are in apparent contradiction with the above considerations as they demonstrate in the short term an improvement in insulin sensitivity, glucose homeostasis and Type II Diabetes (70). The most commonly advanced hypothesis is that protein exerts beneficial effects on glucose homeostasis by reducing body weight. Indeed all these studies aimed at decreasing body weight. Compared to high-fat or high-carbohydrate diets, high-protein diets tend to demonstrate enhanced weight loss, subsequent to increased energy expenditure and satiety (71). Consequently, improvement of features of the metabolic syndrome, such as insulin sensitivity and type II diabetes, seems to be linked more to an effect of protein intake on satiety and body weight loss than to an intrinsic property of protein administration.

### 1.6.3 Dietary proteins: Dyslipidemia and lipotoxicity

When insulin sensitivity is considered, studies pertaining to the effect of proteins on plasma lipids are again very discrepant in the literature, and it is almost impossible to distinguish between the beneficial effects of protein *per se* and indirect effects mediated by fat loss and body weight reduction (72).

Studies often suggest that improvements in the features of the metabolic syndrome, such as the lipidic profile, were first linked to a decrease in body weight. Clifton *et al* (65) pooled results from three randomized clinical trials (73-75) in which overweight/obese subjects (BMI> 26 $kg/m^2$) were assigned to *ad libitum* diets, among which one was high in protein (i.e. 25% of energy as protein or the high-protein Zone diet) during at least 6 months. Computation of the results demonstrated that the high-protein diet showed greater fat and weight loss, better improvement in lipidic profile, in total cholesterol and triacylglycerol and reduced associated CVD risk factors. However the conclusion of other studies were more prudent as they observed favourable effects of high-protein diets on changes in triglyceride and HDL-cholesterol concentrations but less favourable changes in total and LDL-cholesterol than conventional, lower proteins diets (76).

Intriguingly, studies were in agreement when attributing dyslipidemia, characterised by high plasma concentration of triglycerides and VLDL-cholesterol and low levels of LDL-cholesterol and HDL-cholesterol, to an increase in carbohydrates intake. Indeed, low-carbohydrates diets reverse these abnormalities (67). A meticulous study, by Krauss *et al* (77) designed to assess the effect of different carbohydrate intakes, independently of weight loss, on lipid profile clearly demonstrated that moderate carbohydrate restriction had beneficial short term effects on dyslipidemia. It can then be argued that with high-protein diets, it is more the reduced proportion of carbohydrate than the increased amount of protein administration *per se* which improved dyslipidemia and associated cardiovascular risk factors.

Even though this PhD work addresses the global question of the effects of dietary proteins on dyslipidemia and whole body lipid metabolism, it specifically focuses on their potential effects to prevent ectopic fat accumulation in the liver, hypothetically by increasing hepatic

fat oxidation or altering hepatic *de novo* lipogenesis. This question has recently gained much interest, but actual knowledge is still very scarce. An important limitation to the study of liver metabolism in humans, contrary to adipose or muscle tissues, is the difficulty to have direct access to the tissue and then to liver gene expression. Liver biopsies are considered too invasive to be ethically admitted for research purposes. Consequently, the techniques that are used to approach liver metabolism are mainly indirect, such as labelled tracers. Imaging tools are more informative about the structure of the organ, and recent advances in NMR-spectroscopy have provided more information about the composition of liver metabolites concentrations and liver parenchyma structure.

Until recently little or no data were available in humans. However the role of dietary proteins on liver fat and lipotoxicity has begun to be addressed over the last few years in rodents.

The group of Torres, from the Department of Fisiologia de la Nutricio in Mexico conducted many studies in rodents on the prevention of lipotoxicity by dietary proteins and believed that effects might differ according to the type of proteins (78, 79).

In a recent review of their work, they reported that the type of dietary protein has a significant contribution to the process of lipotoxicity through the modulation of insulin secretion and the regulation of adipocyte metabolic function (80). Modulation of insulin secretion appeared to be central in improvement of hepatic lipotoxicity. Soy protein administration, compared to casein, is associated with an improvement in insulin sensitivity, decreased insulin secretion and a decreased insulin-glucagon ratio, probably linked to a decrease in the sterol regulatory element binding protein (SREBP-1). SREBP-1 is involved in the regulation and expression of more than 30 genes dedicated to the synthesis and uptake of cholesterol, FFA, triglycerides and phospholipids in the liver. SREBP-1c acts as a promoter for expression of enzymes such as fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), ATP citrate-lyase. Soy protein intakes also showed a decrease in adipocyte hypertrophy, hyperleptinemia and free fatty acids, leading to a decrease in the influx of lipids to the liver while improving their oxidation. Lipids and ceramide depots were decreased, which reduces hepatic lipotoxicity (81) (**see Figure 3**).

Another study in C57BL6 mice investigated the effects of dietary essential amino acids (EAA) on high-fat diet induced obesity (DIO) (82). Results were concordant with those of Torres *et al.* in the sense that EAA supplements reduced plasma insulin concentrations, which were associated with a decrease in SREBP-1 expression, leading to a decrease in hepatic steatosis and *de novo* lipogenesis.

Taking current knowledge gained from such studies in rodents and basic fundaments of protein metabolism in humans together, it is possible to propose an hypothesis that dietary protein intake may impact on insulin secretion and insulin sensitivity and consequently might affect regulation of hepatic gene expression, such as SREBP-1 and hepatic fat metabolism.

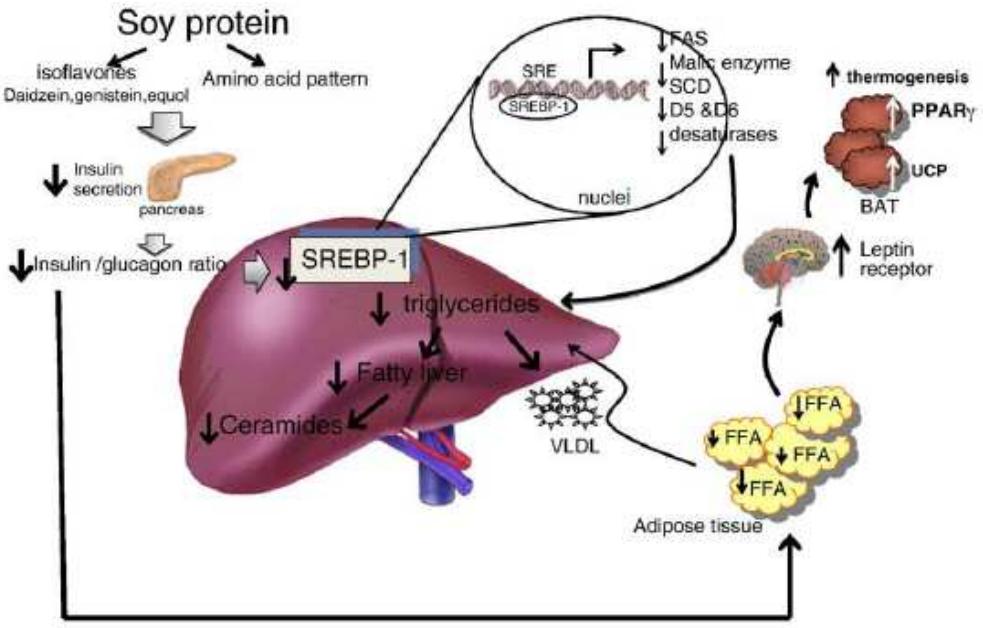


**Fig 3. The mechanism of action of soy protein (79)**
The amino acid pattern and isoflavones decrease the insulin-glucagon ratio, reducing SREBP-1 expression and therefore hepatic lipogenesis. Furthermore, soy protein decreases adipocytes hypertrophy and the release of fatty acids (FFA), which reduces the influx of FFA into the liver. These changes lower the accumulation of hepatic lipids and ceramide, which reduces lipotoxicity. In addition, soy protein increases the thermogenic capacity of brown adipose tissue.

### 1.6.4 Dietary proteins and Renal Function

A major consideration point of consideration before recommending an increase of dietary protein for treatment of obesity and the metabolic syndrome is the potential adverse effects of proteins on renal function and acid-base balance (83). Population-based studies, preliminary prospective studies, animal models of kidney disease, and clinical trials, although controversial, tended to find associations between dietary protein intakes and progression of kidney disease (84). Indeed, experimental data indicate that acutely or chronically increased protein intake modulates renal function by increasing renal plasma flow (RPF) and glomerular filtration rate (GFR) which leads to hyperfiltration (55).

It is generally accepted that glomerular hyperfiltration can cause progressive kidney damage in individuals who already suffer from kidney disease, however there is controversy regarding the effects in individuals with normal kidney function (84). The effects of chronically increasing protein intake on GFR has been extensively reviewed by King *at al.* (55). Interactions between direct and systemic effects on renal processes seem to best explain this phenomenon. The author proposed a hypothetical model to explain the increase in GFR. Direct and indirect effects might act together and lead to an increase in preglomerular arterial pressure and RPF which might in turn result in an increase in GFR.

Addressing the effects of high-protein diets on glomerular function is even more conflicting as the most frequently reported parameter is GFR, the precursor of which depends largely upon the method used. The gold standard method to investigate GFR is constant intravenous infusion of inulin, which is an ideal filtration marker that is completely filtered and is not reabsorbed by the tubular cells. This method is, however, invasive and time consuming and cannot be applied to large scale screening. Consequently, serum creatinine concentration is a common surrogate measure of renal function in clinical practice. However, it is an imperfect filtration marker as it is both flittered and reabsorbed in the renal tubular cells and its production is not constant among individuals.

A major point with intervention studies addressing the effects of protein is the length of dietary intervention. Many studies are performed over short periods of time, which may be insufficient to lead to significant kidney dysfunction. Wagner *et al* demonstrated that one week on a high-protein diet increased GFR in elderly subjects but did not cause clinically detectable acidosis or renal failure (83). In a prospective cohort study involving 1642 women, Knight *et al.* demonstrated decreased kidney function in women with pre-existing renal failure, but not in those with normal renal function (85).

Acquiring knowledge and understanding about the effects of high-protein diets on renal function becomes of major importance given the popularity of high-protein weight loss diets. A recent clinical trial, by Frank *et al*, addressed this question when investigating the effects of 7-day high-protein or normal protein diet on renal hemodynamic and selected clinical-chemical factors in 24 healthy young males. They concluded that renal hemodynamic and urinary excretion of uric acid, sodium and albumin was already impaired after one week on the high-protein diet and recommend that more attention is paid to the potential adverse effects of high-protein diets on the kidney (86).

### 1.6.5 Summary; Dietary proteins: enough evidence for practice?

Current knowledge tends to attribute a beneficial role of protein in promoting optimal health, by favouring lean body mass retention, weight control, reducing inflammation, improving insulin sensitivity, bone and cardiovascular health (87). These effects on weight control seem to be linked to an increase in protein-induced energy expenditure and satiety. The questions of insulin sensitivity and renal functions are more controversial, as discussed above, and further studies of the long term safety of such diets is imperative (72).
When referred to the review of Brehm & D'Alassio, it seems to be reasonable to recommend diets moderately increased in protein and modestly restricted in carbohydrates and fat, and especially saturated fat, with a special focus on long-term compliance and safety of chronic high protein intakes (72). However it's still on debate.

### *1.7 HYPOTHESIS*

Current knowledge associates the development of steatohepatitis with obesity and glucose intolerance, subsequent to hypercaloric high-fat and/or high-carbohydrate intakes and low physical activity. The satietogenic and thermogenic effects of dietary protein intakes are also well recognized. Therefore we hypothesized that

1) protein might decrease hepatic steatosis by reducing IHCL content
2) by reducing hepatic steatosis, protein might improve glucose homeostasis
3) protein might exert beneficial effects by improving lipid metabolism at the hepatic and whole body level

### *AIMS OF THIS WORK*

This aim of this work was to investigate the metabolic effects of dietary protein intake on hepatic lipid metabolism and glucose homeostasis. This question was assessed by evaluating the effect of dietary protein modulation on

- amount of triglycerides in the liver (IHCL)
    - $^{1}$H-NMR
- exogenous lipid oxidation
    - by labelled $^{13}CO_2$ in breath after ingestion of $^{13}$C-labelled triolein
- net lipid oxidation and whole body energy expenditure,
    - by indirect calorimetry
- hepatic beta-oxidation,
    - by plasma concentrations of beta-hydroxbutyrate
- triglycerides concentrations,
    - by chylomicron-TG and VLDL-TG clearance
- glucose tolerance
    - by two-steps euglycemic-hyperinsulinemic clamp
    - OGTT
    - HOMA-IS
    - glucose turnover

# *CHAPTER 2*
# *METHODOLOGY*

## *2.1 ISOTOPE TRACER METHODOLOGY (88)*

### 2.1.1 What is a tracer?

Robert L Wolfe and David L Chinkes defined in their famous work "Isotope Tracers in Metabolic Research", a tracer as : "a compound that is chemically and functionally identical to the naturally occurring compound of interest (the tracee) but is distinct in some ways that enables detection" (89). An ideal tracer should be detected with sufficient precision when administered in trace amounts, should not alter or interact with the metabolism and kinetics of the tracee, but should however perfectly reflect its metabolism. It is assumed that the tracer and the tracee do not differ in their kinetics or their metabolism and that, once the tracer is taken up by tissues, it does not re-enter the systemic circulation (90).
There are 3 general ways in which tracers are used in metabolic research; a) to trace the kinetics and metabolism of an injected compound, such as the kinetics of a labelled pharmacological agent, b) to measure its rate of incorporation into another compound, such as the rate of synthesis of a product (example: protein synthesis), c) to measure the rate of appearance of a substrate into plasma by the "tracer dilution" technique, such as for endogenous glucose production or lipolysis.

A tracer is made by labelling a molecule otherwise identical to the tracee with one or more atoms that are distinct from the most abundant form of the atom. Indeed elements are composed of atoms that are chemically identical, but slightly different in mass. Such atoms are called *isotopes*. The difference in mass is due to a different number of nuclear neutrons, which, however, do not affect its chemical properties. As an example $^{12}C$ is the most abundant atomic mass for the carbon element at 99%, $^{13}C$ is its naturally occurring stable isotope and $^{14}C$ is its radioactive one.

There are two major types of tracers; those which are labelled with *radioactive* isotopes and those which are *stable*. They have major distinct properties.
*Radioactive isotopes* undergo spontaneous disintegration until they reach a more stable state and form an atom of another element. Disintegration is accompanied by emission of three types of radiations, α, β and γ. Consequently the abundance of a radioactive tracer is

measured by a scintillation counter. It is expressed as decay or disintegrations per minute (dpm)/per unit mass and is called Specific Activity (SA). Even if the use of radioactive isotope as tracers is a convenient method for determining its abundance in biological samples, the major limitation for studies in human subjects is the health risk associated with the process of disintegration. Ionizing radiations are accompanied by the transfer of large amounts of energy which can disrupt molecular function, and lead to cell death and tissue damage depending on the altered functions.

*Stable isotopes* present the advantage of not undergoing spontaneous decay. Their most obvious advantage is probably that they are safe for human use, presenting almost no associated risk when used in tracer amounts. An isotopic effect, leading to a different metabolism of the tracee relative to the tracer, has sometimes been reported. However studies in vivo have provided little evidence, and these effects should be minimized. Stable isotope also present the advantage that there exists a stable isotope for almost every element such as $^{15}N$, $^{18}O$, $^{18}C$ (91). Actually, recent advances are supporting the use of labelled stable isotopes to investigate the effects of diet on nutrients metabolism (92).

Interestingly the use of stable isotopes as metabolic tracers preceded the use of radioactive isotopes by almost 20 years. In the early 1930s, Schoenheimer and Rittenberger at the University of Columbia, pioneered research in metabolism using the isotope tracer technique and stable isotopes, such as deuterium to trace fat metabolism (93) and $^{15}N$-glycine to demonstrate the dynamic nature of the protein pool (94). In the 1950s-1960s, with the advent of scintillation counting, most metabolic studies used radioactive tracers to determine the kinetics of substrate metabolism and metabolic rates. Since the 1970s the use of stable isotopes regained interest; the wide availability of tracers labelled with stable isotopes, the improvement of sample analysis with the advent of the quadruple gas chromatography mass spectrometer (GCMS) and an increase in awareness of human safety during clinical investigations stimulated the use of stable isotopes.

## *2.2 GAS CHROMATOGRAPHY – MASS SPECTROMETRY (GC-MS)* (95, 96)

### 2.2.1 GC-MS principles

This is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. It is composed of two major building blocks; the gas-chromatograph (GC) and the mass spectrometer (MS) (**Figure 4**). In these experiments, isotopic enrichment analysis are carried out by a Hewlett-Packard (HP) 5890 Series II Gas – Chromatograph interfaced with a (HP) 5971 quadrupole mass spectrometer, Palo Alto, CA, USA).

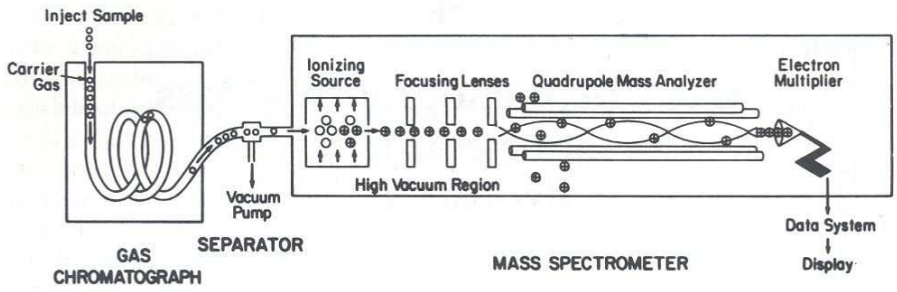


**Fig.4** Schematic diagram of the components of GCMS system

The gas-chromatograph provides a simple, rapid and reproducible method for high-resolution separation of volatile compounds. Organic compounds must generally be converted to derivatives that are thermally stable, chemically inert, and volatile at temperature below 300°C. Derivatization is achieved by masking polar groups (N-H/O-H) and replacing the hydrogen atoms by acylation, alkylation or sylation. The gas-chromatograph contains a capillary column which allows elution and separation of compounds as a function of their mass and chemical properties. Gas chromatography is a microanalytical, very sensitive, method, which requires only small volumes of samples and allows detection even at concentrations of $10^{-12}$.

The mass spectrometer refines this preliminary fragmentation by splitting each molecule into ionized fragments in the ionization chamber, and detecting these fragments using their mass to charge ratio (m/z). Detection of an ionized molecule relies on the principle that its

particular path can be controlled by electric and magnetic field in a mass-dependant fashion, which occurs in the mass analyser. Several methods can be used to ionize the molecules, among which electron impact and chemical ionization exist. Electron impact ionization consists of bombarding the molecules with free electrons emitted from a filament. Consequently, the molecules are fragmented in a typical, characteristic and reproducible spectrum of smaller molecules, leading to a spectrum of m/z ratios. This is called "hard ionization" in contrast to "soft ionization" which results from molecular collision with an introduced gas. Chemical ionization is achieved by introducing a reagent gas, usually methane or ammonia, into the ionization chamber which reacts with and ionizes the molecule.

Detection of ionized particles occurs by measuring their acceleration in the mass analyser. There are a variety of mass analyzers, but metabolic studies frequently employ the quadrupole. The quadrupole mass filter consists of four parallel circular rods, electrically connected in pairs, which radiate an electrodynamics field when exposed to an alternating current. Selectivity of the ionic masses is reached by modulating the amplitude of the quadrupole voltage. A range of ionic masses or a single selected ion mass resonates through the filter while all other masses are pumped away. The ions that enter the quadrupole field drift through the field axis and are subject to oscillations with a frequency that is related to the mass charge ratio of the particles reaching the collector. As a linear brushing of voltage is imposed, masses are detected as a function of crescent values of m/z ratio.

Detection of ions occurs through the monitoring of the electrical current that it is generated in proportion to the abundance of each ion. For computerization, it is amplified by an electron multiplier which is then converted into a logical signal that is interpreted by the computer.

In the case of metabolic tracer studies an accurate measure of isotopic ratio is to monitor only selected peaks associated with a specific substance, which is called selective ion monitoring (SIM). 6,6-$^{2}$H-penta-acetyl glucose was analyzed with a gas chromatograph-mass spectrometer (Hewlett Packard, Palo Alto, CA) in the chemical ionization mode with selective monitoring of m/z 331 and 333. 1,1,2,3,3-$^{2}H_5$ glycerol enrichments were measured on acetylated derivatives with selective ion monitoring at 164.1 and 159 m/z.

### 2.2.2 Gas chromatography-isotope ratio mass spectrometry (GC-IRMS) (95, 96)

Isotope Ratio Mass Spectroscopy (IRMS) is a specialization of mass spectrometry methodology, in which a pyrolysis chamber is inserted between the GC outlet and the mass spectrometer inlet. IRMS methods are used to measure the relative abundance of an isotope in gaseous form such as $^{13}CO_2$ versus automatically equilibrated reference $CO_2$ and express the enrichment in Atom Percent Excess. To measure the isotopic enrichment in a solid or liquid, molecules, separated by GC, are first combusted and heat-cut in the pyrolysis chamber and directed then into the ionization chamber. Ionized gas is accelerated in a flight tube and isotopes separate to their mass into Faraday collectors. The current of each ion beam is then measured using a 'Faraday' detector or multiplier detector. Compared to GCMS, which allows detection of almost limitless variety of samples, in IRMS analysis of pure gas is limited and sample preparation, for any compound other than pure gas, is more tedious, however it reaches a greater precision ($1 \times 10^{-5}$).

## *2.3 TRACERS METHODOLOGY APPLIED TO THIS WORK*

Only *stable isotopes* were used in the present studies to assess metabolism *in vivo*. Among the different ways of tracing metabolism, we either measured the rate of incorporation of the tracer into another compound, assessed its rate of appearance into plasma by the "tracer dilution" technique, or sometimes both while using the same tracers. We also evaluated oxidation of $^{13}C$-labelled substrates by the recovery of $^{13}CO_2$ in expired gas.

### 2.3.1 Primed continuous infusion of tracers and oral single dose ingestion

Some tracers were constantly infused throughout the study, after a preliminary bolus injection, whereas others were administrated orally as a single dose. All tracers were furnished by Cambridge Isotope Laboratories, Cambridge, MA. The isotopes were dissolved in isotonic saline and subsequently sterilized and tested for sterility and lack of pyrogenicity.

***6,6-$^2H_2$-glucose:*** *[HOCD2(CHOH)4CHO]*

6,6-$^2H_2$-glucose was infused during most of the studies. In the two-step clamp investigations, it was used to assess endogenous glucose production in the fasting state (bolus: 2mg/kg; followed by a continuous infusion of 20 µg/kg/min) for 120 minutes, hepatic insulin sensitivity in the first step (insulin infusion: 0.3 mU/kg/min; 6,6 $^2H_2$-glucose 40 µg/kg/min) for 90 minutes and whole-body glucose uptake/insulin sensitivity in the second step (insulin infusion of 1.0 mU/kg/min; 6,6 $^2H_2$-glucose 80 µg/kg/min) for 90 minutes.
In postprandial studies it was infused continuously throughout the study (bolus 2 mg/kg, continuous infusion 40 µg/kg/min), at baseline to assess endogenous glucose production (EGP), and during the postprandial phase to assess effects of proteins on glucose turnover, rate of appearance (Ra) and disappearance (Rd).

***1,1,2,3,3- $^{2}H_5$-glycerol:*** [(HOCD2)2CDOH]

$^{2}H_5$-glycerol was used in postprandial studies to assess the effects of protein on adipose tissue lipolysis according to the dilution principle. The bolus injection was 1 µmol/kg and the continuous infusion 0.1 µmol/kg/min.

We also attempted to measure the incorporation of the tracer in TG-VLDL, to trace VLDL synthesis and kinetics, however results were inconclusive as sample collections were to scarce to model VLDL-TG secretion (97). The methodology was improved in later protocols to trace VLDL-TG synthesis.

**2.3.2 Oral administration**

Simultaneously, we wanted to trace the effects of chronic and/or acute intakes of protein on exogenous fat susceptible to affect hepatic lipid metabolism during their metabolism.

