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Abbreviations used: APR, Average Protein Requirement; BMI, Body Mass Index; BW, Body Weight; SG, sleeve gastrectomy; RYGB, Roux-en-Y gastric by-pass; TBW, Total Body Water; NEFA, Non Esterified Fatty Acids; GH, Growth Hormone; IGF-1, insulin-like growth factor-1; CRP, C-Reactive Protein; IL-6, Interleukin 6; GLP-1, Glucagon-Like-Peptide; FFM, Fat-Free Mass

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ABSTRACT

Context. Following bariatric surgery, protein deficiency intakes are reported in morbidly obese patients, whereas post-bariatric protein requirements are not specifically defined with validated method in this population.

Objective. To assess average protein requirement (APR) in obese subjects, before, 3 months and 12 months after bariatric surgery using the validated method of nitrogen balance.

Design and setting. Prospective longitudinal study conducted in 21 morbidly obese patients (BMI 43.9±1.4 kg/m²) before (M0), 3 months (M3) and 12 months (M12) after sleeve gastrectomy or Roux-en-Y gastric by-pass. An additional larger cross-sectional study was performed to validate APR before surgery in non-operated matched obese patients (n=106). APR was evaluated at M0, M3, M12 by measuring 3 days dietary intakes together with losses of nitrogen in urine and stools.

Main outcome measure. APR was defined as the mean value of protein intake required to achieve balance nitrogen equilibrium.

Results. Before surgery, APR in morbidly obese patients was 0.76 [95%CI, 0.66-0.92] g/kg Body Weight (BW)/d in the experimental group, and 0.74 [0.70-0.80] g/kg BW/d in the validation group. APR was 0.62 [0.51-0.75] g/kg/d at M3 and 0.87 [0.75-0.98] g/kg/d at M12, with no difference between surgical procedures. Spontaneous protein intakes were respectively 0.80±0.05, 0.43±0.03 and 0.71±0.04 g/kg BW/d respectively at M0, M3 and M12.

Conclusion. This study demonstrates a temporal change in protein requirement after bariatric surgery whatever the type of surgery. Spontaneous protein intakes following bariatric surgery does not cover protein requirements for most patients, suggesting that specific dietary protein recommandations have to be adapted in obese patients with bariatric surgery.

Key words : *obesity, nutrition, protein requirements, bariatric surgery, recovery*

TRIAL REGISTRATION clinicaltrials.gov Identifier: NCT01249326

INTRODUCTION

Bariatric surgery is considered as one of the most effective treatment of clinically severe obesity, resulting in long-term body weight loss, control or remission of comorbidities (type 2 diabetes, hypertension, cardiovascular diseases, cancer) and improved quality of life and mortality [1,2]. Following bariatric surgery, weight loss is accompanied by a substantial loss of fat and fat-free mass implying many metabolic and functional consequences like reduced resting metabolic rate, fatty acid oxidation and skeletal integrity. In relation with nutrients deficit, these modification also increase the risk of weakness, fatigability and vulnerability to critical stress in the post-operative period, suggesting that nutritional intakes, especially protein intakes are key nutrients for protecting from these deleterious outcomes. Inappropriate protein intakes after bariatric surgery might contribute to a greater loss of fat-free mass. For instance, fat-free mass has been shown to be spared with high dietary protein intakes following caloric restriction [3,4]. In bariatric surgery patients, a better lean body mass preservation was reported when protein intakes was over 60g/d [5], or when the ratio of protein to energy was higher than 20% [6]. In addition, previous studies have demonstrated severe post-bariatric protein deficiency with a very high proportion of patients having dietary protein intakes below 60g/day even 4 months after bariatric surgery [6-8]. The current guidelines for nutritional support in bariatric surgery patients state that protein intakes within meals, including supplementation, should be in the range of 60-120g/day. Many others programs recommend a range of 60 to 80 g/d of total protein intakes. However, these international recommendations are not based on studies specifically designed to assess protein requirement. Recommendations are mostly based on clinical outcomes like plasma albumin alterations after bariatric surgery: albuminemia inferior to 35 g/L occuring in patients having a poor protein intake (< 60 g/d) [9,10]. Dietary protein needs in obese patients after bariatric surgery have yet to be defined with robust, internationally recognized and validated methods such as

nitrogen balance, the only method used currently to assess average protein requirement in many different populations [11,12]. Therefore, the aim of this study was to determine the average protein requirement in obese patients using the classical nitrogen balance method before, three months and one year after bariatric surgery, in order to implement clinical data with accurate therapeutic targets aiming at limiting protein deficiency for establishing suitable recommendations for morbidly obese patients with bariatric surgery.

MATERIAL AND METHODS

The trial was a longitudinal, prospective follow-up study of protein requirement of severely obese patients candidates for a sleeve gastrectomy (SG) or a Roux-en-Y gastric by-pass (RYGB) (Experimental group, n=21). Protein requirement in severely obese patients was also calculated and validated in a large cohort of obese patients not subjected to bariatric surgery (Validation group, n=106). The study protocol was conducted in accordance with the guidelines in the Declaration of Helsinki and was approved by the Ethical Committee of the Auvergne region (agreement no. AU 817; March 2010).

Populations of Patients

Experimental group

Thirty-three obese patients planned to undergo SG or RYGB procedures were recruited from the departments of clinical nutrition and digestive surgery of Clermont-Ferrand University Hospital (France), and from the department of digestive and general surgery of Edouard Herriot Hospital (Lyon, France). Adult men and/or women aged between 18 to 60 years, with a body mass index (BMI) higher than 40 kg/m², and having obtained the agreement of the medical staff for SG or RYGB, were eligible for the study. Among the 33 patients, five patients withdrew from the study during inclusion visit (**Figure 1**). Five patients were

excluded because of taking drugs incompatible with the study (anti-inflammatory drugs). The remaining 23 patients, i.e. 16 patients scheduled for SG (13 women) and 7 patients scheduled for RYGB (5 women), were included in the study. After complete medical and biological assessment, no known neuromuscular disease, no progressive cardiovascular disease, no cancer, no uncontrolled hyperadrenocorticism and thyroid dysfunction, no severe infection or acute/chronic inflammatory disease in the 3 months prior to inclusion, and no treatment by corticosteroids, immunosuppressives, anabolics, growth hormone were detected. Included patients were not smoking more than 10 cigarettes per day, not drinking more than 2 alcoholic drinks per day and not practicing physical activity more than 5 hours per week. One patient operated on SG suffered from post-operated complications and dropped out 3 months after surgery. One patient had to be operated again on RYGB 10 months after SG and dropped out before the end of the study. Overall 21 patients were included in analysis for the entire study (Figure 1).