***1,1,1- $^{13}C_3$-triolein:*** [C3C54H104O6]

The meal was composed of 10 % long chain triglycerides (LCT, mainly as olive oil) labelled with 1% $^{13}$C-trioleate. Dietary LCT are first delivered to the periphery by chylomicrons and thereafter to the liver as captation of remnant-chylomicrons. The rate of appearance of $^{13}CO_2$ in expired gas was also assessed to evaluate whole body lipid oxidation.

## *2.4 PRINCIPLES AND CALCULATIONS*

### 2.4.1 Primed continuous infusion

Calculations differ according to whether the organism is studied in steady state conditions or not. In steady state conditions, meaning when the concentrations of the tracer and tracee do not vary over the time, the dilution of the tracer can be used to determine the rate of appearance (Ra) of the tracee in the organism, like endogenous glucose production (EGP). As, in steady state conditions, tracee concentrations do not change over time, the Ra of the substrate equals its rate of disappearance (Rd), then EGP equals glucose uptake by the tissues.

In the 2-step hyperinsulinemic euglycemic clamp studies, it is assumed that steady-state equilibrium is reached at each step after one hour of infusion. Then the Ra and Rd of glucose metabolism were estimated during the 30 last minutes. The equation used (98) was based on Steele's development (99), and reported as follow in Wolfe's book (95).

At the isotopic equilibrium, the relative concentration of isotope is equal in the infusion mixture and the extracellular fluid (ECF). Thus,

$$[\text{tracer}/(\text{tracer}+\text{tracee})]_{\text{INFUSATE}} = [\text{tracer}/(\text{tracer}+\text{tracee})]_{\text{ECF}}$$

Therefore, when multiplicated by the infusion rate,

$$[F/Ra] = [\text{tracer}/(\text{tracer}+\text{tracee})]_{\text{ECF}}$$

where $IE = [\text{tracer}/(\text{tracer}+\text{tracee})]_{\text{ECF}} * 100$ or $IE = [m/((m) + (m^{+1}))]$

Thus ,

$$[F/Ra] = IE * 0.1$$

Solving for Ra yields,

$$Ra = F/IE * 0.1$$

In order to account for the contribution of the isotope to total Ra, the Ra of unlabeled glucose is calculated by subtracting the rate of isotope infusion from the total Ra.

Therfore,

$$Ra = [F/IE * 0.1]-F$$

In order to compare between subjects, Ra is expressed in relation to body weight, thus

$$Ra = [[F/IE * 0.1]-F]/w$$

| | |
|---|---|
| *At the isotopic equilibrium:* | Ra = Rd = GTo |
| *Before glucose perfusion:* | Ra = GTo = EGP |
| *During the clamp:* | Ra = GTo |
| | EGP= GTo – FGcor |

where Ra, Rate of appearance (mg/kg/min), Rd, Rate of disappearance (mg/kg/min), GTo, Glucose Turnover (mg/kg/min), F, infusion rate of 6,6-$^2H_2$ – glucose (mg/min), IE, Isotopic Enrichment, w, body weight (kg), FGcor, is glucose infusion rate corrected for the variation in plasma glucose concentration (mg/kg/min).

Most of our studies investigated metabolism in non-steady state conditions, looking at the postprandial response of metabolites. Consequently the rate of appearance can be calculated from the dilution of the tracer at any given time, corrected for changes in tracer dilution per unit of time and similarly the Rd is calculated from the Ra, corrected for changes in tracee dilution per unit of time. The equations used were adapted from the non-steady state equation of Steele based on the single-pool kinetic model (99). The use of non-steady state conditions is based on various assumptions regarding the distribution and volume of the metabolite pools of which their adequacy in reflecting the complexity of substrate kinetics has been extensively questioned (100). It has been admitted that even if the single-pool model is imperfect and fails to accurately measure kinetics, it can be used to provide reasonable approximations when the equations are corrected to account for the non-ideal natural comportment of the substrates. Therefore Steele advanced the concept of a rapidly mixing pool that was a fraction ($p$) of the total extracellular pool of the substrate of interest ($V$). $pV$ values are defined as functions of the variations of Ra and substrate concentrations over time. Again, many studies were performed in an attempt to find a single value that enables a reasonable approximation of the true value in a variety of circumstances (101).

Although the model is imperfect, there has not yet been any other approach that is unequivocally better.

Therefore the Steele's equation for non-steady state conditions is still widely used and generally accepted (101, 102).

$$Ra = [F - pV\,[(C_1 + C_2)/2][(IE_2 - IE_1)/(t_2 - t_1)]]/[(IE_2 + IE_1)/2]$$

$$Rd = Ra - [pV\,[(C_2 - C_1)/(t_2 - t_1)]]$$

where Ra is the rate of appearance (mg/kg/min), F is the specific isotope infusion rate (mg/kg/min), $p$ is the pool fraction, $V$ is the volume of distribution (l/kg), $C_1$ and $C_2$ are tracee concentrations at sampling $t_1$ and $t_2$ and $IE_1$ and $IE_2$ are the excess isotopic enrichments of tracee at time $t_1$ and $t_2$.

The $pV$ value chosen for Ra glucose was of 0.150 (l/kg) with a *pool fraction (p)* of 0.75 and a volume of distribution ($V$) of 0.2 l/kg (101) (103). The volume of distribution used for Ra glycerol was 0.23 l/kg (104) (10).

**2.4.2 Oral single dose administration**

In postprandial studies, meals were labelled with $^{13}C$, such as $^{13}C$-trioleate. It was thus possible to assess the rate of oxidation of the labelled substrate by measuring total and labelled carbon dioxide production but also to follow semi-quantitatively pathways of its metabolism by measuring plasma concentrations of newly synthesised $^{13}C$-substrates. Exogenous substrate oxidation was calculated with reference to the equation used for quantifying the oxidation of a triglyceride load as described by Binnert *et al* (105).

## *2.5 INDIRECT CALORIMETRY*

### 2.5.1 Principles

Indirect calorimetry is a widely used technique to assess energy expenditure and substrate oxidation *in vivo*. The fundamental principle is based on the measurement of whole-body oxygen consumption and carbon dioxide production over given periods of time. It is based on the recognition that each of the three major macronutrients, when oxidized, reacts with a known amount of oxygen to produce a know amount of carbon dioxide and water since under normal conditions neither oxygen nor carbon dioxide are stored. Their measurement represents all of the oxidative process occurring in the body. When the specific respiratory quotient of the three macronutrients (carbohydrates, lipids and proteins) and the rate of protein oxidation, estimated from the nitrogen-end product (urine urea) is known, it is possible to calculate the net oxidation rate of the three substrates individually as well as total energy expenditure (106).

An important point to underline is that indirect calorimetry represents only net but not actual rates of substrates oxidation. It estimates the net disappearance rate of a substrate regardless of the metabolic interconversions that the substrate may undergo before its disappearance from its metabolic pool (107). For example, fatty acid oxidation, oxidation of ketone bodies and oxidation of glucose formed from glycerol, are all included in net lipid oxidation, while oxidation of fatty acids produced from amino acids or from glucose compute for net protein and net glucose oxidation respectively (107) (108).

Calculation of net substrate oxidation is done according to the work of Livesey and Elia, in which they paid particular attention to the effects of different composition of conventional foods on the accuracy of the equations of indirect calorimetry (109). They assessed to what extent both errors in the measurement of $\dot{V}O_2$ and $\dot{V}CO_2$ and in the assumptions about carbohydrate, fat and protein oxidation, may affect results. They concluded that the accuracy should be considered to be at the very best within 5% of the true value and that considerably larger errors are expected under special circumstances.

*The equations used for substrates metabolism were as follow (108)*

Glucose oxidation (mg/min): $(4.650 * \dot{V}CO_2)-(3.311\ \dot{V}O_2) - 3.581*N$

Lipid oxidation (mg/min): $(1.720 * \dot{V}O_2)-(1.720* \dot{V}CO_2) - 1.776*N$

Protein oxidation (mg/min): $6.25 * N$

where $\dot{V}O_2$ is oxygen consumption (ml/min), $\dot{V}CO_2$ is carbon dioxide production (ml/min), N is urinary nitrogen excretion (mg/min) over the measurement period, and 6.25 is the conversion factor for nitrogen excretion to protein oxidation.

*Calculation of energy expenditure relies on the use of Weir Formula (110)*

EE (kcal/min): $\left[[(3.941* \dot{V}O_2)+(1.106* \dot{V}CO_2)]*1.44\right]-(2.17* N)\right]/1440$

where $\dot{V}O_2$ is oxygen consumption (ml/min), $\dot{V}CO_2$ is carbon dioxide production (ml/min), N is the urinary nitrogen excretion (g/day) among period of gas analysis, and 1440 is the number of minutes per day.

In case where high-protein meals, diets or amino acids are administered the urea pool size will vary during the measurements and correction factors have to be introduced. The actual metabolic nitrogen production rate is equal to urinary urea excretion to which is added the change in urea nitrogen pool size (111).

*Protein oxidation was assessed as follow*

Protein oxidation (mg/min)= $6.25 * \left[N + [((Ut_2-Ut_1)*28*0.6* w)/(t_2-t_1)]\right]$ *(111)*

where N is the urinary nitrogen excretion (mg/min) during the period of gas analysis, $Ut_2$-$Ut_1$ are the plasma concentrations of urea (mmol/l) respectively at time $t_1$ and $t_2$, w is body weight (kg), 0.6 *w is the urea distribution volume and 28 is computed to the molecular weight of the 2 molecules of nitrogen present in urea.

Net protein oxidation was adjusted by taking into account that nitrogen estimated from urinary urea represents almost 90 % of protein catabolism in reference to Boden *et al* (112) and 85% in Study II and III in reference to Minehira *et al* (113). Protein oxidation was adjusted as follow

Study I: Protein oxidation = 6.25 * (N/90*100)

Study II/III: Protein oxidation = 6.25 * (N/85*100)

**2.5.2 Pitfalls in indirect calorimetry**

Calculations of indirect calorimetry are based on the assumption that respiratory oxygen and carbon dioxide exchanges are equal to what happens at the metabolic and cellular levels, meaning to the rate of metabolic oxygen utilization and carbon dioxide productions. This implies that any perturbation of this "steady-state" condition will result in erroneous results. This assumption is correct for oxygen consumption in almost all circumstances but is more critical for carbon dioxide, due to the large size of the endogenous $CO_2$/bicarbonate pool, involved in acid-base buffering. Hyperventilation leads to an overestimation of carbon dioxide production, which will have implications on the calculation substrate oxidation and lead to an overestimation of carbohydrate oxidation over a short period of time. However this pitfall can be resolved if measurements are made long enough, as the period of hyperventilation will be followed by one of hypoventilation and substrate oxidation calculated throughout the whole period of unstable measurements will be representative of "true" substrate oxidation.

### *2.6 NUCLEAR MAGETIC RESONANCE SPECTROSCOPY*

The objective of in vivo NMR spectroscopy is to identify and quantify important chemicals in various body tissues.
In this work this technology was used to assess the effects of different diets over periods of 4 to 6 days on the accumulation of fat in the liver, which is called intrahepatocellular lipids (IHCL).

Briefly the principle of this technology rely on the property of stable nuclides, those which contain odd numbers of protons and/or neutrons to have a magnetic moment and angular momentum, i.e. a non zero spin. The most commonly studied nuclei used for *in vivo* NMR spectroscopy are protons ($^{1}H$), sodium ($^{23}Na$) or phosphorus ($^{31}P$). The advantages of $^{1}H$ spectroscopy are that it is easier to perform, it is more widely available, and it provides a much higher signal-to-noise ratio than sodium or phosphorus.

Principles of NMR usually involve two sequential steps which lead to the emission of specific signals from a molecule in a tissue or metabolites. The first property relies on the alignment (polarization) of the magnetic nuclear spins in an applied, constant magnetic field and the second on the fact that this alignment is disturbed by applying an electro-magnetic field, usually induced by a radio frequency (RF) pulse. The required perturbing frequency is dependent upon the static magnetic field and the nuclei of observation. Frequencies are specific for metabolites and depend on the configuration of protons within the chemical. Consequently metabolites are first identified by their frequencies and are expressed as shift in frequency (in parts per million [ppm]) relative to a standard usually water which is abundant in tissues. In other words chemical are identified by comparing their frequency location/shift to that of water (**see Figure 5**).

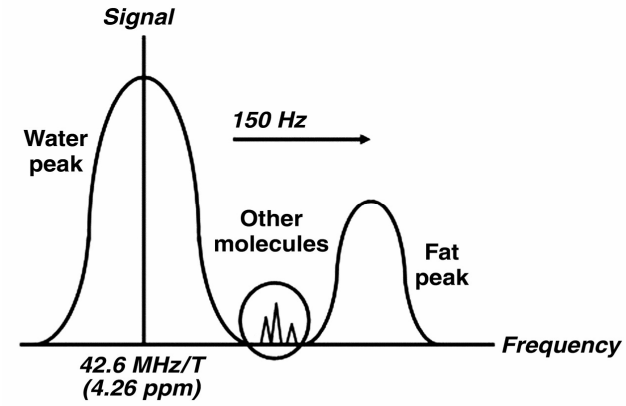


**Fig 5**: **This diagram shows metabolites frequency relative to water**. The peaks in $^{1}$H-NMR spectra correspond to different metabolites and are identified primarily by their frequencies. The dominant peaks in liver are those of water and lipids. (114).

The position of the $^{1}$H-NMR peak on the x-axis (frequency) is used to characterize the actual metabolites or chemicals, whereas the y-axis (signal high/intensity) and the peak line width give additional chemical information used to determine the "area" and quantify the amount of the observed molecule in the sampled voxel of tissue.
Because there is no absolute scale for the y-axis an internal denominator or ratio is necessary for objective quantification of the metabolite concentrations. Also calculation of the area under the spectra is not straightforward and depends on multiple instrumental and biophysical parameters (114).

In magnetic resonance imaging, the same frequency variation is first Fourier transformed and then used for spatial localization of the signal to a voxel to create a cross-sectional image. It is then possible to quantify the volume of a specific organ for example.
In the present work, all $^{1}$H-MRS data were performed by our collaborators in Bern, in the research group of Ch. Boesch, Department of Clinical Research, MR Spectroscopy and Methodology, University Bern, Switzerland. More methodological information can be obtained in the following papers (115-117).

# *CHAPTER 3*
# *STUDY I*

## High-protein intake reduces intrahepatocellular lipid deposition in humans

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**3.1 Study I, why?**

In the 1990's much research focused on the metabolic effects of fructose. Indeed the arrival of high fructose corn syrup (HFCS) on the market, drastically increased consumption of total sugar and fructose also, which was observed to coincide with an increase incidence of obesity and insulin resistance.

Our research group has been particularly interested in understanding the development of insulin resistance with fructose overfeeding, which was assessed by the gold standard method recognized for measuring insulin sensitivity the "hyperinsulinemic euglycemic clamp". These studies demonstrated that a reduction in insulin sensitivity was observed only when large amounts of fructose was supplemented to a standard maintenance diet (3g fructose/kg body weight) during 7-day (118). At the same time, and with the development of $^{1}$H-NMR technology, research began to pay attention to the correlation between ectopic fat depot (IHCL) in the liver, obesity, dietary pattern, inactivity and insulin resistance. In our group, Lê KA had particularly investigated this aspect in her work and observed that high fructose overfeeding increased *de novo* lipogenesis and IHCL in healthy subjects and moreover in subjects with a family history of Type II diabetes (116, 118). However recently, one of our last studies demonstrated that short-term overconsumption of glucose increased *de novo* lipogenesis to the same extent as that of fructose (119).

Beside high fructose/carbohydrate intakes, hypercaloric high-fat diets may also play a role in the development of NAFLD and insulin resistance. In rodents, high-fat feeding is now currently used as an intervention for producing models of hepatosteatosis in rodents. In humans, epidemiological data found correlations between high-fat diets, obesity insulin resistance and NAFLD, but interventional studies were lacking (120).

Consequently we assessed the effects of short term, 4-day, hypercaloric high-fat diet (+30% energy as saturated fat) on ectopic fat depots in the liver and development of insulin resistance in ten healthy young men. We were inspired by the work of Samuel *et al* (121) who demonstrated that short-term high-fat feeding (3 days) in rats was sufficient to specifically induce accumulation of triglycerides in the liver in conjunction with the development of hepatic insulin resistance. Samuel also demonstrated that increasing energy expenditure and fat oxidation through mitochondrial uncoupling by supplementation of 2,4-Dinitrophenol

(16mg/kg/day) counteracted the effect of high fat feeding by preventing ectopic fat depot and insulin resistance. Therefore we decided to try to reproduce the effects of 2,4-Dinitrophenol by supplementing the high-fat diet with 1.5g protein/kg body weight, since protein intake is known beside to increase postprandial thermogenesis.

The following study was a randomized controlled trial, in which insulin sensitivity and IHCL were assessed after three different controlled diets; a reference isocaloric controlled diet (C), a high-fat diet (HF) and a high-fat high-protein diet (HFHP).

Our results partly reproduced those of Samuel, since high-protein intake was able to prevent an increase in IHCL, but we failed to demonstrate a reduction in insulin sensitivity.

*Personal contribution*

I was involved in the design and development of the protocol. I carried out the recruitment of subjects, the food supplementation to all volunteers and the clinical trial with the help and supervision of D.Faeh and the research nurses from the Cardiomet Clinical Investigation Center (Cardiomet CIC). I conducted data analysis, and received supervision and help from L.Tappy for the writing and revision of the manuscript.

*Am J Clin Nutr 2009; 90:1002-10*

# High-protein intake reduces intrahepatocellular lipid deposition in humans

Murielle Bortolotti[1], Roland Kreis[2], Cyrille Debard[3], Bertrand Cariou[4], David Faeh[1], Maud Chetiveaux[4], Michael Ith[2], Peter Vermathen[2], Nathalie Stefanoni[1], Kim-Anne Lê[1], Philippe Schneiter[1], Michel Krempf[4], Hubert Vidal[3], Chris Boesch[2], Luc Tappy[1,5].

[1]Department of Physiology, University of Lausanne, Lausanne, Switzerland

[2]Department of Clinical Research (AMSM), University of Bern, Bern, Switzerland

[3]INSERM U870 and INRA U1235, F-69600 Oullins, France /Université Lyon 1, Faculté de Médecine, Hospices Civils de Lyon, F-69003 Lyon, France

[4]INSERM, U915, F-44000 Nantes, France/Université de Nantes, Faculté de Médecine, Institut du Thorax, F-44000 Nantes, France

[5]Department of Endocrinology, Diabetes, and Metabolism, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

*ABSTRACT*

**Background**

High-sugar and fat intakes are known to increase intrahepatocellular lipids (IHCL) and to cause insulin resistance. High protein intake may facilitate weight loss and improve glucose homeostasis in insulin-resistant patients, but its effects on IHCL remain unknown.

**Objective**

We aim to assess the effect of high-protein intake on high-fat diet-induced IHCL accumulation and insulin sensitivity in healthy young males

**Design**

Ten volunteers were studied in a cross-over design after 4 days on either hypercaloric high-fat diet (HF), hypercaloric high-fat high-protein (HFHP) diet, or control, isocaloric diet (C). IHCL were measured by $^{1}$H-MR spectroscopy, fasting metabolism by indirect calorimetry, insulin sensitivity by hyperinsulinemic-euglycemic clamp, plasma bile acids (BAs) concentrations by ELISA and GC-MS and expression of key lipogenic genes was assessed in subcutaneous adipose tissue biopsies.

**Results**

HF increased IHCL by 90 ± 26% and plasma tissue-plasminogen-activator-inhibitor-1 (tPAI-1) by 54 ± 11% (both, $p < 0.02$). It inhibited plasma free fatty acids by 26 ± 11% and β-hydroxybutyrate by 61 ± 27% (both, $p < 0.05$). HFHP blunted the increase in IHCL and normalized plasma β-hydroxybutyrate and tPAI-1 concentrations. Insulin sensitivity was not altered while the expression of SREBP-1c and key lipogenic genes were increased by HF/HFHP ($p < 0.02$). BAs concentrations remained unchanged after HF, but increased by 50 ± 24 % after HFHP ($p = 0.14$).

**Conclusions**

Proteins significantly blunted the effects of a high-fat diet on IHCL and tPAI-1 through effects presumably exerted at the level of the liver. Protein-induced increase in BAs concentrations may be involved.

***INTRODUCTION***

In animals (122) and healthy humans (116) high-fat or high-fructose diets lead to the development of several features of the metabolic syndrome, such as increased plasma triglycerides, hepatic and extrahepatic insulin resistance, and liver steatosis. In contrast, increasing protein intake has been suggested to exert beneficial metabolic effects by promoting weight loss and improving glucose homeostasis in insulin-resistant patients (66, 123). The effects of dietary protein on hepatic lipids remain however unknown. The aim of this study was therefore to investigate the potential beneficial effects of a high-protein diet on hepatic steatosis induced by a high-fat diet. For this purpose we monitored liver fat (intrahepatocellular lipid: IHCL) concentrations in healthy subjects consuming a control, balanced isocaloric diet (C), or hypercaloric diets with a high-fat (HF) or high-fat and high-protein (HFHP) content. Since adipose tissue metabolism may secondarily impact on hepatic lipid content, the effects of these diets on subcutaneous adipose tissue gene expression were also studied. In addition, bile acids (BAs) have been recently recognized as important regulators of hepatic lipid metabolism, and possibly of total energy expenditure (124-126). Therefore, circulating BAs concentrations were also monitored.

*SUBJECTS AND METHODS*

**Participants**

Two groups of healthy male volunteers were included in this study. Ten volunteers, aged 24 ± 1 y, and with a mean BMI of 22.4 ± 0.6 kg/m², took part in the main protocol, and were studied in a cross-over design after an isocaloric diet, or a hypercaloric high-fat diet, or a hypercaloric high-fat, high-protein diet. Another group of 6 volunteers, aged 25 ± 1 y were studied in a complementary cross-over study after a high-fat high-cholesterol (HF-high chol) and a high-fat low-cholesterol diet (HF-low chol). All subjects were sedentary, non-smokers and had no family history of diabetes. The experimental protocol was approved by the Ethical Committee of Lausanne University School of Medicine. All participants provided written informed consent at inclusion.

**Study design**

For the main study (effects of high fat and high fat high protein diets), each of the 10 subject was studied on three occasions in a cross-over design. They received in randomized order during four days either a standard isocaloric control diet (C) (calculated using the equation of Harris-Benedict (127) multiplied by a Physical Activity coefficient of 1.4), or a hypercaloric high-fat diet (HF) or a hypercaloric high-fat high-protein diet (HFHP). The diets were designed to provide the same total carbohydrate intake for all three diets, and the same fat intake for HF and HFHP (**Table 1**).

**TABLE 1**
Nutrient content of the experimental diets ($n$ = 10)[1]

| | C diet | HF diet | HFHP diet |
|---|---|---|---|
| Energy (kcal) | 2248 | 2923 | 3370 |
| Carbohydrate (kcal) | 1237 | 1237 | 1237 |
| Total fat (kcal) | 674 | 1349 | 1349 |
| Protein (kcal) | 337 | 337 | 784 |
| Saturated fat (%) | 33.5 | 51.6 | 52.6 |
| Monounsaturated fat (%) | 37.0 | 27.6 | 26.5 |
| Polyunsaturated fat (%) | 16.5 | 8.8 | 7.9 |
| Cholesterol (mg) | 226 | 376 | 653 |

[1] C, control; HF, high-fat; HFHP, high-fat, high-protein.

In HF and HFHP, the extra fat intake was provided mainly associated with dairy products like (butter, milk and cheese) and the extra protein as eggs, ham, salami and tuna. The study was performed on an out-patient basis, and subjects received all their food as pre-packed food items with instruction on how and when to prepare and consume them, and were instructed not to consume any other food or drinks. Experimental periods were separated by a washout period of at least two weeks.

At the end of the fourth day of each period, IHCL concentrations were measured by proton magnetic resonance spectroscopy ($^1$H-MRS). On the morning of the fifth day, an adipose tissue biopsy was obtained by needle aspiration from the subcutaneous periumbilical adipose tissue under local anaesthesia. Thereafter, an infusion of 6,6 $^2H_2$-glucose (Cambridge Isotope Laboratories, Cambridge, MA; bolus: 2mg/kg; continuous: 20 µg/kg/min) was started. Blood samples were collected at baseline and after 90, 105 and 120 min to measure concentrations of basal hormones, substrates and 6,6 $^2H_2$-glucose enrichment, and at time 60 to measure plasma lipids, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). After this 120-min baseline measurement period, a 2-step hyperinsulinemic-euglycemic clamp (0.3 mU/kg/min from 120 to 210 min, then 1.0 mU/kg/min from 210 to 300 min) with variable 6,6 $^2H_2$ glucose infusion was performed, and plasma hormone and substrate concentrations were measured at 30 min intervals. Substrate oxidation and energy expenditure were continuously measured throughout the test by open circuit indirect calorimetry (Deltatrac II, Datex Instrument, Helsinki, Finland) using the equations of Livesey and Elia (109). Net protein oxidation was calculated from urinary urea nitrogen excretion rate, assuming that urea excretion accounted for 90% total urinary nitrogen excretion (112). Urine collected overnight was used for basal protein oxidation, while urine collected at the end of the clamp was used for protein oxidation during hyperinsulinemia.

Since the HFHP diet was considerably enriched in cholesterol compared to the other diets due to consumption of eggs and meat-based products, a second complementary cross-over study was performed in 6 volunteers. In this study, subjects received during 4 days either a high fat-low cholesterol (HF-low chol) diet or a high fat-high cholesterol (HF-high chol) diet. The high fat supplementation (+ 30% total energy as fat) was similar to that used in the previous study and a high cholesterol intake was ensured by adding 30 g egg yolk/day (ca 300 mg cholesterol/day). At the end of this period, variations in body weight were assessed, IHCL were measured by proton magnetic resonance spectroscopy ($^1$H-MRS), and a fasting

blood sample was obtained for measurement of plasma glucose, FFA, triglycerides, cholesterol, HDL cholesterol bile acids, AST and ALT.

**Analytical procedures**

Plasma metabolites (glucose, free fatty acids, triglycerides, ß-hydroxybutyrate) and insulin were determined by classical enzymatic methods and radioimmunoassay respectively, as previously reported (116). Adiponectin, tPAI-1, TNF-α, IL-1, IL-6 were analysed by immunoassay using a multiplex assay (LINCO Research, St Charles, MO, USA). Total plasma bile acid concentrations were measured by Elisa using a kit from (Randox Laboratories, Crumlin, Co Antrim, UK). Individual bile acids (BAs) concentrations were measured by gas chromatography-mass spectroscopy (GC-MS) (128). Plasma isotopic enrichment of 6,6 $^{2}H_{2}$-glucose was calculated by GC-MS, as previously described (129). Total glucose turnover was calculated using Steele's equations for steady state conditions (99). Endogenous glucose production (EGP) was calculated as (total glucose turnover)-(exogenous glucose infusion rate).

**$^{1}$H Magnetic resonance spectroscopy**

For the main study (effects of HF and HFHP) $^{1}$H-MRS examinations were performed on a clinical 1.5 T MR scanner (Signa; General Electric Medical Systems, Waukesha WI, USA) using a flexible receive RF coil in combination with the body transmit coil. Data acquisition and processing was similar to that described in reference (116). In short: single-voxel $^{1}$H-MR spectra were acquired with an optimized point-resolved spectroscopy (PRESS) sequence (echo time 20 ms, 2-kHz bandwidth, 1024 points). On the basis of coronal (spoiled gradient recalled echo sequence, 60° flip angle, 1.5 ms echo time, 0.11 s repetition time, 8 mm slice thickness, 2.5 mm gap between slices, 48 cm field of view, 512 x 192 matrix size) and axial (fast spin echo sequence, 9 ms echo time, 4 s repetition time, 4 mm slice thickness, 1 mm gap between slices, 40 cm field of view, 512 x 128 matrix size) MR images obtained under breath hold in expiration, a volume of interest of 55 $cm^{3}$ was placed in a lateral area of the liver and repositioned at the same location in follow-up examinations. Positioning of the receive coil made use of external anatomical landmarks. Thirty-two acquisitions with water pre-saturation were recorded and stored individually for each spectrum. The repetition time (5 – 6 s) was adjusted such that the subjects could breathe normally and acquisitions could occur

during brief breathing arrests in expiration (130). Effects of residual motion were found to be evidenced by small shifts in resonance frequency. They were accounted for by aligning all individual scans that fell into a frequency window of 12 Hz and by discarding those acquisitions with a lipid peak that was shifted by more than 6 Hz. Spectra were processed, fitted, and quantitated similarly to earlier descriptions (116) using prior knowledge fitting (**Figure 1**). Quantitation to obtain IHCL in units of mmol/kg was based on the median water signal from 8 separate acquisitions obtained without water suppression, a $T_2$ of 50 ms for this water signal (as determined earlier), and a liver water content that was experimentally determined for each investigation, using proton density weighted MR images acquired with the body coil in breath hold and with a water reference standard placed on the subjects breast (spoiled gradient recalled echo sequence, single slice of 10 mm, 12°flip angle, 1.8 ms echo time, 200 ms repetition time, 21 s total scan time).

For the complementary study on the influence of cholesterol intake, all MRS measurements were performed on a 3T MR scanner (Trio, Siemens, Erlangen, Germany) with slightly adapted measurement conditions: localization with a stimulated echo sequence (echo time 20 ms, repetition time 5 s) without water presaturation. Three spectra were recorded in each session. Data processing and model fitting was performed with jMRUI (131) including realignment of spectra, elimination of the water peak (Henkel Lancosz singular value decomposition using 3 components) for the determination of the lipid peak areas and peak area fitting using prior knowledge parameter relations. T2 relaxation was corrected with values taken from the literature. IHCL values obtained at 3T with somewhat modified methodology are probably systematically somewhat different from those obtained in the main study at 1.5 T. However this influence is expected to be small and does not affect the conclusions of the study because both parts of the MR investigations were self-contained.

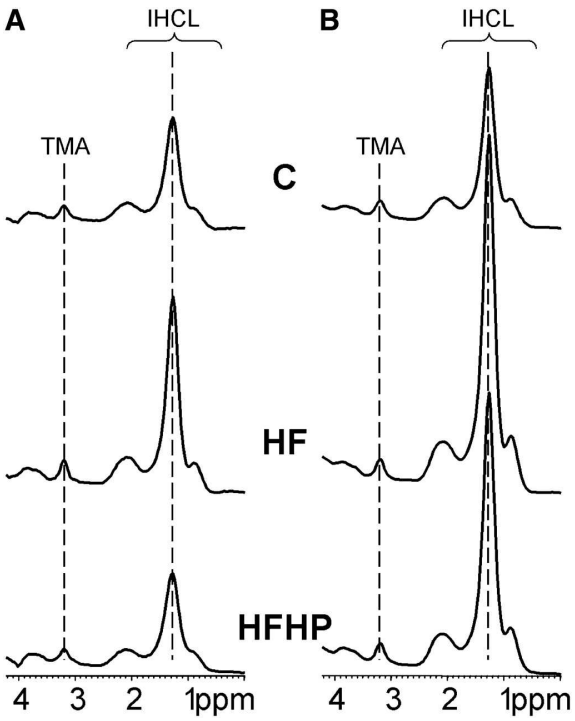


**FIGURE 1.** Localized proton magnetic resonance spectroscopy spectra of liver showing data quality (A) and overall results (B) for intrahepatocellular lipids (IHCL). The 3 rows show the spectra for the 3 dietary regimens (C, control; HF, high-fat; HFHP, high-fat, high-protein), Panel A shows data from a single subject, and panel B shows the averaged spectra from all subjects ($n$ = 10). Trimethylammonium (TMA) compounds were unaffected by diet. The dashed line indicates the peak of methyl protons used for quantitation.

**Gene expression in adipose tissue**

The real-time quantitative PCR assay for mRNA has been previously described and validated (132). Hypoxanthine phosphoribosyltransferase (HPRT) mRNA was measured by real-time quantitative PCR as a reference gene and the mRNA level of the genes of interest was expressed as a percentage ratio referred to the expression of HPRT. Gene expression of selected genes involved in lipid, carbohydrate and energy metabolism were measured. The complete list is shown in **Table 2**.

**Statistical analysis**

All data were expressed as mean ± standard errors of the mean (SEM). The non parametric Wilcoxon signed-paired rank test with Bonferroni's correction was used to test the null hypothesis between all three paired dietary conditions (C, HF, HFHP). A non parametric one-way analysis of variance was performed to assess differences between HF-high chol and HF- low chol). SEM of percentage changes from referred values was calculated as the mean of the relative SEM to mean value for both variables. The software used was STATA version 9.1 (Stata Corp, College Station, TX, USA).

*RESULTS*

**Effects of high fat and high fat high protein diets**

All three diets were well tolerated, with neither reported problems of compliance to the diets nor side effects. Compliance was verified by interview, and a 49 ± 8% increase in net protein oxidation corroborated that compliance to HFHP was good.

**Substrates, intrahepatocellular lipids and liver enzymes**

Compared to C, HF did not change substrate concentrations, except for a 26 ± 11 % reduction of FFA ($p < 0.05$), a 61 ± 27 % reduction in ßOHB ($p < 0.05$) and a 22 ± 14% reduction in plasma VLDL-TG ($p < 0.05$) (**Figure 2**). HFHP suppressed FFA to the same extent as HF ($p < 0.02$), but normalized plasma VLDL-TG and ßOHB concentrations. HF nearly doubled IHCL concentrations ($p < 0.02$). Compared to HF, HFHP led to a significant reduction of IHCL by 22 ± 32 % ($p < 0.02$) (**Figure 2**). AST and ALT were not altered by either diet except for a 28 ± 15 % increase of ALT with HFHP ($p < 0.02$) (**Table 3**).

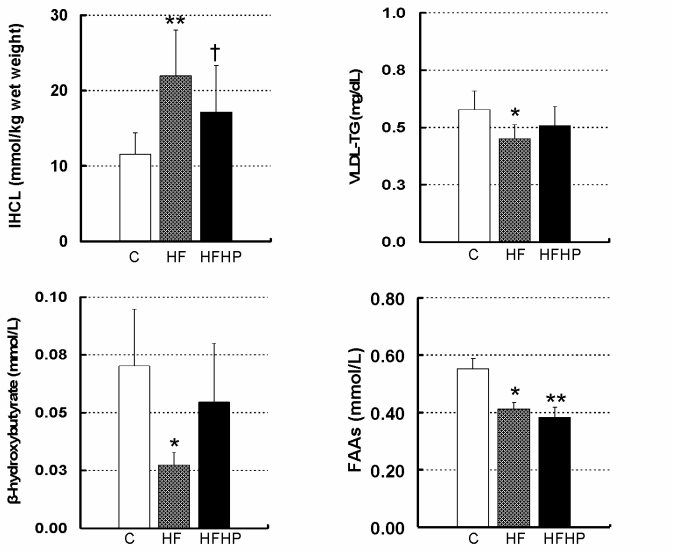


**FIGURE 2.** Mean (±SEM) effects of a balanced isocaloric diet (control, C), a hypercaloric high-fat (HF) diet, or a hypercaloric high-fat, high-protein (HFHP) diet on intrahepatocellular lipid (IHCL), VLDL-triglyceride (TG), plasma $\beta$-hydroxybutyrate, and nonesterified fatty acid (NEFA) concentrations in healthy men ($n$ = 10). Wilcoxon's signed-rank test with Bonferroni's correction was used for pairwise comparisons. *,**Significantly different from control: *$P < 0.05$, **$P < 0.02$. †Significantly different from HF, $P < 0.02$.

**Insulin sensitivity**

Fasting endogenous glucose production was not affected by the HF and HFHP diet. HFHP increased fasting glucagon concentrations by 14 ± 9 % compared to HF ($p < 0.05$), but did not alter insulin concentrations. Total glucose turnover, endogenous glucose production, and the percent inhibition of glucose production were similar under all conditions at both low and high insulin infusion rate, indicating that HF and HFHP did not significantly alter hepatic or extrahepatic insulin sensitivity (**Table 3**).

**TABLE 3**
Body weight, glucoregulatory hormones, and insulin sensitivity ($n = 10$)[1]

| | | | | *P* for pairwise comparison | | |
|---|---|---|---|---|---|---|
| | C diet | HF diet | HFHP diet | C vs HF | C vs HFHP | HF vs HFHP |
| Body weight (kg) | 73.8 ± 2.3 | 73.9 ± 2.6 | 74.9 ± 2.3 | >0.2 | 0.005 | 0.034 |
| Glucose (mg/dL)[2] | 93.8 ± 1.3 | 96.5 ± 2.2 | 93.5 ± 1.4 | 0.092 | >0.2 | >0.2 |
| Insulin ($\mu$U/mL)[3] | 8 ± 1 | 9 ± 1 | 10 ± 1 | >0.2 | >0.2 | 0.097 |
| Glucagon (pg/mL) | 56 ± 4 | 56 ± 5 | 64 ± 6 | >0.2 | 0.169 | 0.022 |
| AST (U/L) | 26 ± 3 | 24 ± 2 | 26 ± 3 | >0.2 | >0.2 | >0.2 |
| ALT (U/L) | 22 ± 3 | 23 ± 3 | 28 ± 6 | 0.172 | 0.018 | 0.120 |
| Insulin sensitivity | | | | | | |
| EGP ($mg \cdot kg^{-1} \cdot min^{-1}$) | | | | | | |
| Basal | 2.13 ± 0.12 | 2.17 ± 0.11 | 2.17 ± 0.11 | >0.2 | >0.2 | >0.2 |
| First step | 0.82 ± 0.16 | 0.84 ± 0.22 | 0.68 ± 0.15 | >0.2 | >0.2 | >0.2 |
| Second step | 0.04 ± 0.03 | 0.00 ± 0.00 | 0.13 ± 0.06 | >0.2 | >0.2 | >0.2 |
| TO ($mg \cdot kg^{-1} \cdot min^{-1}$) | | | | | | |
| First step | 2.87 ± 0.13 | 3.05 ± 0.18 | 3.19 ± 0.27 | >0.2 | >0.2 | >0.2 |
| Second step | 5.77 ± 0.54 | 5.84 ± 0.59 | 6.25 ± 0.45 | >0.2 | >0.2 | >0.2 |
| IGP (%) | 62 ± 8 | 62 ± 9 | 69 ± 6 | >0.2 | >0.2 | >0.2 |

[1] All values are means ± SEMs. C, control; HF, high-fat; HFHP, high-fat, high-protein; TO, total glucose turnover; EGP, endogenous glucose production; IGP, inhibition of glucose production, AST, aspartate aminotransferase; ALT, alanine aminotransferase. Wilcoxon's signed-rank test with Bonferroni's correction was used for pairwise comparisons.

[2] To convert mg/dL to mmol/L, multiply by 0.0555.

[3] To convert $\mu$U/mL to pmol/L, multiply by 6.945.

**Adipokines, energy metabolism and lipid profile**

HF and HFHP significantly increased plasma leptin concentrations (HF: 2.7 ± 0.4 ng/mL, HFHP: 3.1 ± 0.6 ng/mL, both $p < 0.02$ vs C: 2.3 ± 0.4 ng/mL). Adiponectin concentrations were not altered by either diet (HF: 15.8 ± 2.3 µg/mL, HFHP: 15.9 ± 2.4 µg/mL, C: 14.2 ± 1.7 µg/mL, ns) (not reported in tables or figures). Energy expenditure, as net carbohydrate, lipid and protein oxidation rates were not altered by HF compared to C. HFHP increased net protein oxidation by 49 ± 8% ($p < 0.02$), and decreased net lipid oxidation by 28 ± 13% ($p < 0.05$), but did not significantly alter net carbohydrate oxidation or energy expenditure (**Table 4**). Both HF and HFHP increased total, LDL and HDL cholesterol concentrations compared to control condition (all, $p < 0.02$). Total ($p < 0.05$) and VLDL-TG ($p < 0.02$) were slightly decreased by HF compared to C (**Table 4**).

**Inflammatory markers**

Plasma-tissue-plasminogen-activator-inhibitor-1 (tPAI-1) concentrations were increased by 54 ± 11 % with HF ($p < 0.02$), but were completely normalized with HFHP. In contrast, TNF-α, IL-1ß and IL-6 were not significantly altered by either diet (**Figure 3**).

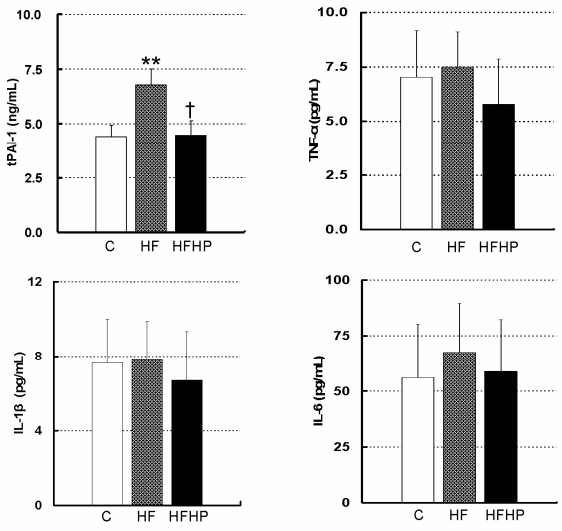


**FIGURE 3.** Mean (±SEM) effects of a balanced isocaloric diet (control, C), a hypercaloric high-fat (HF) diet, or a hypercaloric high-fat, high-protein (HFHP) diet on plasma concentrations of tissue-type plasminogen activator inhibitor-1 (tPAI-1), tumor necrosis factor-α (TNF-α), interleukin-1$\beta$ (IL-1$\beta$), and IL-6 in healthy men ($n$ = 10). Wilcoxon's signed-rank test with Bonferroni's correction was used for pairwise comparisons. **Significantly different from control, $P < 0.05$. †Significantly different from HF, $P < 0.02$.

**TABLE 4**
Energy metabolism and plasma lipid profile ($n$ = 10)[1]

| | | | | $P$ for pairwise comparison | | |
|---|---|---|---|---|---|---|
| | C diet | HF diet | HFHP diet | C vs HF | C vs HFHP | HF vs HFHP |
| Energy metabolism | | | | | | |
| Energy expenditure (kcal · kg$^{-1}$ · h$^{-1}$) | 1.05 ± 0.02 | 1.06 ± 0.03 | 1.06 ± 0.03 | >0.2 | >0.2 | >0.2 |
| Carbohydrate oxidation (mg · kg$^{-1}$ · min$^{-1}$) | 1.36 ± 0.16 | 1.49 ± 0.10 | 1.41 ± 0.11 | >0.2 | >0.2 | >0.2 |
| Lipid oxidation (mg · kg$^{-1}$ · min$^{-1}$) | 0.66 ± 0.09 | 0.64 ± 0.04 | 0.48 ± 0.06 | >0.2 | 0.028 | 0.028 |
| Protein oxidation (mg · kg$^{-1}$ · min$^{-1}$) | 0.65 ± 0.05 | 0.54 ± 0.03 | 0.97 ± 0.07 | 0.079 | 0.015 | 0.008 |
| Plasma lipid profile (mg/dL) | | | | | | |
| Total triglycerides[2] | 73 ± 8 | 61 ± 6 | 68 ± 9 | 0.032 | >0.2 | 0.053 |
| VLDL triglycerides[2] | 51 ± 7 | 40 ± 5 | 45 ± 7 | 0.017 | >0.2 | 0.169 |
| Total cholesterol[3] | 139 ± 9 | 154 ± 10 | 160 ± 9 | 0.059 | 0.007 | 0.139 |
| LDL cholesterol[3] | 80 ± 7 | 92 ± 7 | 96 ± 6 | 0.013 | 0.007 | >0.2 |
| HDL cholesterol[3] | 36 ± 2 | 42 ± 3 | 41 ± 2 | 0.006 | 0.005 | >0.2 |

[1] All values are means ± SEMs. C, control; HF, high-fat; HFHP, high-fat, high-protein. Wilcoxon's signed-rank test with Bonferroni's correction was used for pairwise comparisons.
[2] To convert mg/dL to mmol/L, multiply by 0.01129.
[3] To convert mg/dL to mmol/L, multiply by 0.02586.

**Gene expression in adipose tissue**

HF increased significantly the expression of key genes related to the lipogenic pathway such as those coding for the master regulator SREBP-1c (+70 ± 12 %), hexokinase II (+32 ± 10%) ($p < 0.02$), and fatty acid synthase FASN (+ 133 ± 20%) ($p < 0.05$). In contrast the expression of most genes related to lipolysis and lipid oxidation (HSL, ATGL, PDE-3b, CD36 and CPT-1) was not affected, while expression of PLIN, a protein involved in lipid droplet coating, was decreased ($p < 0.02$). There was also no effect of HF on genes related to cholesterol utilisation and metabolism (ABCA-1, LDLR) except for LXRα (+17 ± 7%). The expression of adiponectin and PPARG was also not modified after HF diet. HFHP tended to further increase the expression of lipogenic genes, particularly SREBP-1c, increased importantly the expression of CD36, but reduced that of CPT1, a gene related to fatty acid oxidation (both, $p < 0.02$). It increased PPARG mRNA concentrations (+45 ± 15%) ($p < 0.02$). Finally, HFHP significantly increased the expression of LXRα ($p < 0.02$) and LDLR ($p < 0.05$) (**Table 2**).

**TABLE 2**
Gene expression in adipose tissue from healthy subjects after 4 d of each dietary condition ($n = 8$)[1]

| | | | | *P* for pairwise comparison | | |
|---|---|---|---|---|---|---|
| Lipid metabolism | C diet ($n = 7$) | HF diet ($n = 8$) | HFHP diet ($n = 8$) | C vs HF | C vs HFHP | HF vs HFHP |
| Lipolysis | | | | | | |
| Hormone-sensitive lipase | 1856 ± 185 | 1616 ± 168 | 1487 ± 211 | >0.2 | 0.028 | >0.2 |
| Adipose triglyceride lipase | 2296 ± 241 | 2267 ± 226 | 2358 ± 219 | >0.2 | >0.2 | >0.2 |
| Perilipin | 8699 ± 986 | 6539 ± 716 | 8212 ± 1713 | 0.018 | >0.2 | >0.2 |
| Phosphodiesterase 3b | 44,563 ± 6017 | 47,592 ± 5674 | 44,679 ± 5393 | >0.2 | >0.2 | >0.2 |
| Lipogenesis | | | | | | |
| Sterol regulatory element-binding protein-1c | 636 ± 54 | 1085 ± 159 | 1349 ± 208 | 0.018 | 0.018 | 0.036 |
| Fatty acid synthase | 144 ± 29 | 336 ± 69 | 276 ± 41 | 0.043 | 0.063 | 0.161 |
| Hexokinase II | 377 ± 37 | 496 ± 54 | 520 ± 69 | 0.018 | 0.018 | >0.2 |
| Lipid oxidation | | | | | | |
| Fatty acid translocase | 6743 ± 891 | 7511 ± 619 | 8407 ± 854 | >0.2 | 0.018 | >0.2 |
| Carnitine palmitoyltransferase | 67 ± 8 | 58 ± 9 | 52 ± 8 | 0.063 | 0.018 | >0.2 |
| Cholesterol-related | | | | | | |
| Liver X receptor-α | 234 ± 18 | 275 ± 18 | 331 ± 30 | 0.028 | 0.018 | >0.2 |
| ATP-binding cassette A | 80 ± 13 | 83 ± 15 | 81 ± 12 | >0.2 | >0.2 | >0.2 |
| LDL receptor | 37 ± 5 | 34 ± 5 | 48 ± 7 | >0.2 | 0.028 | 0.012 |

[1] All values are means ± SEMs. Data are expressed as a percentage ratio referring to the expression of hypoxanthine phosphoribosyltransferase. C, control; HF, high-fat; HFHP, high-fat, high-protein. Wilcoxon's signed-rank test with Bonferroni's correction was used for pairwise comparisons.