Validation group

The patients (n = 106) belonging to the *Validation group* were morbidly obese with a BMI > 40 kg/m². They were hospitalized in the clinical nutrition department of Clermont-Ferrand University Hospital (France) from October 2012 to December 2014 for the initial treatment of their weight management. All participants underwent a medical assessment which included biological analysis, anthropometric data, body composition assessment, evaluation of resting energy expenditure through indirect calorimetry. Exclusion criteria were chronic renal failure with a creatinine clearance < 60 ml/min using the Modification of Diet in Renal Disease (MDRD) equation [13].

Study protocol

After inclusion, patients were investigated during three periods: before (M0), 3 months (M3) and 12 months (M12) after surgery. For each period, two nitrogen balance measurements (nitrogen intakes – nitrogen losses) were performed at two different levels of protein intakes ("high" and "low") at 15-day intervals. Each nitrogen balance lasted 3 days and were spaced by 6 days (d1 to d3 and d10 to d12). The day following the first nitrogen balance (d4), patients came to the laboratory for parameters measurement. Patients were weighted to the nearest 0.1 kg with the use of manual-weighing scale (Seca 709; Seca, Hamburg, Germany). Height was measured to the nearest 0.5 cm with the use of a standardized wall-mounted height board. The metabolic, hormonal and inflammatory status of the patients were analysed from a blood sampling performed in the fasting state.

Body composition (fat and fat-free mass) was evaluated with total body water (TBW) measurement using whole body deuterium water dilution. Resting energy expenditure was measured using indirect calorimetry (Deltatrac®, Datex Instrument, Geneva, Switzerland).

Nitrogen intakes

Experimental group. For each nitrogen balance, nutritional intakes were assessed using a 3-days food survey and Nutrilog software. Daily protein intakes in grams (gP) was converted to nitrogen intakes (gN), (1g of N corresponds to 6.25g of protein). The 3-days average nitrogen intakes were performed to calculate global daily nitrogen intakes. At d4, for each study period, the dietitians have analysed protein intakes of the first nitrogen balance. The food diary was started one day before the first nitrogen balance and was purchased at the last day of the second nitrogen balance to evaluate protein intakes compliance. If the dietitians considered these protein intakes as "low" (i.e. protein intakes < 0.8 g/kg/d), they advised the patient to increase their protein intakes of at least 0.20 g/kg/j for the next 9 days, including the period of the second nitrogen balance evaluation. If protein intakes at the first nitrogen

balance period was considered as “high” (i.e. protein intakes > 1.2 g/kg/d), the patients had to decrease their protein intakes of at least 0.20 g/kg/j for the next 9 days, including the period of the second nitrogen balance evaluation.

Validation group. Estimation of daily protein intakes was provided for each patient by 5 day-food records analysis previously to the patient stay in the department of clinical nutrition. A mean of daily protein intakes was then calculated for each patient, expressed per kg of body weight and then converted in nitrogen intakes. Then nitrogen loss measurements were based on urinary nitrogen loss and estimated losses using the accurate data from the experimental results.

Nitrogen excretion

Experimental group. Simultaneously with the 3-days food survey, nitrogen losses were assessed using a 3-day urines (3x24h) and 2-day stools collections (2x24h). Each day, a urine sample of 10 mL was collected from pooled urine after stirring and stored at -20°C for later use. 24-hour stools were collected in plastic bags, placed in sealed plastic boxes and stored at -20 °C for later use. Just before analysis, 24-hour feces were mixed and a sample was used for analysis. Concentration of nitrogen was measured by chemiluminescence (Antek 7000, Alytech, Juvisy Sur Orge, France) in urine samples and by refractometry method in faeces samples. Averages of 3-day nitrogen losses in urine and 2-day nitrogen losses in stools were calculated. Because of the extreme difficulty in measuring miscellaneous nitrogen losses, these sources were estimated at 0.008 g of nitrogen per kg of body weight per day as previously described [14].

Validation group. The relationship between 24-hour urinary urea and total nitrogen losses from the experimental group study was checked to estimate nitrogen balance in morbidly

obese patients and to establish a new specific predictive equation of total nitrogen loss from daily urinary urea.

Nitrogen balance-Protein requirement

Nitrogen balance was calculated from the difference between average daily nitrogen intake and total nitrogen loss for each period of measurement [11,12]. Average protein requirement (APR) - i.e. average protein amount required to maintain a neutral nitrogen balance for a given population - for obese people before and after bariatric surgery, was calculated from the linear regression of 24- hour nitrogen balance related to protein intake in grams per kg of body weight per day [11,12].

Blood parameters

Venous blood samples were collected for metabolites, hormones and inflammatory factors analyses. For triglycerides (TG), total-cholesterol (T-Chol), HDL-cholesterol (HDL-C), non esterified fatty acids (NEFA), glucose, insulin, albumin, prealbumin, adiponectin, leptin, growth hormone (GH), insulin-like growth factor-1 (IGF-1) and C-Reactive Protein (CRP) analysis, blood samples were collected into serum tubes and placed at room temperature for 20 minutes before centrifugation. For Interleukin 6 (IL-6), Tumor necrosis factor α (TNFα), unacylated and acylated ghrelin, blood samples were collected in EDTA-plasma tubes. For acylated ghrelin, para-hydroxymercuribenzoic acid in HCl (PHMB, 1mM in the final sample volume) was immediately added to plasma to prevent degradation of acylated ghrelin by protease. For Glucagon-like peptide-1 (GLP-1) collection, EDTA-plasma tubes with dipeptidyl peptidase IV inhibitor were used. After collection, all blood samples were centrifuged for 10 min at 4 °C at 4500 rpm. Plasma samples were immediately stored

at -80 °C until analysis. For each metabolic exploration day, a total of 30 ml of blood was sampled.