**Plasma bile acid concentrations**

Total bile acid concentrations were 64 ± 22 % and 50 ± 24 higher after HFHP than after HF and C respectively. Although not significant, this difference prompted us to proceed with measurement of individual bile acids by GC-MS (**Figure 4**). Compared to C and HF diets, HFHP diet specifically increased plasma cholic acid (CA) concentration by 269 ± 27 % ($p < 0.02$) and 248 ± 27 % ($p < 0.05$), compared to C and HF respectively. A similar, albeit less pronounced profile was observed for chenodeoxycholic acid (CDCA) (+ 125 ± 19 % HFHP vs C, $p < 0.02$; + 108 ± 23 % HFHP vs HF, $p < 0.05$) and deoxycholic acid (DCA) (+ 63 ± 19 % HFHP vs C, $p < 0.05$; + 46 ± 21 % HFHP vs HF, $p < 0.05$). The degree of conjugation was also assessed under all 3 dietary conditions. The conjugated CA concentrations were decreased by 40 ± 21 % upon HFHP compared to HF, $p < 0.05$ (HFHP: 28.6 ± 5.9 %, HF: 47.8 ± 9.2 %). Circulating BAs concentrations were not correlated with IHCL concentrations under any of the dietary conditions tested.

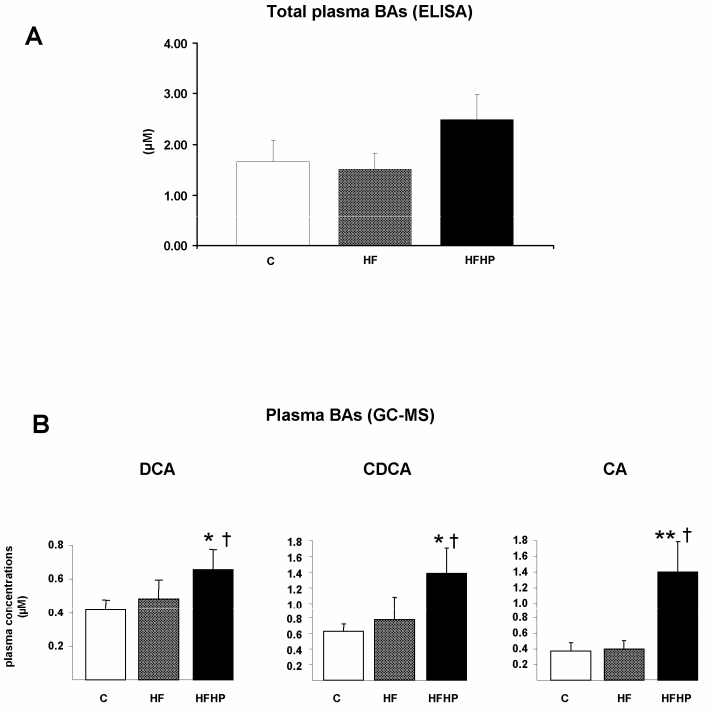


**FIGURE 4.** A: Mean (±SEM) effects of a balanced isocaloric diet (control, C), a hypercaloric high-fat (HF) diet, or a hypercaloric high-fat, high-protein (HFHP) diet on total bile acids (BAs). B: Mean (±SEM) concentrations of deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), and cholic acid (CA) in healthy men ($n$ = 10). ELISA, enzyme-linked immunosorbent assay; GC-MS, gas chromatography–mass spectrometry. Wilcoxon's signed-rank test with Bonferroni's correction was used for pairwise comparisons. *,**Significantly different from control: *$P < 0.05$, **$P < 0.02$. †Significantly different from HF, $P < 0.02$.

**Effects of high fat high cholesterol vs high fat low cholesterol diet**

The concentrations of substrates, AST, ALT and IHCL after these two diets were similar to those observed in the first study after the HF diet, except for glucose, and bile acids concentration ($p<0.02$) (**Table 5**). Body weight did not change between pre and post high fat supplementation (HF-low chol delta : -0.02 ± 0.01 kg; HF-high chol delta: +0.4 ± 0.1 kg). Also there was no difference between high- and low cholesterol diets in any of the parameters tested (**Table 5**).

**TABLE 5**
Effects of a high-fat (HF) diet with a high cholesterol (HF-high chol) or a low cholesterol (HF-low chol) content ($n = 6$)[1]

| | HF-low chol | HF-high chol | Pairwise comparison |
|---|---|---|---|
| Body weight (kg) | 73.4 ± 2.2 | 73.2 ± 2.1 | >0.2 |
| Glucose (mg/dL)[2] | 85.5 ± 1.5 | 85.8 ± 1.3 | >0.2 |
| NEFA (mmol/L) | 0.280 ± 0.025 | 0.378 ± 0.053 | 0.120 |
| Bile acids (μmol/L) | 2.80 ± 0.24 | 2.62 ± 0.39 | >0.2 |
| Hepatic metabolism | | | |
| IHCL (μmol/kg wet wt) | 27 ± 11 | 25 ± 10 | >0.2 |
| AST (U/L) | 22 ± 2 | 25 ± 3 | >0.2 |
| ALT (U/L) | 24 ± 2 | 25 ± 3 | >0.2 |
| Plasma lipid profile (mg/dL) | | | |
| Total triglyceride[3] | 97 ± 30 | 89 ± 16 | >0.2 |
| Total cholesterol[4] | 175 ± 12 | 182 ± 8 | >0.2 |
| HDL cholesterol[4] | 54 ± 6 | 55 ± 9 | >0.2 |

[1] All values are means ± SEMs. IHCL, intrahepatocellular lipid; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NEFA, nonesterified fatty acid. A nonparametric one-factor ANOVA was used for paired comparisons.

[2] To convert mg/dL to mmol/L, multiply by 0.0555.

[3] To convert mg/dL to mmol/L, multiply by 0.01129.

[4] To convert mg/dL to mmol/L, multiply by 0.02586.

**DISCUSSION**

A high fat diet is known to induce intrahepatic fat deposition in rodents (121). Consistent with these findings, we also observed that high fat feeding produced a significant 90% increase in IHCL after only 4 days in healthy volunteers. There was however no change in glucose production or its suppression by hyperinsulinemia. This may appear surprising in regard of the association between IHCL concentrations and hepatic insulin sensitivity observed in many studies (121, 133, 134) and suggest that IHCL are not directly related to hepatic insulin resistance (135). Surprisingly, we observed a major 26% reduction in fasting plasma FFA. The analysis of adipose tissue documented an increased expression of FASN, while the expression of genes involved in lipolysis (HSL, ATGL, PDE3, perilipin), and lipid oxidation (CPT1) were not significantly affected during HF. Similar observations were also reported by Meugnier et al.(135), who found a decrease in FFA concomitant to a marked induction of genes related to the lipogenic pathway in skeletal muscle in response to 4 weeks of high-fat diet in healthy subjects. Altogether, these results indicate that, in humans, short term high-fat feeding shifts the balance between lipogenesis and lipolysis toward lipogenesis in adipose tissue, thus stimulating the deposition of excess dietary lipids. Under such conditions, plasma FFA concentrations remain low, and a high flux of plasma FFA can therefore not be responsible for intrahepatic fat accumulation. We observed instead a major 61 ± 27 % decrease in fasting plasma ßOHB concentrations, which strongly suggests that suppression of beta-oxidation and ketogenesis contributed to the accumulation of triglyceride in liver cells. Altogether, these results suggest that hypercaloric, high-fat intakes stimulate net fat storage in adipose tissue and in the liver, resulting in IHCL deposition and in adipose tissue triglyceride storage.

Insulin sensitivity was not altered during hyperinsulinemia on a high-fat diet, which contrasts with the hepatic insulin resistance observed in rodents very early after being put on a high-fat diet (121), but is in agreement with the data observed after 4 weeks of high-fat diet in a group of young healthy individuals (135) . Since the study was performed on an outpatient basis, it is possible that poor diet compliance would be responsible. This appears however, unlikely since urinary urea excretion increased as expected during protein overfeeding. Changes in body fat were not monitored, but the fat overload provided in this study was not expected to significantly change body fat content over such a short period (136).

Hepatic insulin resistance may nonetheless develop with longer exposure to a hypercaloric high-fat diet leading to a substantial increase in body fat.

Increasing the dietary protein intake produced a 22 % decrease in IHCL. High-protein intake also increased plasma βOHB to normal concentrations, but did not reverse the suppression of plasma FFA. In adipose tissue, the effects of HFHP were quite similar to those observed after HF alone, with an induction of lipogenic genes, consistent with a switch of adipose tissue metabolism toward net fat storage. This pattern even tended to be enhanced with HFHP. Furthermore, HFHP increased the expression of PPARG. Given the role of PPARG in adipose tissue differentiation, and the metabolic effects of PPARG activation by TZDs (137), it is possible to speculate that, in the long term, enhanced expression of PPARG by dietary protein may prevent HF-induced insulin resistance by promoting fat deposition in adipose rather than ectopic fat depots.

HF and HFHP had concordant effects in adipose tissue, i.e. a switch toward lipogenesis and energy storage, but a divergent effect in the liver, as HF increased while HFHP reduced IHCL. This suggests that high-protein intake primarily affected liver metabolism through stimulation of hepatic beta-oxidation and ketogenesis. The mechanisms by which protein exerted these effects remain speculative, but several tracks can be proposed for future investigation. Firstly, high-protein intake increases total amino-acid degradation at the whole body level. Since the bulk of amino-acid catabolism takes place in the liver and is an energy-requiring process, high protein intake may have merely increased hepatic lipid oxidation through an increase in hepatic energy expenditure (138). Secondly, protein increased plasma BAs, and BAs may inhibit lipogenesis and favour hepatic lipid oxidation through stimulation of LXR and FXR (139, 140). Since HFHP was also enriched in cholesterol, we initially considered the possibility that this increase was merely secondary to stimulation of BAs synthesis by enhanced dietary cholesterol intake. We therefore performed an additional study comparing the effects of diets providing the same overload of saturated fat, one with high and the other with low cholesterol. The results indicated that the dietary cholesterol content did not significantly affect IHCL and BA concentrations, and hence the increase in BAs was most likely to be attributed to dietary proteins. Thirdly, a high protein intake causes an increased day-long secretion of glucagon which may stimulate hepatic ketogenesis (79, 141). Finally, numerous metabolic genes are regulated by amino-acids or their metabolites (142) and it is possible that the expression of genes involved in lipid synthesis and oxidation, or in lipoprotein metabolism were altered in liver cells after high protein intake (143).

A high-fat diet is known, in rodents, to increase pro-inflammatory cytokine release through activation of the transcription factors Ikkβ et NFκB (144). Furthermore, these pro-inflammatory cytokines are thought to play a role in the development of fat-induced insulin resistance (145,146) . It was therefore a secondary aim of this study to evaluate the effects of high fat and high fat, high protein intake on several markers of inflammation. High fat intake significantly increased tPAI-1 but failed to alter several plasma pro-inflammatory cytokines, while HFHP seemed to restore initial levels. tPAI-1, a fibrinolytic inhibitor, is produced by several cell types in the organism, amongst which hepatic stellate cells and adipocytes. A rise in tPAI-1 in adipose tissue has been consistently associated with insulin resistance and obesity (147) in several large epidemiological studies, and hence may be an early marker of insulin resistance (148). In addition, tPAI-1 is thought to be involved in the development of hepatic fibrosis (149). In patients with NAFLD, increased plasma tPAI-1 was strongly correlated with liver fat, while tPAI-1 expression was observed in liver cells. Furthermore, liver tPAI-1 expression was linked to an increase in TNF-α and TNF-α receptor II, suggesting a link between intrahepatic fat accumulation and inflammation (150). Our observation therefore suggests that dietary protein modulates fat-induced inflammation.

Our study has several limitations which have to be considered. First, the effects of a high-protein intake were documented in a group of healthy subjects in whom an increase in IHCL was produced by a short term hypercaloric high fat feeding. Although this procedure almost doubled baseline IHCL, the increase in liver fat was small compared to the fatty liver infiltration observed in obese patients with NAFLD. Furthermore, the mechanisms responsible for deposition of fat in the liver may not be identical in NAFLD patients and our experimental model (151). Second, an increase in protein intake may possibly improve liver metabolism while exerting unwanted effects on other systems at the same time. In this regard, deleterious effects of protein on glomerular filtration rate and kidney function are of special concern (152, 153).

In summary, our findings indicate that a high protein intake significantly prevents intrahepatic fat deposition induced by a short term hypercaloric, high fat diet in humans. It remains to be evaluated whether modulating the dietary protein intake, may be included in therapeutic or preventive strategies for NAFLD without adverse events.

***ACKNOWLEDGMENTS***

The authors' responsibilities were as follow- MB and LT: supervision of all the data in the study and responsibility for the integrity of the data; MB, DF, KAL, PS and LT design and development of the protocol; MB, DF, NS, PS : recruiting subjects, carrying out the clinical trial, and conducting data analysis; RK, MI; PV, and CB: measurements of IHCL by $^{1}$H-MR spectroscopy, development of the technique and data analysis; CD and HV: analysis of adipose tissue biopsy samples, data analysis; BC, MC and MK: analysis of plasma bile acids concentrations and data analysis; MB, DF, KAL, PS and LT analysis and interpretation of data; MB,PS and LT: writing of the manuscript, which was reviewed and modified by all authors. This study was supported by the Swiss National Foundation for Science (grant 310000-109737 to LT and CB). None of the authors reported any conflicts of interest.

# *CHAPTER 4*
# *STUDY II*

## Effects of whey protein supplementation on intrahepatocellular lipids in obese female patients

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### 4.1 Study II, why?

Results obtained from Study I determinated the design of the next two protocols which are briefly summarized in the following figure (**Figure 6**)

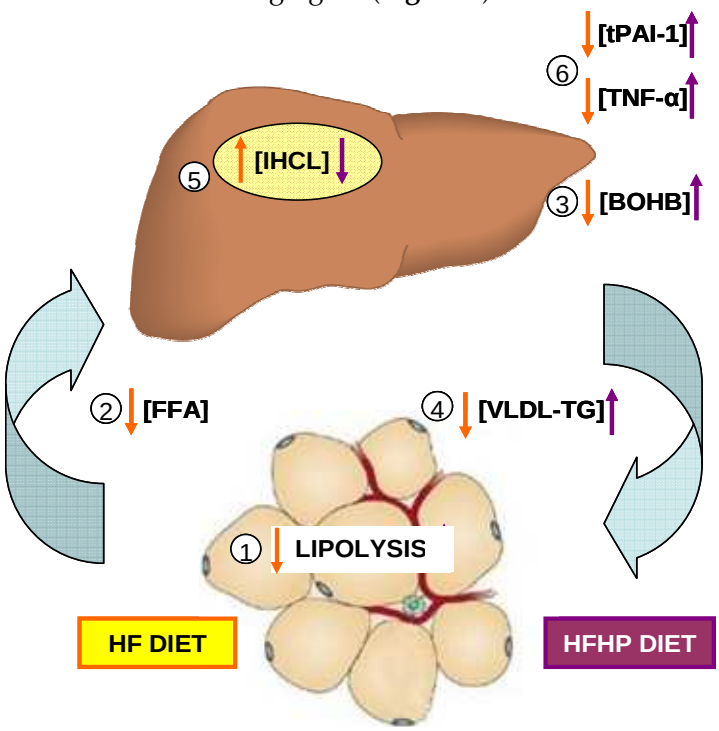


**Fig 6: Summary of the main results of Study I**.
*Abbreviations* HF, high-fat diet; HFHP, high-fat high-protein diet; FFA, free fatty acid; BOBH, beta-hydroxybutyrate, VLDL-TG, very low density lipoprotein-triglycerides; IHCL, intrahepatocellular lipids, TNF-α, tumor necrosis factor alpha; tPAI-1, tissue plasminogen activator inhibitor-1.

*Proposition of mechanism*

**High-fat diet** (1) inhibits lipolysis in adipose tissue, (2) which leads to a decrease in FFA plasma concentration. Since (3) BOHB and (4) VLDL-TG plasma concentrations are decreased, hepatic fat oxidation and TG export might be reduced by HF intake, (5) which altogether results in an increase in IHCL. (6) Elevation of tPAI-1 and TNF-α plasma concentration, which reflects some grade of inflammation are associated with the increase in IHCL.
**High-fat high-protein diet** (1) (2) does not change FFA plasma concentration when compared to HF. Since (3) BOHB and (4) VLDL-TG plasma concentrations are restored to normal values, HP intake might counteract effects of HF diet probably by increasing hepatic fat oxidation and TG export, (5) which altogether results in a decrease in IHCL, (6) tPAI-1 and TNF-α plasma concentrations.

Given the role of lipotoxicity and NAFLD in the development of features of the metabolic syndrome and insulin resistance, these encouraging results incited us to further investigate the metabolic effects of high protein intake on hepatic lipid metabolism.
We were therefore interested in focusing our research on the clinical implications of protein supplementation. Indeed NAFLD is frequently increased in obese patients and is considered to be the hepatic component of the metabolic syndrome, tightly associated with impaired glucose tolerance and dyslipidemia. As reported previously, protein intake has been shown to decrease steatosis and improve glucose homeostasis in animal models of steatohepatosis as well as in humans. Therefore we evaluated the effects of a 4-week of supplementation with 60g/day whey protein on steatosis (by $^{1}$H-NMR) and glucose homeostasis (by OGTT) in eleven non diabetic obese women, otherwise left *ad-libitum*. Since protein intake might affect renal function, we also controlled renal function every week by calculating creatinine clearance from 24-hour urine collections.

These preliminary results confirmed our expectations as intrahepatic and fasting plasma triglycerides were reduced in obese subjects after the 4-week whey protein supplementation. Since the obese women were allowed to consume a spontaneous diet, a satiating effect of the protein supplementation, leading to a decrease in their daily food intake cannot be excluded. Theses preliminary results suggest that a high protein diet may in the long term reduce the risk of steatohepatitis and cardiovascular disease in obese patients. The present study did not observe any adverse renal effects.

*Personal contribution*

I was involved in the design and development of the protocol. I supervised three masters' students, M.Corazza, E.Maiolo and E.VanDijke who carried out the experimental part of the study; i.e. the recruitment of subjects, supplementation of whey protein and the clinical trial. I conducted data analysis. I received major supervision and help from L.Tappy and R.Kreis, who carried out the whole $^{1}$H-MRS methodology part, during the preparation and revision of the manuscript.

*Submitted to Diabetes Care*

# EFFECTS OF WHEY PROTEIN SUPPLEMENTATION ON INTRAHEPATOCELLULAR LIPIDS IN OBESE FEMALE PATIENTS

Murielle Bortolotti, M.Sc [1]; Elena Maiolo, M.Sc [1]; Mattia Corazza, M.Sc [1]; Eveline Van Dijke M.Sc[1]; Philippe Schneiter, PhD [1]; Andreas Boss, M.Sc [2]; Guillaume Carrel, MD [1]; Vittorio Giusti, MD [3]; Kim-Anne Lê, PhD [1]; Daniel Guae Quo Chong, M.Sc. [2], Tania Buehler, M.Sc. [2], Roland Kreis, PhD [2]; Chris Boesch, MD& PhD [2]; Luc Tappy, MD [1, 3]

[1] Department of Physiology, University of Lausanne, 7, rue du Bugnon, 1005 Lausanne, Switzerland

[2] Department of Clinical Research, University of Bern, Pavillon 52A, Inselspital, P.O. Box 35, 3010 Bern, Switzerland

[3] Service of Endocrinology, Diabetes and Metabolism, CHUV, Lausanne

*ABSTRACT*

**Objective**

High protein diets have been shown to improve hepatic steatosis in rodent models and in high fat fed humans. We therefore evaluated the effects of a protein supplementation on intrahepatocellular lipids (IHCL), and fasting plasma triglycerides in obese nondiabetic women.

**Research Design and Methods**

11 obese women received a whey protein supplement (WPS) for 4 weeks, while otherwise nourished on a spontaneous diet, IHCL concentrations, visceral body fat, total liver volume ($^{1}$H-NMR), fasting total-triglyceride and cholesterol concentrations, glucose tolerance (standard 75 g OGTT), insulin sensitivity (HOMA IS index), creatinine clearance, blood pressure and body composition (bio-impedance analysis) were assessed before and after 4 week WPS.

**Results**

IHCL were positively correlated with visceral fat and total liver volume at inclusion. WPS significantly decreased IHCL by 20.8 ± 7.7 %, fasting total TG by 15 ± 6.9 %, and total cholesterol by 7.3 ± 2.7 %. WPS increased fat free mass slightly from 54.8 ± 2.2 kg to 56.7 ± 2.5 kg, $p = 0.005$). Visceral fat, total liver volume, glucose tolerance, creatinine clearance and insulin sensitivity were not changed.

**Conclusions**

WPS improves hepatic steatosis and plasma lipid profiles in obese non diabetic patients, without adverse effects on glucose tolerance or creatinine clearance.

***INTRODUCTION***

Non-alcoholic fatty liver disease (NAFLD) is characterized by an elevated intrahepatocellular lipid (IHCL) concentration. Incidence of NAFLD is frequently increased in obese patients, and is considered to be the hepatic component of the metabolic syndrome. It is tightly associated with the metabolic complications of obesity, i.e. insulin resistance, impaired glucose tolerance, and dyslipidemia (9, 11).

Several reports suggest that a high-protein intake may improve NAFLD. In high-fat fed rats, increasing the proportion of protein in the diet reduced hepatic steatosis and dyslipidemia (154, 155) . In healthy human male subjects in whom IHCL concentrations had been nearly doubled by 4-days of hypercaloric high-fat feeding, increasing the dietary protein intake significantly reduced IHCL concentrations (115). These observations suggest that a high protein intake may exert beneficial effects in NAFLD patients. We therefore evaluated the effects of 4-weeks of supplementation with 60g/day whey protein (Whey Protein Supplementation : WPS) in obese non diabetic female patients.

*SUBJECTS AND METHODS*

**Participants**

11 obese female patients, aged 38 ± 2 years, were recruited at the obesity clinic of the Centre Hospitalier Universitaire Vaudois (CHUV), Lausanne, Switzerland. They had a mean body weight of 99.7 ± 5.3 kg, mean height of 1.63 ± 0.02 m, and mean BMI of 37.6 ± 1.8 kg/m$^2$. None had liver or renal disease, nor were they on antidiabetic or antilipemic agents. They were sedentary (less than two sessions of physical activity per week). All reported a daily alcohol intake less than 20 g. The experimental protocol was approved by the Ethical Committee of Lausanne University School of Medicine. Subjects gave their written informed consent before participating in the study.

**Study design**

After inclusion, subjects reported in the morning after an overnight fast to the Cardiomet Clinical Investigation Center (Cardiomet CIC) of the Lausanne University Hospital. Their body weight and blood pressure were measured and their body composition was assessed by bioelectrical impedance analysis. Thereafter, they underwent a standard 75 g oral glucose tolerance test (OGTT) with measurement of plasma glucose and insulin at time 0 and 120 min. Fasting plasma triglycerides, total cholesterol, HDL cholesterol, urea, creatinine, ASAT, ALAT and glucagon were measured. A 24 hour urine collection was obtained for determination of urea and creatinine excretion. Total nitrogen excretion was calculated assuming that urea accounted for 85% of total urinary nitrogen (113)and that extra-renal nitrogen losses were 2g/day. Total energy expenditure and net substrate oxidation rates were measured during 45 min before and over the 120 min after oral glucose ingestion by indirect calorimetry (Deltatrac II, Datex Instruments, Helsinki, Finland)

On the following day, intrahepatocellular lipids (IHCL), visceral fat volume, and total liver volume were measured by clinical Magnetic Resonance (MR) methods at the Department of Clinical Research of University Bern at the Inselspital.

IHCL content was determined on a clinical 3 T MR system (TIM Trio, Siemens Medical, Germany) using a whole body coil for excitation. A volume of interest (2.5 x 2.5 x 3 cm$^3$) was localized in the liver using the body array surface coils for signal detection and a double echo localization sequence combined with Siemens' 2D "prospective acquisition correction"

(PACE) scheme (156), based on a 2D gradient echo image to monitor the position of the diaphragm for triggering in expiration (MRS echo time TE 30 ms, TR according to the breathing cycle between 2.5 and 6s, 4000 Hz spectral width, 2048 data points). MRS was preceded by fast spin echo MRI (HASTE [Half Fourier Acquisition Single Shot Turbo Spin Echo], echo time 89 ms, repetition time 1030 ms, flip angle 150°, nominal resolution 1.7x1.3x5 $mm^3$) in three planes using the same PACE triggering to visualize the liver and to reliably reproduce the placement of the region of interest (ROI) in follow-up examinations. The ROI was placed evading large vessels and proximity to extrahepatic fat. The magnetic field distribution over the ROI was optimized during breath-hold using the manufacturers automated gradient shim routine. For choice of proper flip angle, a $B_1$ mapping scan was recorded in expiration prior to MRS. MR spectra were recorded with water presaturation to determine the lipid and metabolite spectra (32 acquisitions, 60 Hz suppression bandwidth, center frequency at 3 ppm) and without water suppression to acquire the water signal as internal standard (16 scans, center frequency at 4.7 ppm). Automatic fitting of the MR spectra was performed with the home-written software FiTAID allowing for the use of Voigt lines and implementation of prior knowledge restraints (157). The lipid spectrum was modeled using 9 Voigt lines to describe all spectral components and initial model optimizations based on an average spectrum from several subjects, a further 5 lines were used to cover the metabolites and residual water. Absolute quantification was performed in analogy to Bortolotti *et al* (115) and was based on the peak areas of the methylene protons that are not neighbors of an allylic or carboxylic carbon, basic assumptions on lipid composition, the water peak area from non-water-suppressed scans, an assumed liver water content and relaxation corrections based on literature values (131). Results were expressed as volume percentage of lipid.

Volumes of the liver and visceral adipose tissue (VAT) were determined using $T_1$-weighted images of the abdomen, recorded in breath-hold (multi-spin-echo technique, echo train length 7, echo spacing 7.6 ms, repetition time 452 ms, echo time 38 ms,, flip angle 130°, 30 axial slices in 6 slabs covering the pelvis at the lower end and the diaphragm at the upper end, slice thickness of 10 mm, gap between slices 10 mm, 5 slices per breath-hold sequence, acquisition matrix 256 x 147 with a resolution of 2 mm/pixel, body coil was used for excitation and signal acquisition). Volumetry was performed using a semi-automatic implementation of the point counting method, which represents a sparse sampling scheme whereby an operator accepts or rejects points from a regular grid that covers the targeted

anatomic structure in a random orientation (158). Visceral fat was counted on images between pelvis and the upper end of the diaphragm. MR data was acquired and evaluated by operators who were unaware of the preceding dietary regime.

After these initial measurements, the whey protein supplement (WPS) was provided as bags containing 20g commercialized whey protein (WheyProtein94®, Sponser, Wollerau, Switzerland), with instructions to consume the content of one bag diluted in 300 ml water 30 min before breakfast, lunch and dinner. Their food and drink intake was otherwise left *ad libitum*. The study was performed as an open label, unblinded, uncontrolled study.

1, 2 and 3 weeks after the beginning of WPS, volunteers returned to the Cardiomet CIC and a fasting blood sample was obtained for the measurement of plasma triglycerides, total cholesterol, HDL cholesterol, urea, creatinine, ASAT, ALAT and glycemia. 24-hour urine collections were also obtained to measure urea and creatinine excretion. Compliance to WPS was assessed by collecting the empty supplementation bags.

After 4 weeks WPS, all measurements performed at inclusion, OGTT and NMR determination of IHCL, visceral fat volume, and liver volume were repeated.

**Analytic procedures**

After collection, blood and urine samples were sent to the Central Laboratory, CHUV for measurements of fasting plasma total- triglycerides, total cholesterol, HDL cholesterol, urea, creatinine, ASAT, ALAT and 24 hours urea and creatinine excretion. For the other blood parameters, blood was centrifuged at 4°C for 10 minutes, at 3600 rpm, and plasma were stored at -20°C /-80°C until further analysis. Glucose concentrations were measured by the glucose oxidase method with a Beckman Glucose Analyzer II (Beckmann Glucose Analyzer II, Beckmann Instruments, Fullerton, CA). Plasma insulin (RIA kit from LincoMillipore, St CharlesBillerica, MissouriMO, USA) and glucagon (RIA kit from LincoMillipore, St CharlesBillerica, MissouriMO, USA) concentrations were measured by radioimmunoassays, plasma non-esterified fatty acids (NEFA kit from Boehringer MannheimWako Chemical GmbH, Mannheim Neuss, Germany), and plasma beta-hydroxybutyrate (BOHB) concentrations (kit from Boehringer Mannheim, Mannheim, Germany) were measured enzymatically.

**Statistical analysis**

All data were expressed as mean ± SEMs. Parameters measured every week throughout the WPS were analyzed by one way ANOVA for repeated time. An average value (WPS mean) was calculated for the whole WPS period, when time effect was not significant. Values were compared between pre and post WPS by paired *t*-tests. The distribution of IHCL concentrations was markedly skewed, and data were log-transformed for statistical analysis. Correlations between IHCL and other parameters were assessed by the Spearman's rank correlation coefficient test.

***RESULTS***

Characteristics of the subjects before and after WPS are shown in Table 1. Obese patients had a BMI ranging between 30.9 and 52.4 kg/m², and IHCL concentrations ranging between 1.9% and 20.5 % of liver volume. 5 subjects had NAFLD using a cut-off values of IHCL of 5% (15). 3 subjects had two hour plasma glucose concentrations > 140 mg/dl (ca 7.8 mmol/l), indicating impaired glucose tolerance. Average HOMA index was > 2.77 indicating that these group of obese women had significant insulin resistance (159).
Positive correlations were observed between IHCL and liver volume ($\rho = 0.63$, $p < 0.05$), visceral fat volume ($\rho = 0.86$, $p < 0.01$), ALAT ($\rho = 0.73$, $p < 0.01$), and HOMA IS index ($\rho = 0.60$, $p = 0.05$). No correlation was observed between IHCL and BMI or total fat mass.

WPS led to a sustained increase in calculated daily nitrogen excretion (Table 2). Plasma urea concentration also increased while plasma creatinine, daily urinary creatinine excretion and creatinine clearance did not change. Body weight remained unchanged over the 4 week supplementation while body fat mass was slightly reduced and fat free mass was slightly increased. Visceral fat volume and liver volume were not changed (Table 1).

After 4 week WPS, IHCL concentrations had decreased by 20.8 ± 7.7 % ($p = 0.017$), fasting plasma triglycerides had decreased by 15.0 ± 6.9 % ($p = 0.020$) and total plasma cholesterol concentration had decreased by 7.3 ± 2.7 % ($p = 0.024$). Fasting and 2-hour plasma glucose and insulin concentrations were not changed (Table 1).

**Table 1**

**Characteristics of subjects, 11 obese women, before and after the month of WPS at baseline**

| | BASELINE | WPS | *P-value* |
|---|---|---|---|
| **Anthropometric variables** | | | |
| Body weight (kg) | 99.7 ± 5.3 | 100.2 ± 5.4 | NS |
| BMI (kg/m$^2$) | 37.6 ± 1.8 | 37.8 ± 1.8 | NS |
| Fat mass (kg) | 44.8 ± 3.4 | 43.5 ± 3.2 | 0.009 |
| Fat free mass (kg) | 54.8 ± 2.2 | 56.7 ± 2.5 | 0.005 |
| **Blood parameters** | | | |
| Glucose (mmol/L) | 5.2 ± 0.2 | 5.2 ± 0.3 | NS |
| Insulin (µU/mL) | 16.8 ± 2.1 | 17.2 ± 2.9 | NS |
| HOMA IS | 4.0 ± 0.6 | 4.3 ± 1.0 | NS |
| 2h-Glucose (mmol/L) | 7.6 ± 0.9 | 7.4 ± 0.8 | NS |
| 2h- Insulin (µU/mL ) | 111.1 ± 20.6 | 97.1 ± 19.1 | NS |
| Glucagon (ng/L) | 37 ± 2 | 40 ± 4 | NS |
| NEFA (µmol/L ) | 615 ± 57 | 616 ± 60 | NS |
| BOHB (µmol/L) | 58 ± 25 | 41 ± 9 | NS |
| Triglycerides (mmol/L) | 1.65 ± 0.22 | 1.34 ± 0.17 | 0.020 |
| Cholesterol (mmol/L) | 5.65 ± 0.32 | 5.25 ± 0.35 | 0.024 |
| HDL-Cholesterol (mmol/L) | 1.13 ± 0.07 | 1.13 ± 0.05 | NS |
| ASAT (U/L) | 21 ± 1 | 21 ± 1 | NS |
| ALAT (U/L) | 25 ± 3 | 23 ± 3 | NS |
| **Substrate oxidation** | | | |
| Energy Expenditure (kcal/FFM/min) | 0.019 ± 0.001 | 0.018 ± 0.001 | NS |
| Carbohydrate oxidation (kcal/FFM/min) | 1.39 ± 0.31 | 1.33 ± 0.35 | NS |
| Lipid oxidation (kcal/FFM/min) | 0.85 ± 0.12 | 0.63 ± 0.20 | NS |
| Protein oxidation (kcal/FFM/min) | 1.23 ± 0.06 | 1.59 ± 0.16 | 0.019 |
| **$^1$H-NMR** | | | |
| IHCL (vol%) | 7.8 ± 2.2 | 6.3 ± 2.1 | 0.017 |
| Liver volume (cm$^3$) | 1761 ± 138 | 1756 ± 169 | NS |
| Visceral mass (cm$^3$) | 3213 ± 245 | 3184 ± 229 | NS |

All values are expressed as mean ± SEMs. Differences between pre and post whey protein supplementation (WPS) were assessed by the paired parametric *t*-test. *p*-value < 0.05 was considered significant, NS, not significantly different.

**Table 2**
**Evolution of nitrogen and creatinine daily excretion, creatinine and urea plasmatic concentration and creatinine clearance during the month of supplementation.**

| | Week 0 | Week 1 | Week 2 | Week 3 | Week 4 | WPS | *P-value* |
|---|---|---|---|---|---|---|---|
| **Urinary Collection** | | | | | | | |
| Nitrogen excretion [g/day] | 15.6 ± 1.0 | 19.6 ± 1.4 | 20.0 ± 1.7 | 22.3 ± 1.2 | 20.7 ± 2.0 | 20.6 ± 0.8 | 0.000 |
| Urinary Creatinine [mmol/day] | 12.9 ± 0.9 | 12.3 ± 0.9 | 12.4 ± 1.0 | 13.6 ± 0.8 | 12.2 ± 1.1 | 12.6 ± 0.5 | NS |
| **Plasma Collection** | | | | | | | |
| Urea [mmol/L] | 4.9 ± 0.2 | 5.9 ± 0.4 | 5.5 ± 0.5 | 6.1 ± 0.4 | 5.7 ± 0.4 | 5.8 ± 0.2 | 0.002 |
| Creatinine [µmol/L] | 68 ± 4 | 68 ± 2 | 67 ± 2 | 67 ± 2 | 65 ± 2 | 67 ± 1 | NS |
| **Creatinine Clearance [ml/min]** | 137 ± 12 | 127 ± 9 | 131 ± 13 | 141 ± 9 | 131 ± 11 | 132 ± 5 | NS |

Values are expressed as mean ± SEMs. Effects of time during WPS supplementation was assessed by a one way ANOVA repeated for time among weeks 1 to 4. When effect of time during supplementation was not significant, an average value (WPS) was calculated. Difference between week 0 and WPS supplementation was assessed by the paired parametric *t*-test. *p*-value < 0.05 was considered significant, NS, not significantly different.

*DISCUSSION*

Previous studies have shown that a high protein diet reduced hepatic lipid concentrations in high-fat or high-sucrose fed rodents or humans, suggesting that a high protein intake may, directly or indirectly, improve hepatic steatosis (115, 154, 155). We therefore assessed, in obese glucose intolerant young women, whether a 4-week supplementation with whey protein, with an otherwise spontaneous, uncontrolled food intake, would reduce hepatic steatosis and concomitantly improve hyperlipidemia and insulin sensitivity.

IHCL concentrations showed a large interindividual variability in obese subjects, ranging from 1.9 % to 20.5%. IHCL concentrations were correlated with total liver volume and with visceral fat volume, but not with total body fat or BMI, corroborating several reports showing that hepatic fat deposition is tightly linked to visceral obesity (9, 11, 160). Interestingly it seems that when total liver fat is > 5%, liver size increases in proportion to liver fat being 2.4 times larger than the pure fat volume. IHCL were also correlated with HOMA IS, here again corroborating the well known association between NAFLD and insulin resistance (11) (161). IHCL were also weakly correlated with ALAT levels, which are known to be an insensitive marker of hepatic fat (11).

After 4 week of WPS, IHCL decreased significantly by 21 %, and fasting plasma triglycerides and cholesterol concentrations were decreased by 15 % and 8 % respectively. This reduction of IHCL concentrations was not related to changes in visceral fat volume or total liver volume, nor with important changes in body weight or body fat mass, which indicates that the improved IHCL and plasma triglyceride profiles could be attributed to an effect of protein rather than to changes in body composition.

We can only speculate about the possible mechanisms underlying the reduction in IHCL and plasma lipids induced by WPS. Although WPS provided ca 250 kcal/day and body weight did not change a slight but significant decrease in total body fat was observed suggesting that WPS led to a spontaneous decrease in food intake. This hypothesis appears to be corroborated by the evolution of daily urinary nitrogen excretion over time. Pre-WPS daily nitrogen excretion amounted to ca 15 g/day, which corresponds approximately to ca 94 g protein /day assuming 16% nitrogen content in proteins. After WPS, daily nitrogen excretion increased significantly to ca 21 g/day, which corresponds to a total daily protein intake of

129 g/day. This means that, if subjects were hundred percent compliant and consumed the totality of the prescribed 60 g protein supplementation, WPS led to a reduction of spontaneous protein intake from other foods by 27%. This can be readily explained by the well known satiating effects of dietary proteins (56). It is likely that the satiating effects of WPS decreased the intake not only of dietary proteins, but of dietary carbohydrate and fat as well. It is therefore possible that a decrease in carbohydrate and lipid intake was responsible for the decreased IHCL and plasma triglyceride concentrations observed after WPS. In support of this hypothesis, it has indeed been demonstrated, in overfed rats with hepatic steatosis, that increasing the dietary protein content of the diet reduced intrahepatic lipids by decreasing carbohydrate intake (154).

Besides a reduction in spontaneous carbohydrate and fat intake, it is possible that a high-protein intake also reduced hepatic fat via more direct effects. A high protein diet is known to enhance post-prandial thermogenesis, an effect which is linked, at least in part, to the high energy cost of urea synthesis and amino acid conversion into glucose (162, 163). Since these two processes take place in the liver, one can expect that the increased energy requirement of the hepatocyte was met, at least in part, by an increased intrahepatic lipid oxidation. Although not documented in this study, where only fasting concentrations were monitored, feeding high protein meals is also known to increase post-prandial glucagon concentrations (164). The ensuing high glucagon : insulin ratio may therefore have favored lipid oxidation and ketogenesis while inhibiting *de novo* lipogenesis (165) . Finally, other, direct effects of specific amino-acids on intra-hepatic lipid metabolism may be speculated.

Dietary protein metabolism also has complex interaction with glucose metabolism. On one hand, an amino-acid infusion enhances hepatic glucose production (112) and decreases whole body insulin mediated glucose disposal (166). While on the other co-ingestion of protein and glucose have been shown to decrease post-prandial glycemia, an effect which can be attributed to a delayed gastric emptying (167, 168). WPS however failed to significantly alter fasting and two hour plasma glucose and insulin concentrations. Insulin sensitivity was also not grossly altered, as indicated by the HOMA IS index.

In summary, this study demonstrates that 4 weeks supplementation with 3 times 20 g whey protein per day significantly reduced intrahepatic and fasting plasma triglycerides in obese subjects consuming an otherwise spontaneous diet. A satiating effect of the protein

supplementation, leading to lower carbohydrate and or fat intakes, an increased liver energy expenditure, and/or a higher glucagon-insulin ratio, may all be involved in these effects. This preliminary, uncontrolled study suggests that a high-protein diet may, in the long term, reduce the risk of non-alcoholic steato-hepatitis and of cardio-vascular disease in obese patients. While high-protein diet may also have adverse effects, the present study did not find any adverse renal effects.

***ACKNOWLEDGMENTS***

This study was supported by grant 121995 from the Swiss National Foundation for Science to LT and by a grant from Cardiomet/CHUV to KAL. Help with the data acquisition by K.Zwygart is greatly acknowledged. No potential conflict of interest relevant to the article was reported. We thank Uwe Boettcher and Jian Xu of Siemens, Healthcare, for providing and supporting the MRS sequence.

# *CHAPTER 5*
# *STUDY III*

## Effects of dietary protein on postprandial metabolism in healthy humans

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**5.1 Study III, why?**

The results of Study I were encouraging, as discussed in chapter 3. We then designed Study II in an attempt to obtain better insights into the clinical implications of high-protein diets, but we were also interested in the underlying mechanisms which might explain the potential beneficial effects of protein intake. Therefore we designed a third study.

Given the role of lipotoxicity and NAFLD in the development of features of the metabolic syndrome and insulin resistance, we further investigated the metabolic effects of high-protein intake on hepatic lipid metabolism. We hypothesized that protein intake might enhance hepatic fat oxidation and favour VLDL-TG export, leading to a decrease in IHCL and improvement of glucose homeostasis.

The following protocol was designed to allow a deeper comprehension of the underlying mechanisms. We compared the metabolic effects of 2 tests meals with a fixed carbohydrate and fat composition and a varied standard or high protein intake after 4-day of an isocaloric standardized diet. To further evaluate possible long term effects of protein, the metabolic effects of the high protein meal was also tested after 4 days spent on a high-protein diet.
7 healthy young volunteers were allocated to the different diets in a randomized order. Metabolism was assessed over two hours at baseline and during the 6 next hours following meal ingestion.
Measurements with indirect calorimetry provided information on net substrate oxidation rates and energy expenditure, whereas $^{13}$C-triolein incorporated into the test meals was used to calculate exogenous fat oxidation. BOHB plasma concentrations allowed us to estimate hepatic beta-oxidation, whereas plasma concentration of VLDL-TG and Chylomicron-TG gave information about the kinetics of lipid transition through the liver. Glucose metabolism was assessed by continuous infusion of 6,6-$^{2}H_{2}$-glucose, as well as the postprandial plasma glucose and insulin responses.
The results did not support our hypothesis, that a high-protein meal would increase net postprandial lipid oxidation, exogenous lipid oxidation, and stimulate BOHB production, indicating that a high-protein meal does not stimulate whole body or hepatic beta-oxidation. After 4 days on a high-protein diet, a high-protein meal decreased exogenous fat oxidation, impaired postprandial chylomicron clearance and enhanced postprandial triglyceridemia.

These results indicate that under the conditions of our study, a 4-day high protein diet has no effect on insulin sensitivity.

*Personal contribution*

I was involved in the design and development of the protocol. I carried out the recruitment of subjects, the food supplementation to all volunteers and the clinical trial under the help and supervision of the research nurses from the CIC. I conducted data analysis. I received major supervision and help from Ph. Schneiter and L.Tappy during preparation and revision of the manuscript.

*submitted to Clinical Nutrition*

# EFFECTS OF DIETARY PROTEIN ON POSTPRANDIAL LIPID METABOLISM IN HEALTHY HUMANS

Murielle Bortolotti[1], Philippe Schneiter[1], Luc Tappy[1,2].

[1]Department of Physiology, University of Lausanne, Lausanne, Switzerland

[2]Service of Endocrinology, Diabetes, and Metabolism, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

*ABSTRACT*

**Objective**

Protein supplementation may enhance whole body and intrahepatic lipid oxidation. Therefore high protein intake was assessed on postprandial lipemia in healthy men.

**Design**

Seven healthy young male subjects were studied on 3 occasions: after ingestion of a low (0.5g /kg) protein meal, a high (1.5 g/kg) protein meal the carbohydrate and fat composition of which was kept constant and after 4 days on a high (1.5 g/kg) protein diet. Net substrate oxidation, exogenous fat oxidation, glucose and glycerol kinetics, hormones and substrates concentrations were monitored at baseline and during 6 hours after meal ingestion.

**Results**

The high-protein meal delayed plasma glucose, insulin, and glucose appearance, and reduced postprandial NEFA, glycerol, beta-hydroxybutyrate and net lipid oxidation ( $p$ = 0.064 ). It did not affect other variables. After 4 days on a high-protein diet, postprandial exogenous fat oxidation tended to decrease ( $p$ = 0.169 ) whereas postprandial triglyceride concentrations were increased ( $p$ = 0.005 ).

**Conclusions**

High-protein meal did not increase net postprandial lipid oxidation nor exogenous lipid oxidation, and did not stimulate beta-hydroxybutyrate production, indicating that neither whole-body lipid oxidation nor hepatic beta-oxidation were stimulated. After 4 days on a high-protein diet, exogenous fat oxidation decreased and postprandial triglyceride concentrations were enhanced. A high-protein diet may impair chylomicron clearance.

*INTRODUCTION*

The metabolic syndrome, characterized by visceral obesity, altered glucose homeostasis, dyslipidemia, and high blood pressure, is highly prevalent in industrialized countries, and represents a major public health burden (8). Insulin resistance is tightly associated with this syndrome (169), and it is thought to be the result of impaired mitochondrial function, impaired lipid oxidation, and lipotoxicity. Ectopic lipid deposition in the liver, leading to non-alcoholic fatty liver disease, and muscle are frequently encountered in association with the metabolic syndrome (170) and are closely linked to insulin resistance (171).

Both dietary factors (172) and low physical activity (173) are involved in the development of the metabolic syndrome. Among the dietary factors, high sugar (149) and high saturated fat intakes (136) can lead, in animal models and in humans, to the development of several features of the metabolic syndrome, including increased intrahepatic lipids . The effects of dietary protein are in contrast less well known. While amino-acid infusion can clearly impair insulin's hepatic and extra-hepatic actions (174) (166), several observations suggest that a high-protein diet may have beneficial effects on the metabolic syndrome (70). Co-ingestion of protein with carbohydrate tends to decrease postprandial glycemia in healthy subjects and in insulin-resistant patients (164). A high-protein diet has also been shown to improve glucose homeostasis in patients with type 2 diabetes mellitus (175) Furthermore, a hypocaloric high protein diet has also been shown to enhance weight loss and improve glucose tolerance in obese patients (176). Finally, a high-protein diet reduced intrahepatic fat concentrations in carbohydrate fed rats (154), in obese Zucker rats (155), and in healthy human subjects put on a high-fat diet for 4 days (115).

Given the role of lipotoxicity in the development of insulin resistance, the apparent beneficial effects of a high-protein diet on glucose homeostasis and on intrahepatic lipids suggested that protein may improve insulin action by promoting lipid oxidation. To evaluate this hypothesis, we compared the metabolic effects of 2 test meals with a fixed carbohydrate and fat but a standard or high protein load. To further evaluate possible effects of protein, the metabolic effects of the high protein meal was also tested after 4 days spent on a high protein diet. $^{13}$C-triolein incorporated in the test meals was used to calculate exogenous fat oxidation, while indirect calorimetry provided information on total, net substrate oxidation rates.

*SUBJECTS AND METHODS*

**Participants**

7 healthy male volunteers were recruited from students at the University of Lausanne. They had a mean age of 21 ± 0.5 years, mean weight of 74 ± 3 kg, mean BMI of 23.2 ± 0.8 kg/m$^2$ and mean fat mass of 13 ± 0.8 % evaluated from skinfold-thickness measurements using the tables of Durnin and Womersley (177). All were in good physical health based on medical history and a standard physical examination, were not on any medication at the time of the study, had a usual alcohol consumption < 20g/day and were non-smokers. The experimental protocol was approved by the Ethical Committee of Lausanne University School of Medicine and every participant provided informed, written consent.