Metabolites, hormones and inflammatory factors were measured by standard methods. Plasma glucose concentrations was determined using spectrophotometric method with hexokinase and G-6-PDH reagents (Abbott Diagnostics, Rungis, France) on an Architect® analyzer (Abbott Diagnostics, Rungis, France). Insulin concentration was measured using immunoreactive method with Bi-Ins-IRMA kit (Cisbio Bioassays, IBA, Gif/Yvette, France). Adiponectin and leptin were determined by enzyme immunoassay kits (ELISA): Quantikine (R&D Systems, Abingdon, United Kingdom) and Biovendor (Heidelberg, Germany) respectively. T-Chol, TG and HDL-C concentrations were determined using spectrophotometric methods on an Architect® analyzer (Abbott Diagnostics, Rungis, France) with Abbott reactifs (Abbott Diagnostics, Rungis, France). LDL-Cholesterol (LDL-C) was calculated using Friedewald equation (*Friedewald WT, 1972, Clin Chem, 4337382*). NEFA concentration was determined via an enzymatic method (Wako® Chemicals GmbH Neuss, Deutschland) on a Pentra 400 analyzer (Horiba ABX, Montpellier, France). Albumin and pre-albumin concentrations were determined using immunoturbidimetry on an analyzer Architect C8000 (Abbott Diagnostics, Rungis, France), with respectively antiserum reagents Albumin and Prealbumin (Diagam, Ghislenghien, Belgique). GH and IGF-1 were measured by immunoradiometry with respectively the kit HGH-RIACT and IGF1-RIACT (Cisbio Bioassays, Codolet, France). Acylated and unacylated ghrelin were measured using SPIBio ELISA kits: Ghrelin (human acylated) EIA Kit and Ghrelin (human unacylated) EIA Kit (Bertin Pharma, Montigny Bretonneux, France). Total GLP-1 was measured by EDI™ Total GLP-1 ELISA kit (Epitope Diagnostics, San Diego, CA USA). Ultrasensible CRP was determined by immunonephelemetry on an analyzer BN prospec (Global Siemens Healthcare Headquaters, Erlangen, Germany), with antiserum reagent CardioPhase hs CRP (Siemens

Healthcare Diagnostics Products GmbH, Marburg, Allemagne). TNFα and IL-6 concentration were measured using ultrasensible ELISA kits: respectively Human TNF-alpha/TNFSF1A Quantikine HS ELISA Kit: HSTA00D and Human IL-6 Quantikine HS ELISA Kit: HS600B (R&D Systems, Minneapolis, MN, USA).

Body composition

Body composition was assessed by total body water (TBW) measurements using deuterium water dilution [15]. $^{2}H_2O$ (99.9 atom %) was obtained from Eurisotop® (Saint-Aubin, France). The labeled water was sterilized before being drunk by the patient. After collection of baseline urine samples, an oral dose of deuterium oxide (0.07 g/ kg BW diluted in 40 mL of Evian water) was measured and administered to each participant. Then urine samples were collected 4 and 5 hours after deuterium water intake. All urine samples were stored at -20°C until completion of deuterium analysis by isotope ratio mass spectrometry. The fat-free mass compartment was calculated assuming a hydration level of 73.2%. Fat mass was obtained by difference with body weight and expressed as a percentage of body mass.

Resting energy expenditure

Measurements of O_2 consumed and CO_2 expired volumes were performed by indirect calorimetry (Deltatrac®, Datex Instrument, Geneva, Switzerland) over a period of 40 min, at rest, in thermoneutral conditions.

Statistics

Descriptive data, nitrogen intakes and losses are expressed as mean ± standard error of the mean (SEM). Statistical analysis for these data were conducted using NCSS 10 data analysis

software. A repeated measures analysis of variance was used to assess the evolution over time of body weight, body mass index, body composition, resting metabolic rate, blood parameters, nitrogen intakes and losses. When a significant effect was detected, differences among individual means were assessed with Dunnett's two-sided multiple-comparison test with control post-hoc test to determine the difference. The threshold of statistical significance was set at $P<0.05$. Average protein requirements were expressed as mean [95% confidence interval]. Statistical analysis for APR were performed using Stata software, version 13 (StataCorp, College Station, TX, US). Linear regression between protein intakes and nitrogen balance was used to interpolate for APR determination. To take into account between and within subject variability due to several measures for a same subject, random-effects models for correlated data were performed rather than usual statistical tests which would be not appropriate due to the hypothesis of independence data not verified. The normality of residuals from these models was studied using the Shapiro-Wilk test. Then, the predicted values and 95% confidence interval were estimated according to these statistical models.

RESULTS

Patients characteristics

In the experimental group, 15 women and 6 men were included with a mean age of 43.2±2.2 years. Patients characteristics before and after bariatric surgery are shown in **Table 1**. At M3, patients lost a mean of 21.9% of fat mass and 10.3% of fat free mass. At M12, patients continued to loose weight with an additional mean loss of body weight of 15.3±1.7 kg ($P<0.05$ vs 3 months). The additionnal loss of fat mass and fat free mass at M12 in comparison to M3 was respectively 12.3±2.1kg and 3.5±1.1kg. Biological parameters, including glycemic, lipid, hormonal and inflammatory parameters, were significantly improved at M3 and M12 (**Table 2**).

In the validation group, 106 patients were included with a mean age of 45.6±1.3 years. The mean weight of the patients was 119.9±2.4 kg with a mean BMI of 43.4±7.1 kg/m². Body fat was 50.5±2.3kg and fat-free mass was 67.2±2.6kg. The resting metabolic rate in these patients was 2044±62 kcal/d. Fasting plasma glucose was 5.2±0.2 mmol/l, fasting plasma insulin was 18.7±1.4 mUI/l and the HOMA-IR index was 4.6±0.5. Plasma albumin was 37.5±0.3 g/l and prealbumin was 0.245±0.005 g/l.

Nitrogen intakes and losses before, 3 months and 1 year after bariatric surgery

Before surgery, nitrogen intakes (12.31±0.65 g/d) were equilibrated with nitrogen losses (12.70±0.64 g/d) resulting in a not significantly different from zero and neutral mean nitrogen balance (-0.85±0.58 g/d) (**Figure 2**). At M3, nitrogen intakes were significantly reduced compared to the baseline intakes (9.47±0.63 g/d; 3 months after vs before surgery, $P<0.05$). Nitrogen losses were also significantly reduced compared to the nitrogen losses at M0 (9.13±0.54 g/d; 3 months after vs before surgery, $P<0.05$). Mean nitrogen balance was equilibrated for most of the patients (-0.05±0.45 g/d) at M3. At M12, nitrogen intakes increased significantly compared to M3 (11.28±0.62 g/d, $P<0.05$). Nitrogen losses were not significantly increased compared to M3 (10.07±0.80 g/d, P=NS), leading to a mean nitrogen balance close to zero but positive (0.14±0.54 g/d) (Figure 2).