**Study design**

Every subject took part in 3 tests, separated by at least 3 weeks. During the 4 days preceding each test, they consumed a controlled diet, with all food items prepared and provided by the investigators to be consumed at home. Subjects were carefully instructed to prepare and consume all the food provided according to specific instructions, and to refrain from consuming any other foods or beverages during this period. On two occasion, subjects consumed a weight-maintenance diet (control diet: CD) providing 140 % basal energy requirements (calculated using the equation of Harris-Benedict (127) multiplied by a Physical Activity coefficient of 1.4). On the third occasion, subjects consumed the same control diet supplemented with 1.5g/kg/day protein (high protein diet: HPD). The protein supplementation was provided as dairy products (skimmed milk powder, cottage cheese, and yoghurt). Detailed compositions of the diets are given in **Table 1**.

**Table 1: Energy repartition of the 4-day standardized diets, control diet (CD) and high protein diet (HPD)**

| | CD | HPD |
|---|---|---|
| **Total Energy (kcal)** | 2585 ± 32 | 3000 ± 32 |
| **Total Macronutrients** | | |
| Carbohydrates ( kcal) | 1370 ± 22 | 1409 ± 14 |
| Lipids ( kcal) | 810 ± 12 | 768 ± 8 |
| Protein (kcal) | 405 ± 5 | 830 ± 11 |

All values are expressed as mean ± SEM.

After 4 days on one or the other of these two controlled diets, subjects underwent a metabolic test aimed at assessing substrate oxidation and glucose homeostasis in basal conditions and after ingestion of a test meal. One test meal was low in protein (low protein meal, LPM) contained 55 % carbohydrate, 35 % fat labelled with 1% $^{13}$C-triolein (Cambridge Isotope Laboratories, Andover, MA, USA), and 10 % protein as cottage cheese . The other test meal had the same carbohydrate and fat, and labelled triolein content but was enriched with dairy proteins (high protein meal, HPM). The detailed composition of the two test meals is provided in **table 2**.

**Table 2: Energy repartition and composition of the low protein meal (LPM) and high protein meal (HPM)**

| | LPM | HPM |
|---|---|---|
| **Total Energy (kcal)** | 772 ± 19 | 921 ± 24 |
| **Total Macronutrients** | | |
| Carbohydrates (kcal) | 424 ± 11 | 424 ± 11 |
| Lipids ( kcal) | 270 ± 6 | 270 ± 6 |
| Protein (kcal) | 77 ± 3 | 226 ± 7 |
| **Food items** | | |
| White bread (g) | 70 ± 0.0 | 70 ± 0.0 |
| Orange juice (g) | 252 ± 4.4 | 265 ± 3.7 |
| Sirup (g) | 61 ± 4.6 | 23 ± 3.5 |
| Olive Oil (g) | 15 ± 0.2 | 15 ± 0.2 |
| Butter (g) | 13 ± 0.7 | |
| Buttermilk (g) | | 149 ± 1.0 |
| Serac (g) | | 109 ± 6.7 |
| Skimmed milk (g) | | 51 ± 4 |

All values are expressed as mean ± SEMs.

Each subject was studied on three occasions, i.e. after 4 days of controlled diet and with a control test meal (CD-LPM), after 4 days controlled diet with a high-protein test meal (CD-HPM), and after 4 days high-protein diet with a high-protein test meal (HPD-HPM), according to a randomized sequence.

For each metabolic test, subjects came at the Cardiomet clinical investigation unit of the Centre Hospitalier Universitaire Vaudois at ca 7 am after an overnight fast. At their arrival, subjects were asked to void and were transferred to a bed where they remained quiet in a semi-recumbent position for the next 8 hours. An indwelling Teflon® catheter was inserted into a vein of one forearm, two continuous infusions were infused throughout the experiment, one of labelled 6,6 $^{2}H_{2}$-glucose (bolus: 2 mg/kg; continuous infusion: 40 ug/kg/min) and the other of labelled $^{2}H_{5}$-glycerol (bolus: 1 µmol/kg, continuous infusion 0.1 µmol/kg/min) (Cambridge Isotope Laboratories, Andover, MA, USA) . A second indwelling catheter was inserted into a vein of the other forearm for periodic blood sampling. This arm was maintained in a thermostabilzed box heated at 50°C to achieve partial arterialization of venous blood. Respiratory gas exchanges were continuously monitored by means of an open flow, continuous indirect calorimeter with a hood system (Deltatrac II, Datex Instruments, Helsinki, Finland). After a 105 min period allowed for tracer equilibration, and during which basal measurements were performed, the hood was removed for 30 min, the subject was asked to void again before ingesting one of the two test meals in 20 min. Measurements were thereafter pursued for another 360 min.

**Analytical procedures**

Blood samples were collected every 30 min throughout the test for the measurement of plasma glucose (Beckmann Glucose Analyzer II, Beckmann Instruments, Fullerton, CA), urea (Urea Analyzer, Beckmann Instruments Fullerton, CA,USA) plasma insulin (RIA kit from Millipore, Billerica, MO, USA), plasma glucagon (RIA kit from Millipore, Billerica, MO, USA), plasma nonesterified fatty acids (NEFA kit from Wako Chemical GmbH, Neuss, Germany), plasma beta-hydroxybutyrate (BOHB) concentrations (kit from Boehringer Mannheim, Mannheim, Germany) and total plasma triglyceride (TG) concentrations (kit from Biomérieux, Marcy l'Etoile, France). For each blood collection time, one blood sample was ultracentrifuged and TG concentrations were measured in chylomicrons and VLDL

subfractions. Plasma from other blood samples were analysed for 6,6 $^{2}H_{2}$-glucose and $^{2}H_{5}$-glycerol by GC-MS as previously described (129, 178). Breath collections were obtained at 30 min intervals throughout the test and were stored in 10 ml vacutainers until analyzed. Breath $^{13}CO_{2}$ isotopic abundance was determined by continuous flow-isotope mass spectrometry using a Tracermass C/N (sercon, Crewe, UK).

At the end of the test, the subject was again asked to void. Urine voids were collected for determination of urea nitrogen excretion rate.

**Calculations**

Net substrate oxidation rates were calculated from oxygen consumption and carbon dioxide production averaged over 30 minute periods. Urea nitrogen excretion rate was calculated from urinary urea excretion and corrected for changes in the blood urea nitrogen pool size over time (179). Total nitrogen excretion was calculated assuming that urea accounted for 85% of total urinary nitrogen (113) and that extra-renal nitrogen losses were 2g/day. Net substrate oxidation rates were calculated using the equations of Livesey and Elia (109) .

Glucose rates of appearance (GRa) and disappearance (GRd) were calculated using Steele's equation for non steady state conditions using a glucose distribution space of 0.2 and a pool fraction of 0.8 (103). Glycerol rates of appearance (GlyRa) and disappearance (GlyRd) were calculated from the same equation but adjusted with a volume of distribution of 0.23 (l/kg body weight) (104). Exogenous fat oxidation was calculated as described by Binnert et al. (105).

**Statistical analysis**

All data were expressed as mean ± SEMs. The effects of HPD and HPM and their interactions on plasma hormone and substrate concentrations, net substrate oxidation and energy expenditure, and glucose and glycerol turnover were assessed by two–way repeated measures ANOVA with interaction. The parametric *t*-test with Bonferroni's correction was used to test the null hypothesis for postprandial response between all three paired dietary conditions (CD-CM, CD-HPM, HPD-HPM). Significance was assessed at $p \leq 0.05$. The software used was STATA version 9.1 (Stata Corp, College Station, TX)

***RESULTS***

**CD-LPM vs CD-HPM**

When subjects received a normal protein, control diet (CD) for 4 previous days, ingestion of the HPM resulted in a decreased peak glycemia 30-90 after the meal, but a slightly higher glycemia in the latter part of the test, i.e. between 210 and 360 min compared to that observed after the LPM (Fig 1; see figure legend for statistics). Peak plasma insulin concentrations were slightly increased and plasma glucagon concentrations were significantly higher with the HPM ($p = 0.001$) (Fig 1). The effect of the HPM on the last part of the postprandial glycemia curve was mirrored by similar changes in glucose rate of appearance (Ra) and oxidation. After the HPM, glucose Ra and Rd increased to a similar extent during the 60 min following meal ingestion, but returned more slowly to baseline and remained higher from 180 to 300 min compared to that observed after the LPM (Fig 2; see figure legend for statistics). Net glucose oxidation increased to the same extent following ingestions of the LPM and HPM (Fig 3; see figure legend for statistics). Postprandial total energy expenditure (Fig 3) and net protein oxidation (LPM: -7.5 ± 11.2 mg/min, HPM: 16.9 ± 3.2 mg/min, $p = 0.169$) were also higher after HPM.

Plasma NEFA and BOHB concentrations were suppressed to the same extent with LPM and HPM during the initial 120 postprandial period. Thereafter, they increased markedly after LPM, while they remained suppressed with HPM (Fig 4; see fig legend for statistics). Glycerol Ra and net lipid oxidation paralleled the changes in NEFA (Fig 2, 3 and 4; see figure legend for statistics). Ingestion of the HPM led to an increase in total plasma TG and chylomicron TG concentrations (Fig 5; see fig legend for statistics). This increase was however not different between HPM and LPM.

The postprandial increase in breath $^{13}CO_2$ was identical after LPM and HPM. Calculated cumulated exogenous fat oxidation represented only a minor portion of total lipid oxidation, and was similar with LPM (28.7 ± 3.7 %) and HPM (26.2 ± 3.1 %).

**CD vs HPD**

The HPD increased basal protein oxidation to 108 ± 12 mg/min ($p < 0.05$) and decreased basal glucose and lipid oxidation (Fig 3), but otherwise did not significantly alter basal hormone and substrate concentrations or energy expenditure. When the metabolic responses to HPM were compared after CD and HPD, HPD did not significantly alter the glucose,

insulin and glucagon responses (Fig 1), nor the postprandial suppression of NEFA, BOHB and glycerol (Fig 4). The effect of HPM on glucose Ra and glycerol Ra were also the same after HPD and after CD.

After the HPD, the increase in plasma total TG and chylomicron TG induced by HPM ingestion was higher than after both HPM and LPM ingestion after CD (Fig 5). The increase in breath $^{13}CO_2$ over time was also delayed, and cumulated exogenous lipid oxidation was reduced to 21.7 ± 3.4 % ($p$ = 0.169). The increase in postprandial energy expenditure elicited by HPM was greater after HPD ($p$ = 0.0369) than CD ($p$ = 0.3), but postprandial lipid oxidation was similar after HPD.

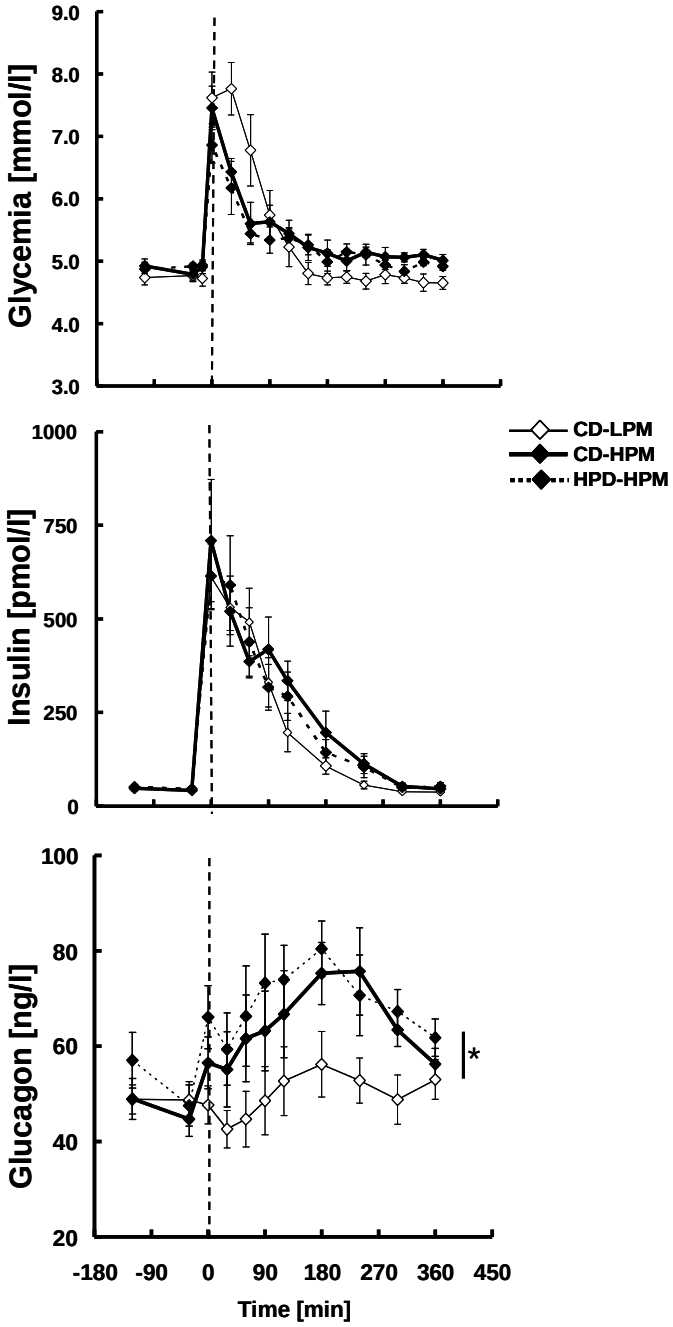


**Fig.1**
Plasma concentrations of glucose (mmol/l), insulin (pmol/l) and glucagon (ng/l) at baseline and after 4 days on a control diet and ingestion of a low protein (LPM: open diamonds)- or high protein (HPM: closed diamonds, full line) - test meal, or after 4 days on a high protein diet (HPD) and ingestion of HPM (closed diamonds, dotted line). Values are means ± SEMs. The dashed line represents end of meal ingestion.
* Significant effect of dietary conditions, without interaction between time and dietary condition, $P \leq 0.05$. Respective $p$ value for time (T) and interaction (I) for glucose (T: $p = 0.00$; I: $p = 0.1$), insulin (T: $p = 0.00$; I: $p = 0.3$) and glucagon (T: $p = 0.01$; I: $p = 0.3$).

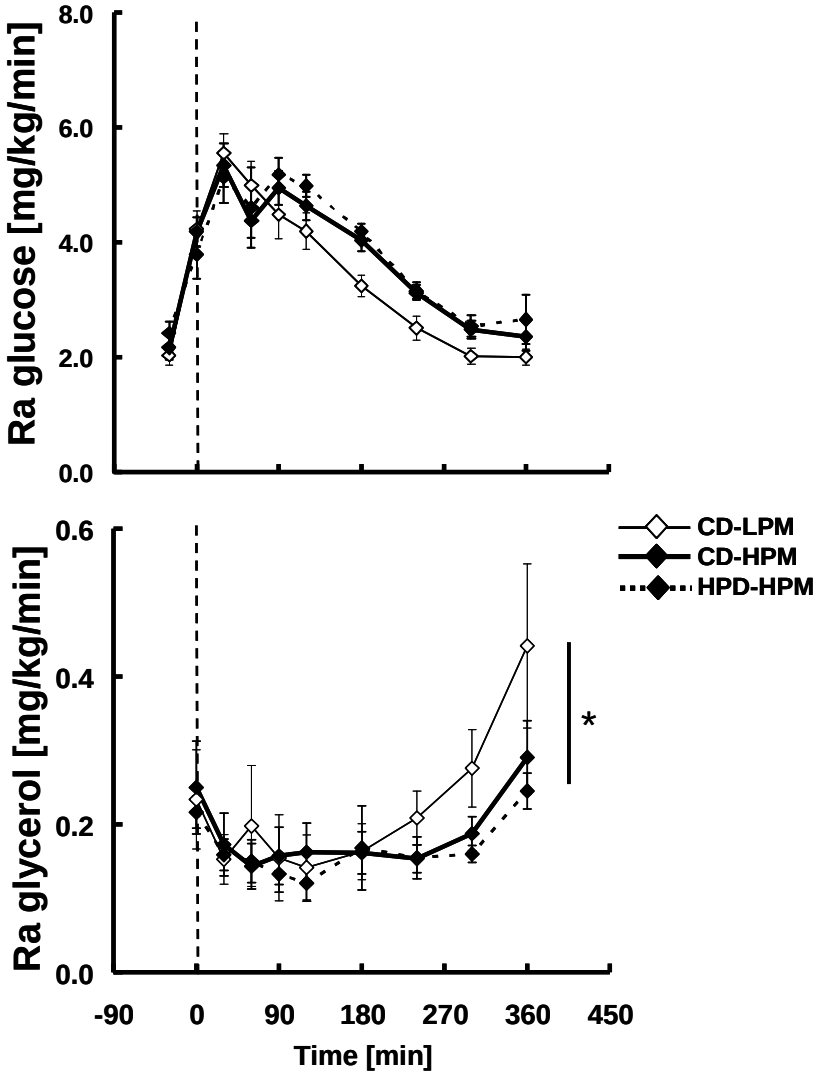


**Fig.2**

Rate of appearance of glucose (Ra) (mg/kg/min) and glycerol (mg/kg/min) at baseline and after 4 days on a control diet and ingestion of a low protein (LPM: open diamonds)- or high-protein (HPM: closed diamonds, full line) - test meal, or after 4 days on a high-protein diet (HPD) and ingestion of HPM (closed diamonds, dotted line). Values are means ± SEMs. The dashed line represents end of meal ingestion.

* Significant effect of dietary conditions, without interaction between time and dietary condition, $P \leq 0.05$. Respective $p$ value for time (T) and interaction (I) for glucose Ra (T: $p = 0.00$; I: $p = 0.3$) and glycerol Ra (T: $p = 0.01$; I: $p = 0.3$).

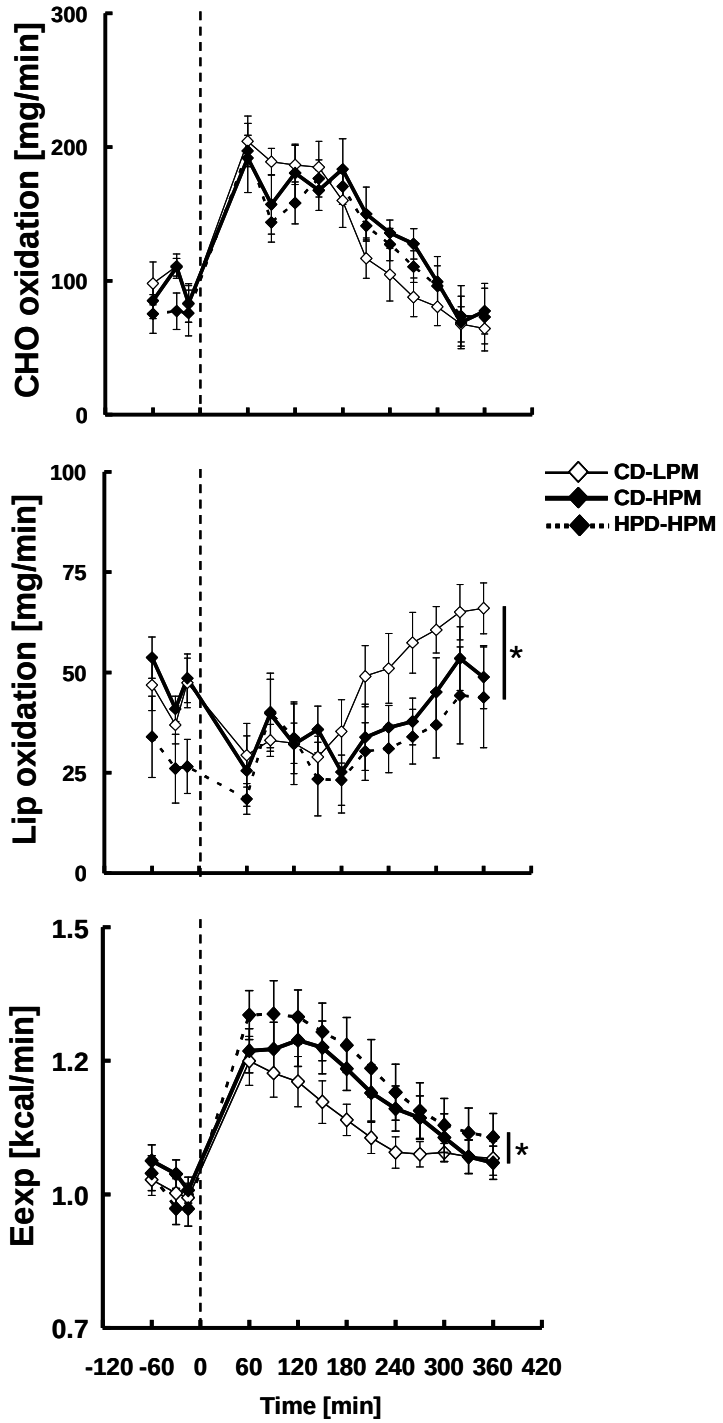


**Fig.3**

Net glucose (CHO) (mg/min), lipid (Lip) (mg/min) oxidation and total energy expenditure (Eexp) (kcal/min) at baseline and after 4 days on a control diet and ingestion of a low-protein (LPM: open diamonds)- or high-protein (HPM: closed diamonds, full line) - test meal, or after 4 days on a high-protein diet (HPD) and ingestion of HPM (closed diamonds, dotted line). Values are means ± SEMs. The dashed line represents end of meal ingestion.

* Significant effect of dietary conditions, without interaction between time and dietary condition, $P \leq 0.05$. Respective $p$ value for time (T) and interaction (I) for CHO oxidation (T: $p = 0.00$; I: $p = 0.4$), Lip oxidation (T: $p = 0.01$; I: $p = 0.3$) and Eexp (T: $p = 0.01$; I: $p = 0.2$).

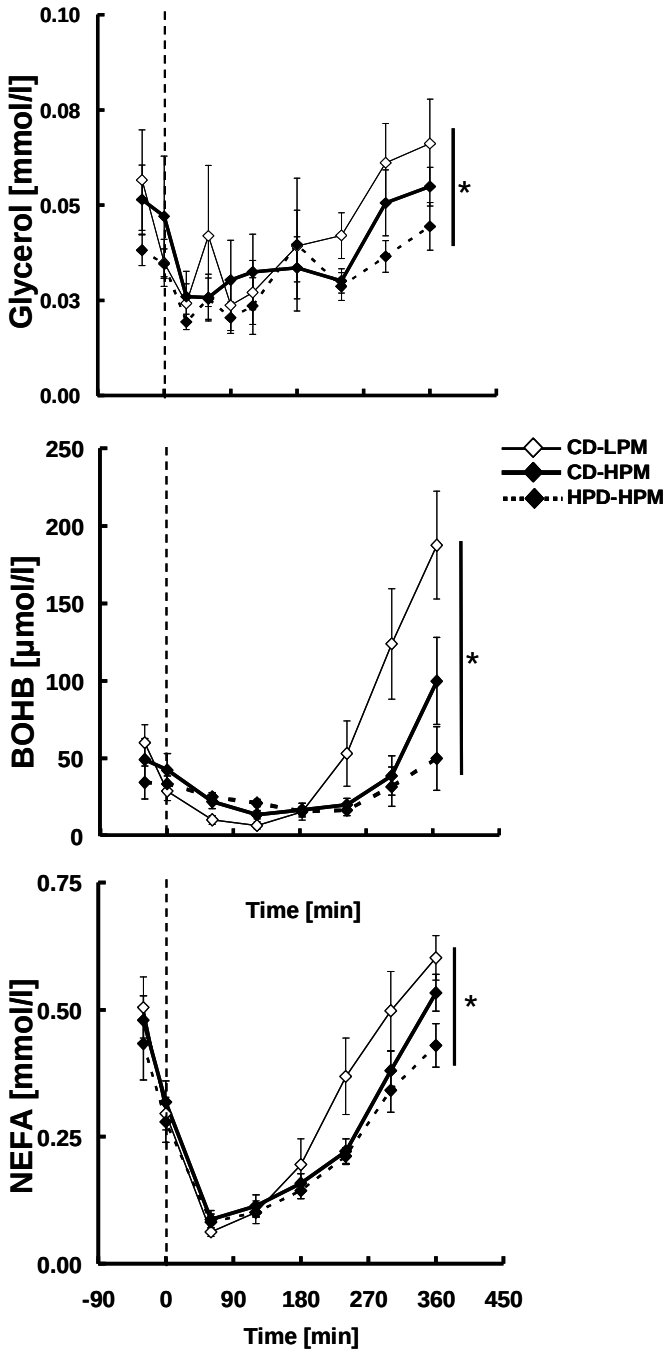


**Fig. 4**

Plasma concentrations of glycerol (mmol/l), beta-hydroxybutyrate (BOHB) (µmol/l) and NEFA (mmol/l) at baseline and after 4 days on a control diet and ingestion of a low-protein (LPM: open diamonds)- or high-protein (HPM: closed diamonds, full line) - test meal, or after 4 days on a high-protein diet (HPD) and ingestion of HPM (closed diamonds, dotted line). Values are means ± SEMs. The dashed line represents end of meal ingestion.

* Significant effect of dietary conditions, without interaction between time and dietary condition, $P \leq 0.05$, excepted for BOHB. Respective $p$ value for time (T) and interaction (I) for glycerol (T: $p = 0.00$; I: $p = 0.3$), BOHB (T: $p = 0.00$; I: $p = 0.04$) and NEFA (T: $p = 0.00$; I: $p = 0.3$).

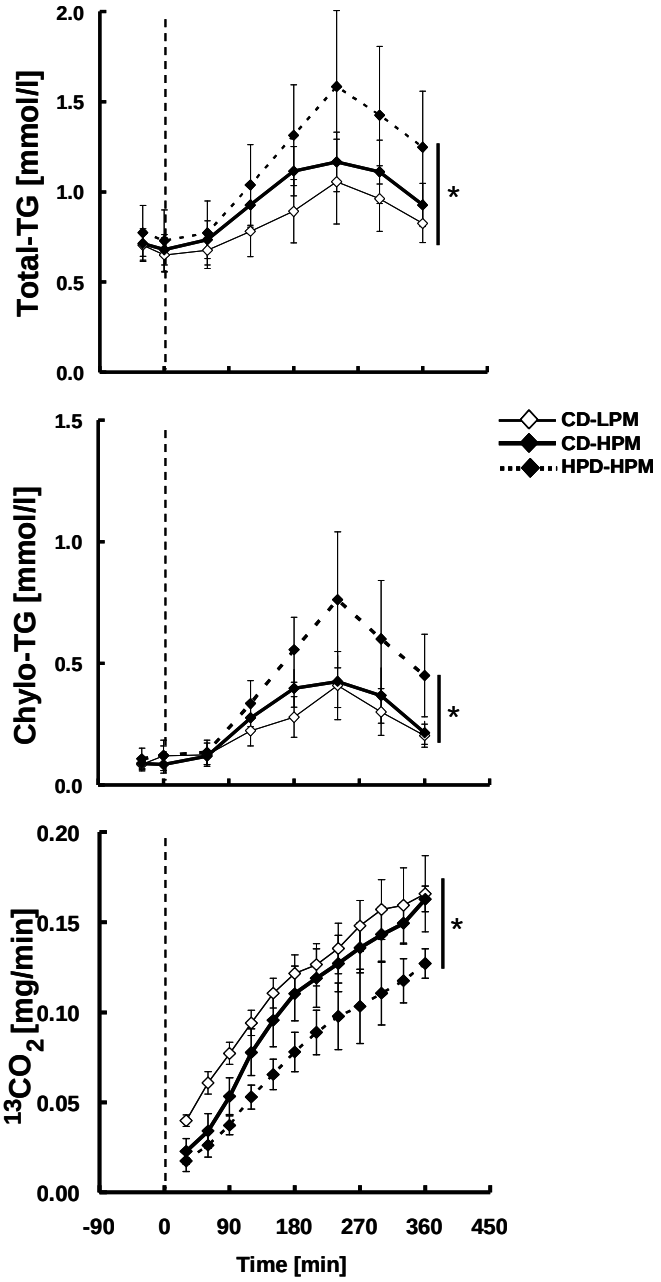


**Fig.5**

Plasma concentrations of total-triglycerides (total-TG) (mmol/l), Chylomicron-TG (Chylo-TG) (mmol/l) and breath $^{13}CO_2$ (mg/min) at baseline and after 4 days on a control diet and ingestion of a low-protein (LPM: open diamonds)- or high protein (HPM: closed diamonds, full line) - test meal, or after 4 days on a high-protein diet (HPD) and ingestion of HPM (closed diamonds, dotted line). Values are means ± SEMs. The dashed line represents end of meal ingestion.

* Significant effect of dietary conditions, without interaction between time and dietary condition, $P \leq 0.05$. Respective $p$ value for time (T) and interaction (I) for total-TG (T: $p = 0.00$; I: $p = 0.4$), Chylo-TG (T: $p = 0.00$; I: $p = 0.3$) and breath $^{13}CO_2$ (T: $p = 0.00$; I: $p = 0.6$)

*DISCUSSION*

A high-protein diet has been shown to reduce intrahepatic fat concentrations in both humans on a high fat diet and in high sucrose fed rats or obese rats, suggesting that dietary protein may either enhance hepatic fat oxidation or decrease hepatic lipogenesis or both (154) (155), (180). Furthermore, several observations indicate that a high-protein diet may improve insulin sensitivity in insulin resistant humans and rodents (164) (175) (176). Since insulin resistance is tightly linked to ectopic lipid deposition in skeletal muscle (181) and the liver (10), this further supports the hypothesis that protein may enhance whole body lipid oxidation. This hypothesis appeared highly plausible since dietary proteins have a high thermic effect and increase postprandial whole body energy expenditure (54). Furthermore, since hepatic conversion of amino acids into glucose and ureagenesis are both stimulated after protein feeding or amino-acid infusion (182), and since both processes have a high energy cost, it is likely that a substantial portion of the thermic effect of protein takes place in the liver. Based on these considerations, we hypothesized that ingestion of a protein enriched meal would enhance whole body lipid oxidation and increase plasma BOHB concentrations (a marker of hepatic beta oxidation and ketogenesis) to a greater extent than a low-protein meal.

Our present observations however do not support this hypothesis. When the effect of the high-protein meal (HPM) containing 25 % protein were compared to those of a control meal containing 10 % protein (LPM), the thermic effect of the meal cumulated over 6 hours was indeed increased, but cumulated fat oxidation, calculated from indirect calorimetry, was not increased, and even tended to be decreased. Furthermore, suppression of plasma BOHB concentrations was more sustained after the high protein meal, consistent with a lowered hepatic beta oxidation.

Plasma glucose, and insulin responses somewhat differed after HPM and LPM. After HPM, peak plasma glucose concentrations tended to be blunted, but glucose concentrations remained elevated for a longer time, and returned more slowly to baseline values. Similar observations have already been reported in the literature and were interpreted as the result of a delayed gastric emptying with high protein meals, resulting in a slower absorption of glucose (168) (183). This explanation is corroborated by our measures of GRa. The GRa return to baseline values was indeed delayed with high-protein meals, consistent with a

delayed gastric emptying. The slight increase in GRa and insulin between 120 and 360 min after a high protein meal may also explain the continuing inhibition of lipolysis and BOHB concentrations observed with high protein meals.

Postprandial net lipid oxidation was not significantly altered by ingestion of a high-protein compared to a low-protein meal. Indirect calorimetry is a robust method to estimate net substrate oxidation under basal conditions and after ingestion of pure glucose or lipids, but it may become relatively inaccurate when protein oxidation changes over time, since acute changes in protein oxidation are not reflected in a single timed urine collection and there is a delay between ureagenesis and urinary urea excretion (184). Although correction of urinary urea excretion for changes in blood urea nitrogen pool size improves the accuracy of indirect calorimetry after protein or amino-acid administration, we nonetheless considered the possibility that the technique be not sensitive enough to detect changes in lipid oxidation induced by dietary protein. We therefore labelled the lipid of the test meals with $^{13}$C-triolein and monitored breath $^{13}CO_2$. This approach provides a measurement of lipid oxidation which does not rely on calculation of protein oxidation or non-protein respiratory quotient. This approach however basically differs from indirect calorimetry by assessing exclusively the direct oxidation of exogenous, labelled lipids ingested with the meal. Thus, changes in intestinal lipid absorption kinetics, hydrolysis of chylomicrons by lipoprotein lipase at the tissue level, and fatty acids tissue uptake and oxidation may all potentially impact on the amount of exogenous lipid oxidation. As with indirect calorimetry, monitoring of breath $^{13}CO_2$ production was not significantly different after low- or high protein meals, and even tended to be lower after a high protein meal. The postprandial suppression of BOHB, which reflect hepatic beta-oxidation, was also identical. The present results provide evidence that dietary protein did not acutely enhance total, hepatic, or exogenous lipid oxidation.

Reduction in intrahepatic lipids was reported after consumption of a high protein diet for several days (115) . We therefore considered the possibility that stimulation of hepatic or whole body lipid oxidation may depend on changes in the cellular substrate pools or in gene expression, which may require several days exposure to a high-protein intake. We therefore also assessed the effects of a HPM ingested after 4-days of diet supplemented with protein. NEFA and BOHB responses to ingestion of a high-protein meal were not altered by consumption of a HPD during the days before the HPM. In contrast, after a HPD, the

postprandial increase in breath $^{13}CO_2$ and exogenous fat oxidation were significantly reduced, while net total lipid oxidation tended to be lower. Furthermore the postprandial plasma total triglycerides and chylomicron triglycerides concentrations were significantly increased. Since GRa time course after ingestion of a high protein meal was similar after a 4-day control diet and a 4-day high protein diet, it is unlikely that the high protein diet markedly changed gastric emptying, and hence this enhanced postprandial triglyceride response is best explained by a decreased tissue extraction of chylomicrons and oxidation of chylomicron-derived fatty acids after the high protein diet. These observations altogether allow to definitively refute the hypothesis that dietary proteins stimulate fat oxidation. Instead, it raises the issue of a possibly impaired chylomicron-triglyceride clearance, with lower postprandial oxidation of exogenous fat and enhanced postprandial hypertriglyceridemia with protein overfeeding.

The high-protein diet used in our experiments was both enriched with protein and hypercaloric compared to CD. Since our experiment did not include a hypercaloric, normal protein diet, we cannot at this stage discard the possibility that the impaired postprandial chylomicron clearance observed after HPD was not the mere consequence of an overfed caloric load during the 4 days of controlled diet, rather than a specific effect of proteins. Similarly, since we did not include a HPD-LPM test in our study, we cannot at this stage evaluate whether a hypercaloric, HPD is sufficient to impair postprandial chylomicron clearance, or whether this effect is more directly related to the protein content of the meal.

In summary, our present observations clearly indicate that increasing the protein content of a mixed meal, whether in an acute setting, or in the course of a 4-day high-protein diet, does not increase whole body net lipid oxidation, oxidation of the lipids ingested with the meal, or plasma BOHB concentrations. This allows to unequivocally refute the hypothesis that a high-protein diet may improve insulin sensitivity and reduce ectopic lipids through stimulation of lipid oxidation. On the other hand , it was observed that a hypercaloric high-protein diet significantly impaired postprandial chylomicron clearance, enhanced postprandial triglyceridemia, and decreased postprandial lipid oxidation. This observation is concerning, and further studies will be needed to evaluate whether this is a specific effect of a high-protein diet or the mere consequence of a calorie excess.

***ACKNOWLEDGEMENTS***

*Acknowledgement to all sources of funding:* This work was supported by research grant 310000_121995 from the Swiss National Foundation for Science to LT

*Acknowledgement to all contributors:* The authors thank the research nurses Marie-Christine Cayeux, Françoise Secretan and Christiane Pellet from the Clinical Investigation Center ( CIC –CHUV) for supervision of volunteers during metabolic investigations and the laboratory technicians, Nathalie Stefanoni, Valentine Rey and Eunika Rossi for analysis of blood samples.

# *CHAPTER 6*
# *DISCUSSION&PERSPECTIVES*

## *6.1 DISCUSSION*

The objectives of the present work were to investigate the effects of dietary protein intake on hepatic lipid metabolism.

In an attempt to cover different points of view, this question was addressed using three protocols involving various feeding conditions. Study I addressed the effects of a 4-day hypercaloric high-fat high-protein diet on insulin sensitivity and on accumulation of fat in the liver (IHCL). The effects of whey protein supplementation on IHCL, insulin sensitivity and lipid metabolism were assessed in obese women in Study II. Finally, the third study (Study III) addressed the effects of a high-protein meal on postprandial lipid metabolism after 4 days on a control or a high-protein diet. The major aspects of each experiment are reported in **Table 1**. In an attempt to provide discussion, the principle baseline variables and results from the 3 studies are summarized in **Table 2**. A schematic summary of the major interesting results for Study I and III is proposed in **Figure 7a** and for Study II in **Figure 7b**.

**Table 1 : Summary of principles baseline parameters for the different dietary conditions among the three studies**

| | STUDY I | | | STUDY II | | STUDY III | |
|---|---|---|---|---|---|---|---|
| | **C** | **HF** | **HFHP** | **B** | **WPS** | **CD** | **HPD** |
| **Protocol design** | | | | | | | |
| Number of subjects | 10 | | | 11 | | 7 | |
| Time of supplementation | 4 days | | | 1 month | | 4 days | |
| Sex | Male | | | Female | | Male | |
| BMI | normal | | | overweight | | normal | |
| **Supplemented diets** | | | | | | | |
| Baseline Energy Requirement (%) | + 140 | + 182 | + 210 | Uncontrolled Alimentation | | + 142 | +166 |
| Energy (kcal) | 2248 | 2923 | 3370 | | | 2585 | 3000 |
| Carbohydrate (kcal) | 1237 | 1237 | 1237 | | | 1370 | 1409 |
| Total fat (kcal) | 674 | 1349 | 1349 | | | 810 | 768 |
| Protein (kcal) | 337 | 337 | 784 | | | 405 | 830 |
| **Types of food items** | | | | | | | |
| | | butter<br>milk<br>cheese | eggs<br>ham<br>salami<br>tuna | | WPS | | skimmed milk<br>cottage cheese<br>joghurt |

*Abbreviations*; control diet (**C**); high-fat diet (**HF**); high-fat high-protein diet (**HFHP**); control diet, low protein meal (**CD-LPM**); control diet, high protein meal (**CD-HPM**); high-protein diet, high-protein meal (**HPD-HPM**); baseline condition (**B**), after whey protein supplementation (**WPS**); BMI, body mass index

**Table 2 : Summary of principles baseline parameters for the different dietary conditions among the three studies**

| | STUDY I (N=10 M)[1] | | | STUDY II (N=11 F)[2] | | STUDY III (N=7 M)[3] | | |
|---|---|---|---|---|---|---|---|---|
| | C | HF | HFHP | B | WPS | CD-LPM | CD-HPM | HPD-HPM |
| **Anthropometric variables** | | | | | | | | |
| Weight (kg) | 73.8 ± 2.3 | 73.9 ± 2.6 | 74.9 ± 2.3* | 99.7 ± 5.3 | 100.2 ± 5.4 | 73.4 ± 2.7 | 73.5 ± 2.8 | 74.1 ± 2.6 |
| BMI (kg/m$^2$) | 22.4 ± 0.6 | 22.4 ± 0.7 | 22.7 ± 0.6* | 37.6 ± 1.8 | 37.8 ± 1.8 | 22.9 ± 0.7 | 22.9 ± 0.8 | 23.1 ± 0.8 |
| Fat mass (%)[a] | 16.2 ± 1.4 | 14.9 ± 1.0 | 16.0 ± 1.4 | 44.5 ± 1.4 | 43.0 ± 1.4* | 13.4 ± 0.8 | | |
| **Blood parameters** | | | | | | | | |
| Glucose (mmol/L) | 5.2 ± 0.1 | 5.4 ± 0.1 | 5.2 ± 0.1 | 5.2 ± 0.2 | 5.2 ± 0.3 | 4.8 ± 0.1 | 4.8 ± 0.1 | 4.9 ± 0.1 |
| Insulin (µU/mL) | 8.3 ± 0.9 | 8.8 ± 0.8 | 10.2 ± 1.5 | 16.8 ± 2.1 | 17.4 ± 2.9 | 7.2 ± 0.6 | 7.0 ± 0.5 | 7.7 ± 1.0 |
| Glucagon (pg/mL) | 56.1 ± 4.4 | 55.8 ± 5.1 | 63.8 ± 6.3 | 37.4 ± 2.4 | 39.8 ± 3.9 | 48.7 ± 3.9 | 44.7 ± 3.7 | 47.6 ± 4.3 |
| Insulin/Glucagon | 0.15 ± 0.01 | 0.16 ± 0.02 | 0.15 ± 0.03 | 0.43 ± 0.05 | 0.42 ± 0.08 | 0.15 ± 0.02 | 0.16 ± 0.02 | 0.17 ± 0.02 |
| HOMA-IS | 1.9 ± 0.2 | 2.1 ± 0.2 | 2.4 ± 0.4 | 4.0 ± 0.6 | 4.3 ± 1.0 | 1.5 ± 0.2 | 1.5 ± 0.1 | 1.7 ± 0.2 |
| NEFA (µmol/L) | 540 ± 58 | 399 ± 43* | 383 ± 53* | 615 ± 57 | 616 ± 60 | 504 ± 60 | 479 ± 48 | 433 ± 72 |
| BOHB (µmol/L) | 70 ± 25 | 27 ± 5* | 55 ± 25 | 58 ± 25 | 41 ± 9 | 60 ± 11 | 49 ± 14 | 35 ± 11 |
| Total-TG (mmol/L) | 0.83 ± 0.09 | 0.69 ± 0.07 | 0.77 ± 0.10 | 1.65 ± 0.22 | 1.34 ± 0.17* | 0.71 ± 0.09 | 0.71 ± 0.07 | 0.77 ± 0.15 |
| Chylomicron-TG (mmol/L) | | | | | | 0.08 ± 0.03 | 0.09 ± 0.02 | 0.11 ± 0.04 |
| VLDL-TG (mmol/L) | 0.58 ± 0.08 | 0.45 ± 0.06* | 0.51 ± 0.08 | | | 0.36 ± 0.06 | 0.36 ± 0.05 | 0.40 ± 0.09 |
| Cholesterol (mmol/L) | 3.61 ± 0.22 | 3.99 ± 0.25 | 4.14 ± 0.22* | 5.65 ± 0.32 | 5.25 ± 0.35* | 3.95 ± 0.28 | 3.89 ± 0.26 | 3.77 ± 0.27 |
| HDL-Cholesterol (mmol/L) | 0.93 ± 0.05 | 1.09 ± 0.07* | 1.07 ± 0.06* | 1.13 ± 0.07 | 1.13 ± 0.05 | 1.14 ± 0.06 | 1.17 ± 0.11 | 1.16 ± 0.11 |
| **Substrates oxidation** | | | | | | | | |
| Energy Expenditure (kcal/min) | 1.05 ± 0.02 | 1.06 ± 0.03 | 1.06 ± 0.03 | 1.03 ± 0.04 | 1.03 ± 0.06 | 1.01 ± 0.03 | 1.04 ± 0.03 | 1.00 ± 0.03 |
| Carbohydrate oxidation (mg/kg/min) | 1.36 ± 0.16 | 1.49 ± 0.10 | 1.41 ± 0.11 | 0.76 ± 0.18 | 0.76 ± 0.20 | 1.27 ± 0.13 | 1.21 ± 0.12 | 0.99 ± 0.14 |
| Lipid oxidation (mg/kg/min) | 0.66 ± 0.09 | 0.64 ± 0.04 | 0.48 ± 0.06 | 0.48 ± 0.07 | 0.35 ± 0.11 | 0.56 ± 0.07 | 0.62 ± 0.05 | 0.38 ± 0.10* |
| Protein oxidation (mg/kg/min) | 0.65 ± 0.05 | 0.54 ± 0.03 | 0.97 ± 0.07* ¶ | 0.68 ± 1.07 | 0.90 ± 0.08* | 0.78 ± 0.15 | 0.78 ± 0.05 | 1.41 ± 0.16¶ |
| Nitrogen excretion (g/day) [b] | 13.1 ± 1.0 | 11.0 ± 0.6 | 19.5 ± 1.5* | 15.6 ± 1.1 | 20.7 ± 2.0* | 11.7 ± 2.2 | 11.7 ± 0.8 | 21.2 ± 2.3¶ |
| **$^1$H-NMR spectroscopy** | | | | | | | | |
| IHCL (vol%)[c] | 1.1 ± 0.3 | 2.2 ± 0.6* | 1.7 ± 0.6¶ | 7.8 ± 2.2 | 6.3 ± 2.1* | | | |

*Table 2 Legend*

All values are expressed as mean ± SEMs.

*Abbreviations*; control diet (**C**); high fat diet (**HF**); high-fat high-protein diet (**HFHP**); baseline condition (**B**), after whey protein supplementation (**WPS**); control diet, low protein meal (**CD-LPM**); control diet, high protein meal (**CD-HPM**); high protein diet, high protein meal (**HPD-HPM**); BMI, body mass index; HOMA-IS, Homeostatsis model assessment-insulin sensitivity; NEFA, non esterified fatty acid, BOHB, beta-hydroxybutyrate; TG, triglycerides; IHCL, intraheaptocellular lipids.

[1] Wilcoxon's paired signed-rank test adjusted with Bonferroni's correction;
[2] paired *t*-test;
[3] paired *t*-test adjusted with Bonferroni's correction ;
[a] fat mass was estimated by skinfold thickness in Study I and III and by bioimpedance in Study II.
[b] Daily nitrogen excretion was estimated from urea urinary excretion during the clamp (Study I), in 24 hours urinary collection (Study II) and during two hours baseline (Study III).
[c] To convert vol % en mmol/kg multiplies by a factor 10.1.
* significantly different from the control condition, respectively in each study, $p \leq 0.05$
¶ significantly different from the HF or CD-LPM condition, respectively for each study, $p \leq 0.05$

Accumulation of fat in the liver was a primary endpoint of assessment in Study I and II. IHCL were evaluated by $^{1}$H- NMR-spectroscopy. In both conditions, i.e. in the condition of a model of steatosis induced by a hypercaloric high-fat intake in healthy humans (Study I) or in the condition of diagnosed NAFLD in obese patients (Study II), supplementing the diet with a protein load led to a significant decrease in IHCL.

After four days of supplementing a hypercaloric high-fat diet with 1.5g/kg/protein, IHCL decreased by 22 % in healthy volunteers, whereas giving a supplementation of 60g protein/day in obese patients (~ 0.6g/protein kg/day) during one month also led to a significant 21 % reduction of steatosis. It is noteworthy that obese patients were however left on an *ad-libitum* diet, which might impact on results (this point will be discussed later in "limitations of the study"). Up until now most research has involved the metabolic effects of low-carbohydrates diets and has reported improvements in hepatic steatosis and features of the metabolic syndrome (185). Consequently Inherent indirect effects of increasing the amount/proportion of dietary protein intake cannot be excluded. However no study had directly investigated the effects of dietary protein on IHCL in humans (186). This observation might be of importance since fatty liver is recognized to be an important component of the metabolic syndrome and reduction in liver fat content might improve the metabolic syndrome.

As a potential mechanism, I first hypothesized that, consequent to the increase in energy expenditure required for the protein metabolism, lipid metabolism and lipid oxidation might be promoted. Increase in energy expenditure following meal ingestion has already been extensively reported (56, 57, 163). We did not report any effect of chronic protein supplementation on energy expenditure at baseline but we did observe a significant effect of acute protein ingestion, which increased energy expenditure 1.2 times and with chronic protein supplementation 1.7 times. Sustained elevation in postprandial thermogenesis requires a higher postprandial energy supply which was hypothesized to be provided by increased lipid oxidation.

Net and whole body lipid oxidation, as energy expenditure, was calculated from gas exchanges, using indirect calorimetry. None of the three studies demonstrated an increase in lipid oxidation at baseline or following a protein load. At baseline, even in the fasting state or

in the postprandial state, increases in protein oxidation tended to favour carbohydrate oxidation at the expense of lipids. In study I chronic protein ingestion increased protein oxidation by 49%, carbohydrate by 4% and tended to reduce lipid oxidation by 28% at baseline. WPS supplementation resulted in a significant 29% increase in protein oxidation and a significant 26% reduction in lipid oxidation. In study II lipid oxidation was significantly reduced by 33 % whereas protein oxidation increased by 81 %.
Altogether these three studies converged to indicate that whole body lipid oxidation tended to be inhibited by high protein diets.