Determination of average protein requirement

In the experimental group, the equation of the relationship between protein intakes and nitrogen balance was used to determine minimum protein intake to reach nitrogen equilibrium, and consequently average protein requirement (APR) for three periods: M0, M3 and M12 (**Figure 3**). In the experimental group, at M0, APR was 0.76 [95%CI, 0.66-0.92]

g/kg BW/d, a value not significantly different from the value obtained in the validation group (n=106 obese patients), i.e. 0.74 [0.70-0.80, *P*=NS] g/kg BW/d (**Figure 4**).

A strong relationship between 24 hours urinary urea (mmol/24h) and total urinary nitrogen losses (g/24h) in the experimental population was established (r^2=0.974) with urea consisting in 86±6% of total urinary nitrogen. So, the equation for estimating total urinary nitrogen loss from urinary urea in this specific population was:

Total urinary nitrogen loss = (0.031 x urinary urea) + 0.43

where total urinary nitrogen loss is expressed in g/24h, urinary urea in mmol/24h. Then, to get an estimation of fecal nitrogen loss, an average of 8% of dietary nitrogen intake (~1g nitrogen/24h) can be applied, added with miscellaneous losses of 0.008g nitrogen/kgBW/d. Finally, when this new formula (urinary+fecal+miscellaneous nitrogen losses) was applied to the validation population, the protein requirement calculation was not different from that obtained after using McKenzie equation (0.79 [95%CI, 0.75-0.84] vs 0.74 [95%CI, 0.70-0.80] g/kg/d, p=NS). Thus, this simplified calculation can be used for the estimation of nitrogen balance in many other clinical conditions including morbidly obese patients.

At M3, APR was measured at 0.62 [95%CI, 0.51-0.75] g/kg/d (Figure 3B) and at M12, APR was 0.87 [95%CI, 0.75-0.98] g/kg/d (Figure 3C).

Spontaneous protein intakes were estimated at 93.21±21.59 g/d (0.80±0.21 g/kg/d), 45.57±16.41 g/d (0.46±0.13 g/kg/d) and 58.01±14.23 g/d (0.71±0.18 g/kg/d) respectively at M0, M3 and M12 after bariatric surgery.

DISCUSSION

This study is the first one to determine protein requirement in morbidly obese patients before, 3 and 12 months after surgery using a validated method such as nitrogen balance. Current protein recommendations after bariatric surgery are based on very few studies and do not rely

on nitrogen balance at two nitrogen levels, whereas it is considered as the reference method for the assessment of protein needs in humans [16]. The most recent recommendation was established by AACE/TOS/ASMBS guidelines in 2013 and set at 60 g/d [17]. This recommendation was graded as D since there were not enough data to support the evidence and no consensus on the outcomes. Other proposed recommendations were 60 to 120 g/d in 2012 [18], > 60 g/d [5], or 90 g/d [19] after gastric by-pass. All these recommendations were based on hypoalbuminemia (< 35 mg/l) [18] or lean tissue mass retention [5]. No data have been reported on the mean protein requirement of morbidly adult obese using nitrogen balance method. This method remains the most robust, solid approach recognized by WHO for determining protein requirement. This method can be easily applied to patients with obesity and following bariatric surgery and allows exploration of the protein balance in the medium and long term, although it has some important shortcomings [14, 20-22]. Alternative methods like indicator amino acid oxidation technique could have been used to evaluate protein requirements [23,24] but these must be adapted to the specificities of protein metabolism in obesity and after bariatric surgery, which still have to be fully investigated. Nitrogen balance measurement is criticized because nitrogen intakes are often over-estimated and nitrogen losses underestimated. In this study, nitrogen intakes were assessed using a 3-day food survey. As obese patients usually tend to underestimate their food intakes, it would lead potentially to an underestimation of the protein requirement. APR of morbidly obese patients at M0 (*experimental group*) was estimated as 0.76 [95%CI, 0.66-0.92] g/kgBW/d. To confirm these data in a larger cohort of morbidly obese patients (*validation group;* n=106), another cross-sectionnal study based on a single measurement approach was performed: a similar value of 0.74 [0.70-0.80] g/kgBW/d for average protein requirement was obtained confirming that APR of 0.76 g/kg/d for morbidly obese patients is higher than the APR proposed for non-obese healthy adults. In the 2007 FAO/WHO/UNU report [12], protein requirement is given as

0.66 g/kg/d, with an approximately 95% confidence interval of 0.63 to 0.69 g/kg/d. Considering a higher body weight consisting in a larger fat mass, a lower APR could be expected. The higher APR per kg body weight in morbidly obese patients could be explained by a higher fat free mass in absolute value, but even when expressed per kg of lean mass in the obese patients, it is still different and higher than in the non-obese populations : it was 1.48 [95%CI, 1.29-1.79], 1.13 [95%CI, 0.93-1.37] and 1.43 [95%CI, 1.24-1.62] g/kgFFM/d, respectively at M0, M3 and M12, whereas extrapolations in non-obese adults considering FFM, protein requirement would be close to 1 g/kgFFM/d (considering 70 to 75% of FFM in non-obese adults and 0.66 g/kg/d as average protein requirement). This important observation confirms that in obese patients, protein requirement, whatever the expression mode, is higher than in non-obese adult population. Considering the difficulties to evaluate FFM in morbid obese patients, it would be thus more relevant and appropiate to express average protein requirement by body weight. On a metabolic point of view, the increased protein demand in morbid obesity could be related to metabolic alterations such as inflammation, insulin resistance, oxidative stress, which induced organs and tissues metabolic impairements and an increase in the production and the activity of a lot of proteins implicated in these perturbations [25].

At M3, average protein requirement decreased to 0.62 [95%CI, 0.51-0.75] g/kg/d with no differences considering the types of surgery. This might be due to the massive changes in body composition after surgery, but also to the metabolic adaptations to low protein-energy intakes. Besides its significant effect on weight loss, bariatric surgery allows the improvement of obesity-related comorbidities, reduction of insulin-resistance and low grade inflammation [26-28] which might contribute to a better protein efficiency compared to the metabolic state previous to surgery. Despite the massive weight loss during the first three months, a neutral nitrogen balance was found likely explained by the strong reduction of nitrogen losses in

parallel with reduction in nitrogen intakes confirming adaptation of the body to lower nitrogen intakes [29,30]. Nevertheless, the nitrogen balance method does not predict the orientation of metabolic fluxes (protein synthesis and degradation, interactions between muscle and splanchnic area) underlying the limitation of the method. Only few authors have evaluated changes in protein turnover after massive weight loss [31] which mainly explain body composition changes. After vertical banded gastroplasty, protein breakdown in patients decreased 3 months after surgery with no change in protein synthesis compared to before surgery, illustrating a slower protein turnover [32]. After bariatric surgery, whatever the types of surgery, the susceptibility for protein malnutrition is very realistic not only as a result of massive weight loss, but also because the patients cannot consume several foods rich in protein [8,33]. Nutritional deficiences in obese patients after bariatric surgeries have been pointed out, notably for protein intakes [8,33]. In Verger et al. study, mean protein intakes 3 months after surgery were 0.38 g/kg/d after gastric bypass and 0.39 g/kg/d after sleeve gastrectomy [33]. Interestingly, Giusti et al. have observed a significant decrease in meat and vegetarian protein intakes at 1 mo after a Roux-en-Y gastric bypass and remained low until 1 y after surgery[34]. In our study, spontaneous protein intakes of the patients at M3 were 0.43±0.03 g/kg/d whatever the types of surgery. Unfortunately no distinction of protein source could be made. These values are inferior to the protein requirement evaluated 0.63±0.38 g/kg/d. In addition, 86% of our obese population do not reach the APR at M3. These observation emphasized the need of nutritional assessment and protein support of these patients after weight reducing surgery, especially during the phase of rapid weight loss.

At M12, the average protein requirement (0.87 [95%CI, 0.75-0.98] g/kg/d) increased compared to the one observed at M3. Whereas weight and fat mass continued to decrease, the loss of FFM has been classically reported to be stabilised one year and up to at least 3 years after bariatric surgery [33,34]. The maintenance of FFM should involved an adaptation of protein

metabolism. Only a few data is available on the evolution of protein metabolism after bariatric surgery. Tamboli et al. have observed higher 24h-urinary levels of 3-methylhistidine 12 months following bariatric surgery, illustrating an increase in muscle protein breakdown [35]. In patients following a Roux-en-Y gastric bypass, urinary urea nitrogen excretion increased 12 months after surgery[34]. Alongside these changes, protein synthesis might also increase to balance the elevation in protein degradation and nitrogen losses in order to ensure the slowing down of FFM loss. Even if the mechanisms of protein metabolism regulation in bariatric surgery have yet to be elucidated, an adaptation of protein metabolism must be seriously considered to explain the higher level of protein requirement at 12 months after surgery.

Some studies indicate that the maintenance of weight loss is achieved among subjects assigned to a higher protein proportion in the diet (25% of energy consumed) after weight loss induced by a low-calorie diet [36]. A mean daily of protein intake of 64.1±2.9 g/d (58.01±14.23 g/d in our study) at 12 months following bariatric surgery has been reported in a previous cohort of 50 patients. This study has shown that a larger protein intake is associated with a better preservation of lean mass after bariatric surgery even 12 months postoperatively [5].

In addition, loss of fat free mass is associated with weight regain [37] due to a diminished resting metabolic rate [38], but also due to the triggering effect of fat free mass loss to promote an increased energy intake in an attempt to restore FFM to an optimal level as refered to the so called “collateral fattening” concept [39]. This suggests the importance to adapt protein intakes to the needs of the patients after surgery to avoid notably excessive loss of FFM. The quality of dietary protein is also of importance for FFM improvement as we demonstrated recently beneficial effects of soluble milk proteins to preserve muscle and lean body mass in old subjects [40,41].

In conclusion, the present study demonstrated an increased protein requirement in severe and morbid obese subjects. Since most of obese patients spontaneously do not reach the protein

intake targets, it is particularly relevant to reconsider protein intake in the monitoring of morbidly obese subjects in pre- and post-operative periods, especially with regard to potential a protein deficit with underestimated consequences such as sarcopenia or immune deficit or post-operative complications. Further studies investigating the impact of protein supplementation in patients receiving bariatric surgery with definite protein targets should then be proposed. The estimation of protein requirements in patients with morbid obesity and following bariatric surgery needs to be precise and validated by similar and alternative methods in order to address new specific recommandations for severely and morbidly obese patients for the regular monitoring of this population, but also on a long term basis after bariatric surgery.

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CG contributed to the design of the study, data analysis, data interpretation, and writing of the report. AM contributed to the design of the study, study oversight, recruitment of participants, data collection, biological analysis, data analysis, data interpretation, and writing of the report. AMD, AB and NF contributed to study oversight, recruitment of participants, data collection. BP contributed to statistical analysis, data interpretation, and writing of the report. KS and MR contributed to the recruitment of participants and surgical procedures. ED and ML contributed to the design of the study, the recruitment of participants, study oversight, data analysis, and data interpretation. PLR contributed to the data interpretation. CL, MM and NF contributed mainly to the data collection and interpretation of the "Validation group" and to the recruitment of participants, study oversight, and data interpretation of the "Experimental group". YB had the idea for the study and contributed to the design of the study, study oversight, data interpretation and writing of the report. All authors read and approved the final manuscript.

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TABLES AND FIGURES

Table 1. Body composition characteristics before, 3 months and 12 months after bariatric surgery.

	Baseline	M3	M12
Body weight (kg)	119.1±5.1	100.0±5.1*	84.8±5.4*#
Body mass index (kg/m²)	43.9±1.4	36.8±1.5*	31.0±1.6*#
Body fat mass (kg)	57.5±3.2	44.9±3.5*	32.6±3.7*#
Body fat mass (%)	48.4±1.3	44.6±1.4*	37.6±2.5*#
Body fat free mass (kg)	61.1±3.1	54.8±2.7*	51.4±3.0*#
Body water (L)	44.7±2.3	40.1±2.0*	37.6±2.2*#
Resting metabolic rate (kcal/d)	1953±97	1657±90*	1549±86*#

Data are mean±SEM (based on isotopically labeled water measurements).
Baseline: before; M3: 3 months after surgery; M12: 12 months after surgery
* significant differences vs baseline, $P<0.05$
# significant differences between M12 and M3, $P<0.05$

Table 2. Biological characteristics of the patients before, 3 months and 12 months after, bariatric surgery.