The third study was partly designed in an attempt to trace the metabolic fate of a lipid load, i.e. exogenous fat oxidation, labelled with $^{13}C$-triolein, with or without concomitant protein ingestion. Results clearly demonstrated that exogenous fat oxidation tended to be reduced by 9 % with a protein load and that chronic ingestion of protein amplified this effect, with a decrease of 24 %. None of the results suggested that chronic protein intake improved exogenous fat oxidation.

We were also interested in hepatic endogenous fat oxidation. It has been demonstrated that fat metabolism/oxidation is highly correlated with BOHB plasma concentrations (187, 188).
Consequently we monitored BOHB plasma concentrations as an indirect marker of hepatic fat metabolism. At baseline, in fasting state, as well as in postprandial state, BOHB concentrations did not significantly increase among the different studies with the protein intakes. It has been previously reported, based on animal studies, that modulation and reduction of the insulin/glucagon ratio might favour hepatic lipid oxidation (79). In none of our studies was this ratio modified whether at baseline or even in the postprandial state.
Altogether, these data clearly do not support the hypothesis that dietary protein decreases liver steatosis by driving substrate metabolism toward lipid oxidation.

At the hepatic level, two other major key regulatory pathways might be involved in the import and the export of fat to the liver. Delivery of fat to the liver is closely related to adipose tissue metabolism. It results from the ability of adipose tissue to take up lipids from the blood, mostly by regulation of chylomicrons and/or VLDL clearance, and to release free fatty acids from adipocytes by lipolysis of adipose tissue triglycerides. Export of triglycerides from the liver mainly occurs through the secretion of VLDL-triglycerides. The three different

studies showed different aspects and led to a divergent understanding of the modulation of hepatic fat transport by proteins.

In Study I, the protein supply was administered together with a hypercaloric high fat intake. High-fat intake led to a decrease in lipolysis in adipose tissue, mirrored by a 26% decrease in fasting NEFA plasma concentrations, and led to an increased activity of adipose lipogenic genes. The addition of protein tended to further enhance these effects of lipids. High-fat intake tended to decrease plasma concentrations of VLDL-TG by 22%, which was interpreted as a reduced export of TG from the liver. As protein prevented the increase in IHCL and restored VLDL-TG plasma concentrations, it appeared plausible that protein might favour export of TG from the liver. At this stage we did not believe that protein might account for a decrease in VLDL-TG clearance.

In the third study, the same protein supplementation, but without a fat overload, did not affect fasting concentrations of NEFA or VLDL-TG in the fasting state, which supported the hypothesis that inhibition of adipose tissue lipolysis and VLDL-TG secretion might be primarily attributed to the fat load. The high protein meal led to significant ~ 3 times increase in postprandial triglycerides responses but only after short-term protein supplementation, which was first expected to be reflected by an increase in VLDL-TG secretion. Surprisingly, elevation of plasmatic triglycerides concentrations were 80% explained by an increase in chylomicron-TG whereas VLDL-TG plasmatic concentrations account for 20% of this change. This observation drove us to propose an effect of dietary protein on adipose tissue to decrease VLDL/chylomicron-TG clearance, through modulation of the activity of the lipoprotein lipase (LPL).

To our knowledge, the effects of dietary protein on LPL have never been reported before. As plasma TG concentrations are linked to an increased risk of cardiovascular disease, benefits of protein ingestion, especially in healthy, normal BMI subjects, might become critical. Observation of changes in chylomicrons-TG concentrations failed to inform about the amount of dietary fat delivered to the liver; does it increase or decrease? At this stage, this still remains an open question and raises interesting perspective for future research (will be detailed later, see perspective section), moreover as little information is available regarding the influence of the amount or nature of dietary proteins on postprandial lipid responses (189).

In the clinical study, the design of protocol and the selection of subjects were very different; subjects were obese women and food provision was uncontrolled over the month. Patients were supplied with 60g/protein per day and left otherwise on a free *ad-libitum* diet. A significant decrease of 21% in IHCL was observed, as well as a significant 15% decrease in total-triglycerides and a 7% decrease in cholesterol. As it will be discussed later (see section compliance of subjects), it is plausible that patients altered their usual diet in response to the satiating effect of protein intake and thus unconsciously decreased their daily carbohydrate and fat intakes. Indeed, they almost all reported a feeling of increased satiety with WPS protein intake.

Altogether, these results did not support the hypothesis that protein intake might decrease steatosis by increasing the export of fat from the liver by increased VLDL-TG secretion. It appears more likely that the delivery of fat to the liver was reduced, which might possibly explain the reduction of IHCL observed in the first study. This speculation will however need further investigations to be supported.

Even if our first endpoint was to assess effects of protein on hepatic fat metabolism, we were also interested in looking at modulation of insulin sensitivity by protein intake. As it was discussed in the introduction, the effects of protein on insulin sensitivity are still controversial, as infusion of amino acids have been reported to induce insulin resistance (66), whereas hypocaloric high protein diets seems to improve insulin sensitivity in obese glucose intolerant subject (70). Moreover fatty liver has been linked to the development of insulin resistance.

We did not report any effects of protein on insulin sensitivity either with the hyperinsulinemic euglycemic clamp, or with HOMA-IS indexes. For the control feeding conditions, subjects were healthy young men. Even if we were able to observe a significant decrease in IHCL concentrations after 4-day of high-fat high-protein diet, elevation of IHCL might be insignificant compared to the content of IHCL in obese patients and too fast to alter insulin sensitivity. This is in accordance with others studies from our research group, investigating the effects of hypercaloric fructose conditions, which have demonstrated that elevation of IHCL are rapidly affected (116) and that insulin sensitivity might develop later (118).

However, clearly in obese patients, an effect of improved insulin sensitivity, or glucose tolerance concomitant to a decrease in IHCL was expected. Here again, we failed to observe such an effect. First, even if reduction in IHCL and total fat mass was significant after the month of supplementation, total body weight was not reduced; therefore these changes might be too small to clearly impact on insulin sensitivity. Second, obese patients were fed on a free *ad-libitum* diet and even if they were asked to record their food intakes before the days preceding OGTT, we had not specifically assessed their usual dietary intakes. Therefore it is possible that measured fasting glycemia and insulinemia were also influenced by the previous day dietary intake.

In the metabolic syndrome, the question about what might be the central cause of this global metabolic disorder, either the fatty liver, or the adipose tissue (9) or the insidious development of insulin resistance is still debated (190). In the first definitions of the metabolic syndrome (WHO, EGIR, 1999) presence of insulin resistance and/or impaired glucose tolerance was the central determinant of the pathology (8), nowadays fatty liver becomes more involved as an initial event (191). Without pretending for an answer, the central metabolic role of the liver in regulating plasma substrate concentrations, makes this organ very susceptible to changes in dietary intakes. Short-term hypercaloric studies may therefore inform us only of the early stage of the development of features of the metabolic syndrome, and insulin sensitivity might possibly develop later.

### *6.2 LIMITATIONS*

Some limits of these studies have to be acknowledged.

The small sample of subjects (ca 7-11 in each study) always raises questions about the reliability of statistics on results. However significance in statistics on small samples is mostly attained only when a similar pattern of response is present among all the subjects. Consequently, with the usual level of a probability of error of 5%, we can be rather confident in the results.

The periods of supplementation might also be arguable, as they were chosen to be really short. It is possible that longer supplementation would have changed results, but we aimed to assess early stages of disease development. Previous reports from our research group have demonstrated that 3 days of carbohydrate or fat overfeeding are sufficient to affect metabolism (192). The question about protein supplementation is somehow different as it has been reported that 7 days are needed to reach nitrogen balance, when protein intakes have been changed (34). Assuming that point, we thought that, as for fat, 4 days of protein supplementation were enough to assess early changes in disease development.
Noteworthy the high-fat high-protein diet provided 50% calories above requirements. For ethical reasons and for the health of subjects, such diets should not be administrated in the long term. In the study III, we wanted to reproduce the same load in quantity of proteins as in Study I. Therefore, periods of supplementation were a compromise between time and effects.

A major point which is also arguable is the compliance of volunteers in such experimental design as they received food and instructions to consume diets at home but were otherwise left on their own during the period of dietary supplementation. They had to record the food consumed, and were asked to be honest by emphasizing the fact that the most important point for us was to know exactly what had been really consumed.
Hypercaloric, high-fat diets were expected to increase steatosis in the liver when strictly consumed, which was indeed observed. Another easy way to assess higher protein consumption was to estimate protein intakes from nitrogen losses in the urine. In the standardized high-protein diets, if they were strictly consumed, we expected an excretion of

nitrogen approximately 32g/day. We failed to report such an increase maybe for two reasons; we did not collect the 24 hours urine, which is necessary to assess daily nitrogen excretion, and probably a new nitrogen balance was not reached after 4 days. However nitrogen excretion calculated in urinary urea samples collected at the end of the clamp in Study I and after two hours baseline in Study III increased significantly by 49% and 80% respectively. In the study with whey protein supplementation in obese patients, as it was explained in the manuscript, we expected a 64% of change in urea production. The results observed were 15% lower, which indicates either that subjects did not take the whole protein supplementation, or likely that they restricted their usual diet as a consequence of the satiating effects of protein. Whatever the reason, we can assume that the protein intake in those patients was increased by 39%, which corresponded to a mean increase of 37 g/protein per day, and that those effects were observed already after one week and did not change during the remaining three weeks of protein supplementation.

Another confounding variable which might affect results is the proportion of energy for macronutrients in the diet. When modifying the intake of a macronutrient either the caloric content of diets is maintained but the proportion of the three macronutrients is then altered, or the proportion of macronutrients does not change but the total intake of calories is increased. Both of them present pitfalls. In the first case it makes it impossible to distinguish between effects resulting from the increase in the proportion of a macronutrient or the decrease in the two others. In the second case, it makes it impossible to distinguish if metabolic changes results from an excess energy intake or from increase in a specific macronutrient.

Consequently one may question our results; will IHCL also increase when saturated fat represent 60% of an isocaloric weight maintenance diet? Will dietary protein also affect chylomicrons-TG clearance when administrated under an isocaloric condition? As we presumed that obese patients reduced their carbohydrate and fat intakes, would the reduction in IHCL and the improvement of plasma triglycerides and cholesterol concentrations also be observed if their habitual diets were strictly maintained? Would results be significantly different if the types of protein were the same in each study?

This last question raises another critical point which may be addressed to that work. As reported in Table 1, the nature of supplemented protein differed between studies. Proteins were mostly supplemented as meat and fish in Study I, whereas it was whey in Study II and casein in Study III. Actually current literature tends to demonstrate that the nature of protein, and even more their specific content in some amino acids, impact on postprandial lipemia (193).

### *6.3 PERSPECTIVES*

All these questions underline the difficulty and impossibility of one single study to answer to complex questions dealing with human nutrition. Instead of solving questions, this work raises more questions and opens new perspectives of research.

First, the effects of protein to decrease lipoprotein clearance were not expected. We presumed that protein ingestion would increase VLDL-TG secretion, but we were really surprised to observe a decrease in chylomicron-TG clearance. The interesting question beyond this is if and how protein might modulate the transport of fat from and to the liver. The uptake of fat from the liver might be indirectly assessed by studying the kinetics of chylomicron, from their synthesis in the intestine to their uptake as chylomicron-remnants from the liver. Detection of apoB-48 has been reported to be ideal for the determination of chylomicron kinetics, being specific to chylomicrons, only synthesized in the intestine, and indicative to particle number (194). This will give insights into the effects of protein on gastric emptying, chylomicron-TG synthesis, and clearance. Labelling the incorporation of $^{13}$C-oleate in VLDL-TG, as previously described by Heath *et al* (195) will give additional information on the flux (kinetics and amount) of fatty acids from the diet into the endogenous pool. To assess more specifically the effects of protein on postprandial lipemia and export of fat from the liver, kinetics of VLDL-TG synthesis might be assessed by measuring incorporation of $^{2}H_5$-glycerol in VLDL-TG after a bolus injection of $^{2}H_5$-glycerol (196, 197).

Another point which needs however to be elucidated is whether it is the hypercaloric load of our diets or the protein content of them which impact on postprandial lipemia. It would then be interesting to follow lipoproteins kinetics in a similar experimental design, i.e. after 4-day of different protein dietary conditions; such as an isocaloric control diet, an isocaloric high protein diet, or a hypercaloric high protein diet with various nature of proteins such as casein/whey/cod or soya proteins.

An important aspect of hepatic fat metabolism which has not been addressed yet is the effects of protein on *de novo* lipogenesis. It is known that hypercaloric high-fructose consumption leads to an increase in *de novo* lipogenesis, resulting in fat accumulation in the

liver, an elevation of plasma TG concentrations, and an increased export of VLDL-TG. TG effects of a hypercaloric high fructose consumption have been extensively addressed by our research group. Therefore the question of the effect of proteins on *de novo* lipogenesis under conditions of hypercaloric fructose consumption is of particular interest for us. It might be proposed to follow the metabolism of a fructose load (0.3g/kg body weight) after 6 days of supplementation of 3g/kg body weight of fructose with or without concomitant protein supplementation. The fructose load might also be ingested with or without protein. Metabolism of a fructose load, labelled with $^{13}$C-fructose, might be allow to specifically assess the effects of protein on $^{13}$C-glucose synthesis, lactate, fructose, but also on incorporation of $^{13}$C-palmitate in VLDL-TG, or apparition of $^{13}CO_2$ in breath.

### *6.4 CONCLUDING REMARKS*

Altogether results of these three studies allow us to clearly refute the hypothesis that supplementation of proteins reduce IHCL by increasing stimulation of whole body fat oxidation, hepatic beta oxidation, or exogenous fat oxidation. The question of the effects of high protein intakes on hepatic lipid metabolism is still open and will need further investigation to be elucidated. The question of the observed decrease in IHCL induced by a high protein intake need to be clarify as it appeared, once explained by a direct effect of protein ingestion on IHCL, and once to be secondary to the satietogenic effects of protein on food intake. In parallel an important point which needs to be investigated is if the variation in hepatic TG concentrations is better explained by an increase in export of fat from the liver or by a decreased uptake of fat from the liver. The effects of protein on postprandial lipemia and lipoproteins kinetics have been little investigated up to now and might be therefore an interesting question of research, the more so that an elevation of plasmatic TG concentrations is highly linked to an increased incidence of cardiovascular disease. Thus the amount and nature of dietary proteins all affect carbohydrate and fat, but the complexity of protein metabolism is far to be solved and completely understood.

### *6.5 SCHEMATIC REPRESENTATION*

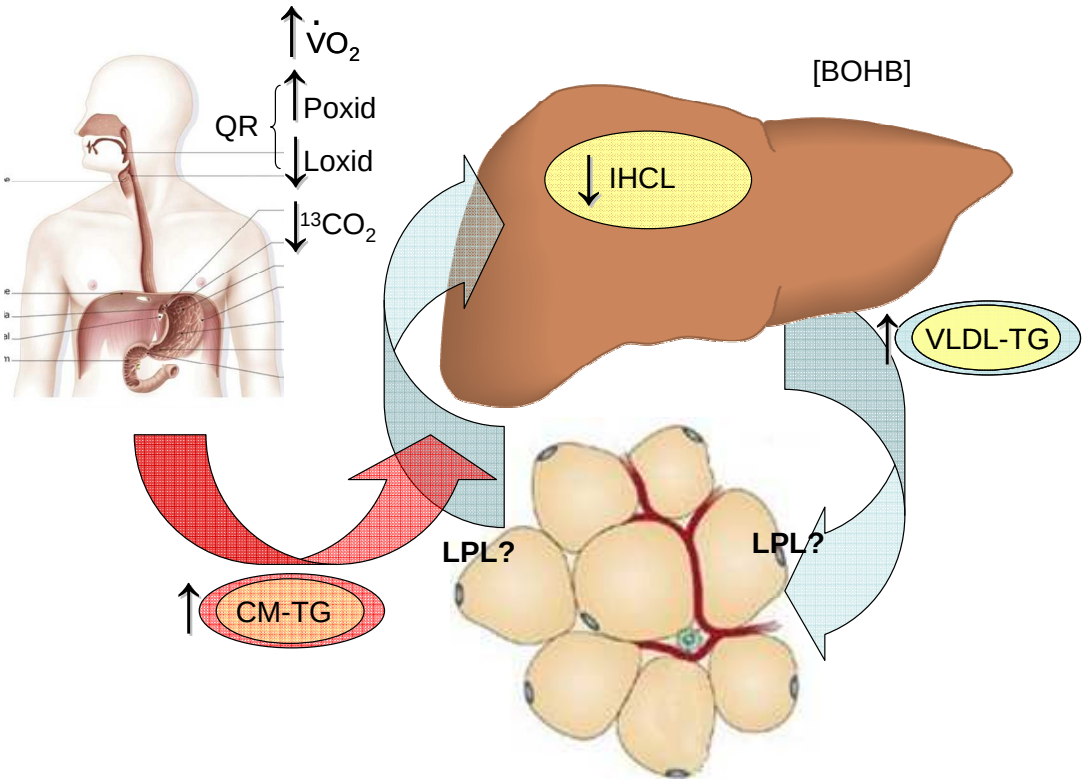


**Fig.7a: General summary of major results from Study I and III** showing effects of 4-day hypercaloric high protein intakes on postprandial lipemia with high protein ingestion. Postprandial thermogenesis is increased ($\dot{V}O_2$), as net protein oxidation (Poxid), whereas whole body lipid oxidation (Loxid), exogenous fat oxidation ($^{13}CO_2$) are decreased and hepatic beta oxidation did not change (BOHB). Chylomicron-triglycerides (CM-TG) clearance is impaired and VLDL-triglycerides (VLDL-TG) concentrations tended to increase. Consequently, the decrease in intrahepatocellular lipids (IHCL) might not be explained by a stimulation of whole-body or hepatic lipid oxidation but rather than by an altered hepatic flux of lipids, by modifying fat importation and/or exportation to the liver. Protein might affect activity of Lipoprotein Lipase (LPL). Respiratory quotient (QR)

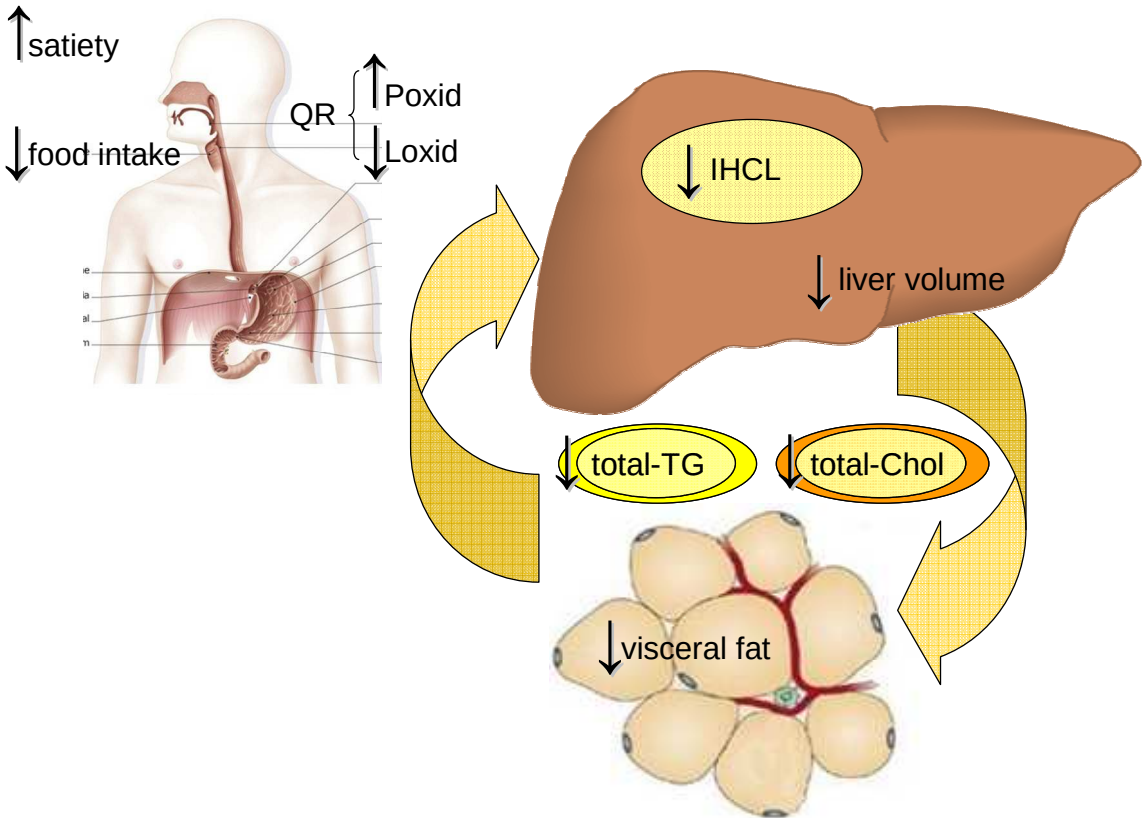


**Fig.7b: General summary of major results from Study II**, showing effects of one month of whey protein supplementation on steatohepatitis and lipemia. Net protein oxidation (Poxid) is increased, whereas whole-body lipid oxidation (Loxid) tends to decrease. Plasmatic concentration of total-triglycerides (total-TG) and total-Cholesterol (total-Chol) are decreased. Visceral fat and liver volume are positively correlated to intrahepatocellular lipids (IHCL). These metabolic effects might probably be consequent to a spontaneous decrease on food intake mediated by the satietogenic effect of protein.

# *CHAPTER 7*
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# *CHAPTER 8*
# *PUBLICATIONS*

***PUBLICATIONS***

In submission

- Bortolotti M, Schneiter P, Tappy L.*Effects of dietary protein on postprandial lipid metabolism*. In submission to *Clinical Nutrition*;

- Bortolotti M, Maiolo E, Corazza M, Van Dijke E, Schneiter P, Boss A, Carrel G, Giusti V, Lê KA, Guae Quo Chong D, Buehler T, Kreis R, Boesch Ch, Tappy L. *Effects of a whey supplementation on intrahepatocellular lipids in obese female patients.* In submission to *Diabetes Care*

Accepted for publication

- Tran Ch, Jacot-Descombes D, Lecoultre V, Fielding BA, Carrel G, Lê KA, Schneiter P, Bortolotti M, Frayn KN, Tappy L *Gender differences in lipid and glucose kinetics after ingestion of an acute oral fructose load.* BJN; 2010

- Sobrecases H, Lê KA, Bortolotti M, Schneiter P, Ith M, Kreis R, Boesch Ch, Tappy L.*Effects of short-term overfeeding with fructose, fat, or fructose+fat on plasma and hepatic lipids in healthy males*

Published

- Bortolotti M, Kreis R, Debard C, Cariou B, Faeh D, Chetiveaux M, Ith M, Vermathen P, Stefanoni N, Lê KA, Schneiter P, Krempf M, Vidal H, Boesch Ch. Tappy L. *High protein intake reduces intrahepatocellular lipid deposition in humans.* Am J Clin Nutr; 2009;

- Le KA, Ith M, Kreis R, Faeh D, Bortolotti M, Tran C, Boesch C., Tappy, L. *Fructose overconsumption causes dyslipidemia and ectopic lipid deposition in healthy subjects with and without a family history of type 2 diabetes*. Am J Clin Nutr 2009;89:1760-5

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- Bortolotti M, Tappy L, Schneiter P. *Fish oil supplementation does not alter energy efficiency in healthy males*. Clin Nutr 2007;26:225-30.

*BOOKS*

- Tappy L, Bortolotti M, Lê K-A, *Macronturients and the Metabolic Syndrome*. Food and health in the new millennium; a concise guide to the role of nutrition in health and disease. Ed. by Rossana Salerno-Kennedy and Claudia Savina. Nova Science Publishers, 2008; 5: 69-82.