	Baseline	M3	M12
Fasting plasma glucose (mmol/L)	6.1±0.3	5.3±0.2*	5.1±0.2*
Fasting plasma insulin (mUI/L)	14.3±2.2	7.0±1.2*	5.4±1.1*
HOMA-IR index	4.0±0.7	1.7±0.3*	1.3±0.3*
Triglycerides (mmol/L)	1.37±0.10	1.08±0.08*	0.99±0.08*
Total-cholesterol (mmol/L)	4.88±0.25	4.20±0.19*	4.60±0.16#
HDL-cholesterol (mmol/L)	1.14±0.05	1.08±0.03	1.41±0.06*#
LDL-cholesterol (mmol/L)	3.12±0.24	2.62±0.19	2.87±0.23
NEFA (µmol/L)	627.9±43.7	737.3±48.1	483.2±58.9#
Albumin (g/L)	40.2±0.6	40.4±0.7	39.7±0.7
Prealbumin (g/L)	0.25±0.01	0.20±0.01*	0.23±0.01*
Adiponectin (µg/mL)	5.2±0.7	6.7±0.6	10.5±1.1*#
Leptin (ng/mL)	53.4±4.1	23.4±3.2*	18.7±4.4*
Acylated ghrelin (pg/mL)	17.0±3.5	8.1±3.1	23.9±7.6#
Unacylated ghrelin (pg/mL)	132.6±17.8	94.0±29.1*	102.3±21.5
IGF-1 (µg/L)	161.2±12.2	136.2±15.9*	165.6±14.4#
GH (mUI/L)	1.5±0.5	5.3±1.4	14.2±3.5*#
GLP-1 (µg/mL)	1.78±0.42	1.30±0.19	0.92±0.15
High sensitive C-reactive protein (mg/L)	6.63±1.32	6.38±2.55	2.70±1.00
IL-6 (pg/mL)	2.36±0.29	2.13±0.20	1.35±0.16*#

Data are mean±SEM
Baseline: before; M3: 3 months after surgery; M12: 12 months after surgery
IGF-1: Insulin Growth Factor-1; GH: Growth Hormone; GLP-1: Glucagon Like peptide 1; IL-6: Interleukine-6
* significant differences vs baseline, $P<0.05$
# significant differences between M12 and M3, $P<0.05$

Figures

Figure 1. Flow chart of the study

Figure 2. Nitrogen intakes, losses and balance before, 3 months and 1 year after bariatric surgery. *M0 : before surgery ; M3 : 3 months post-surgery ; M12 : 1 year post-surgery.*

Figure 3. Relationship between nitrogen balance and protein intakes, and determination of average protein requirements, A) before, B) 3 months and C) 1 year after bariatric surgery. Vertical red lines indicated average protein requirements (g/kg of body weight/d) expressed as mean (value in the middle) [95% confidence interval] (values on both sides of the average).

Figure 4. Determination of average protein requirement in an additional group of obese patients for the validation of protein requirement in a larger population. Vertical red lines indicated average protein requirements (g/kg of body weight/d) expressed as mean (value in the middle) [95% confidence interval] (values on both sides of the average).

Figure 1.

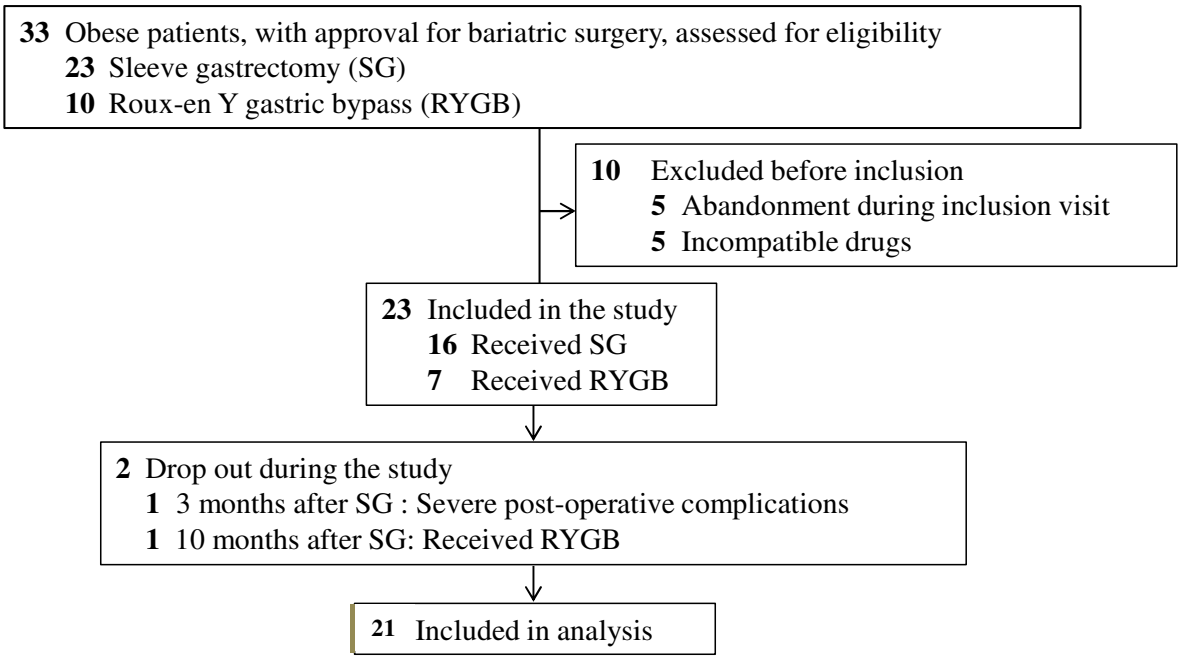

33 Obese patients, with approval for bariatric surgery, assessed for eligibility
23 Sleeve gastrectomy (SG)
10 Roux-en Y gastric bypass (RYGB)
10 Excluded before inclusion
5 Abandonment during inclusion visit
5 Incompatible drugs
23 Included in the study
16 Received SG
7 Received RYGB
2 Drop out during the study
1 3 months after SG : Severe post-operative complications
1 10 months after SG: Received RYGB
21 Included in analysis

Figure 2.

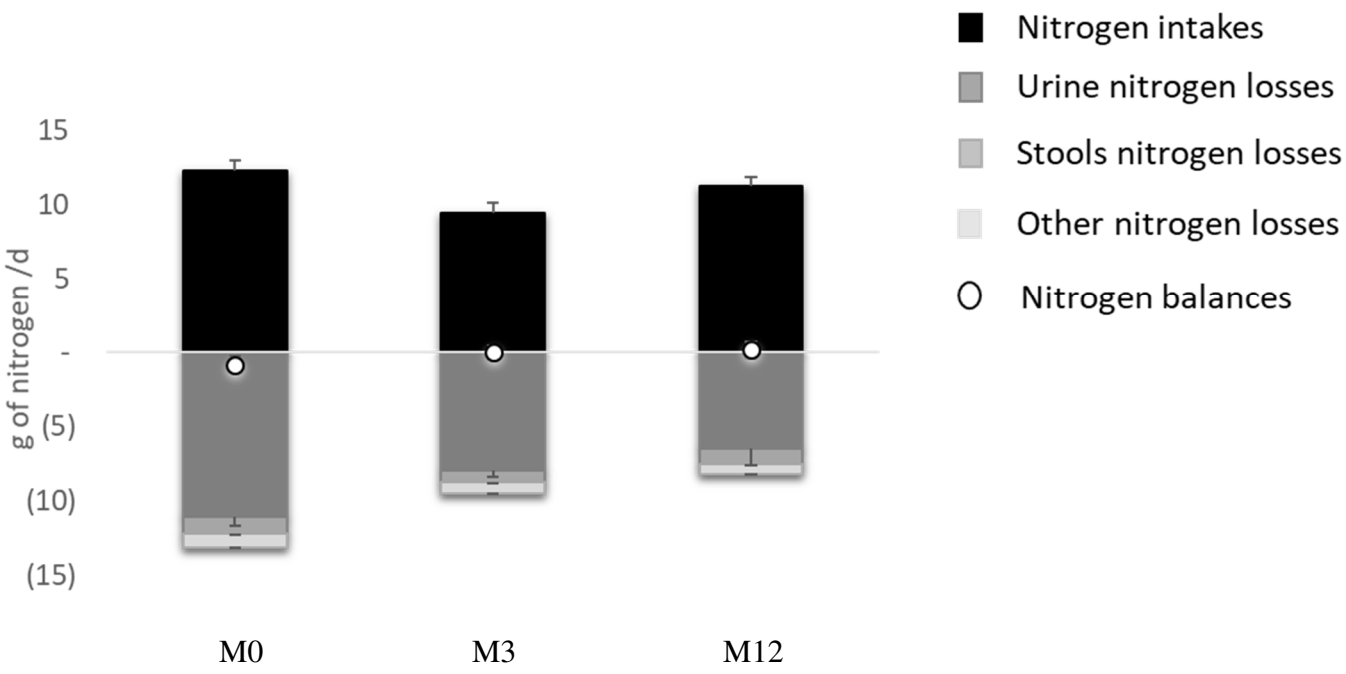
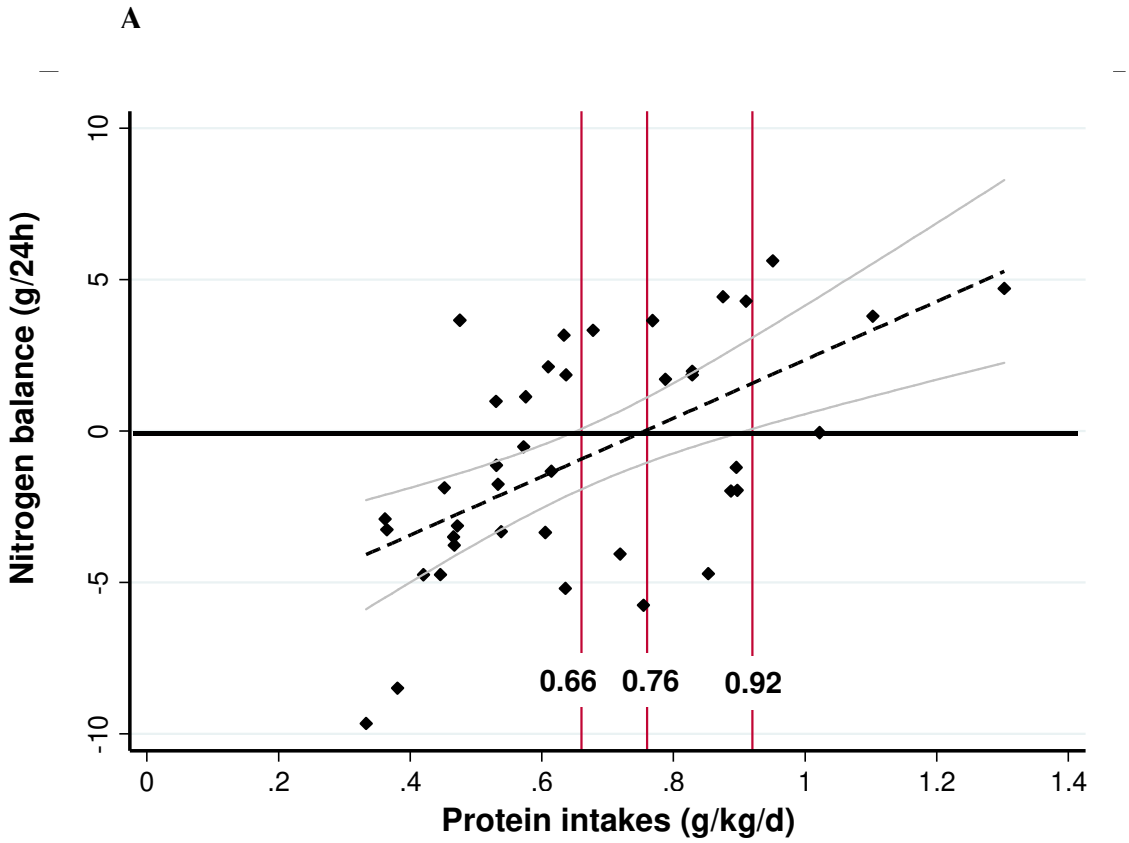
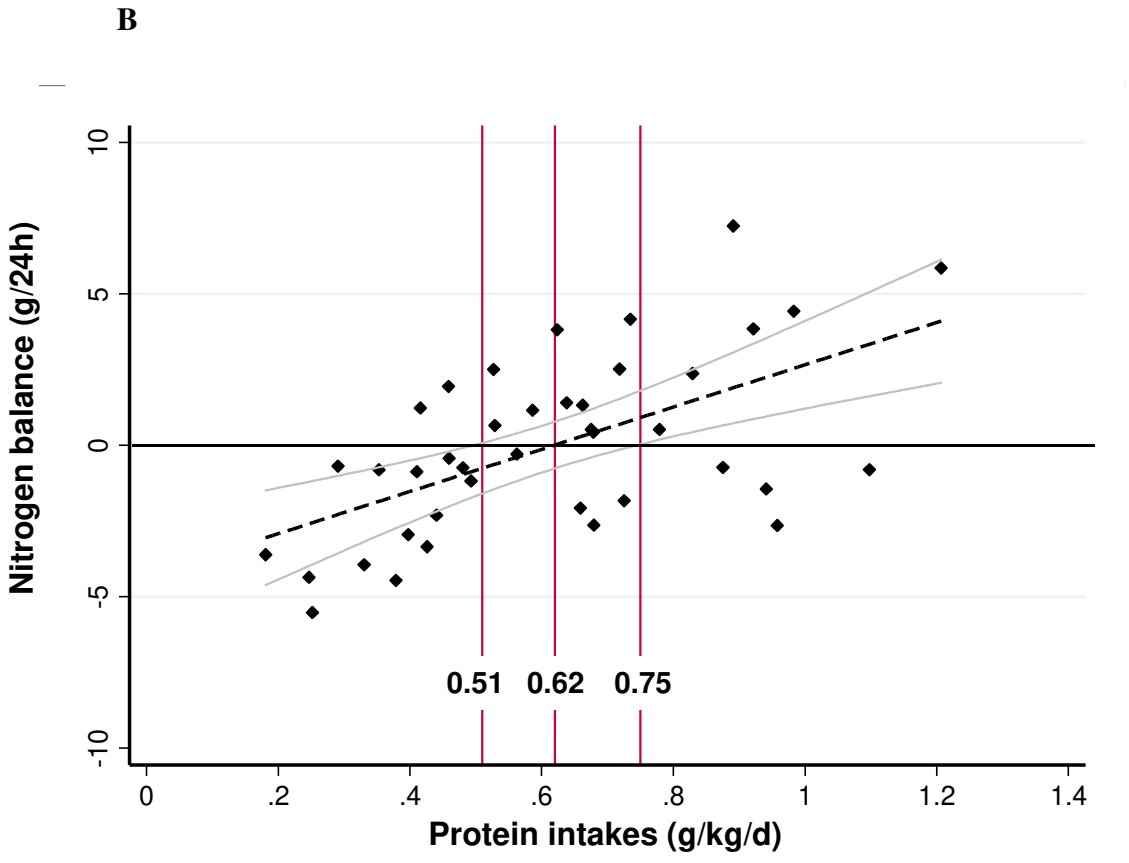

Nitrogen intakes
Urine nitrogen losses
Stools nitrogen losses
Other nitrogen losses
Nitrogen balances
g of nitrogen /d
15
10
5
-
(5)
(10)
(15)
M0
M3
M12

Figure 3.


A
Nitrogen balance (g/24h)
10
5
0
-5
-10
0.66
0.76
0.92
0
.2
.4
.6
.8
1
1.2
1.4
Protein intakes (g/kg/d)


B
Nitrogen balance (g/24h)
10
5
0
-5
-10
0.51
0.62
0.75
0
.2
.4
.6
.8
1
1.2
1.4
Protein intakes (g/kg/d)

C

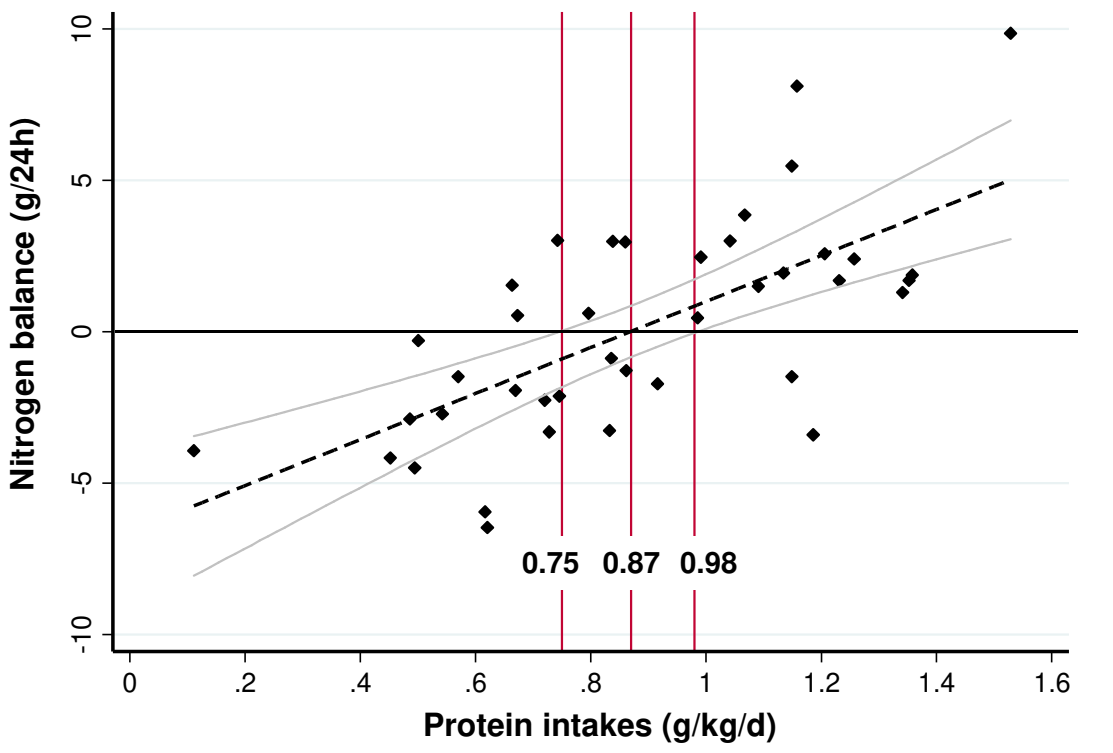
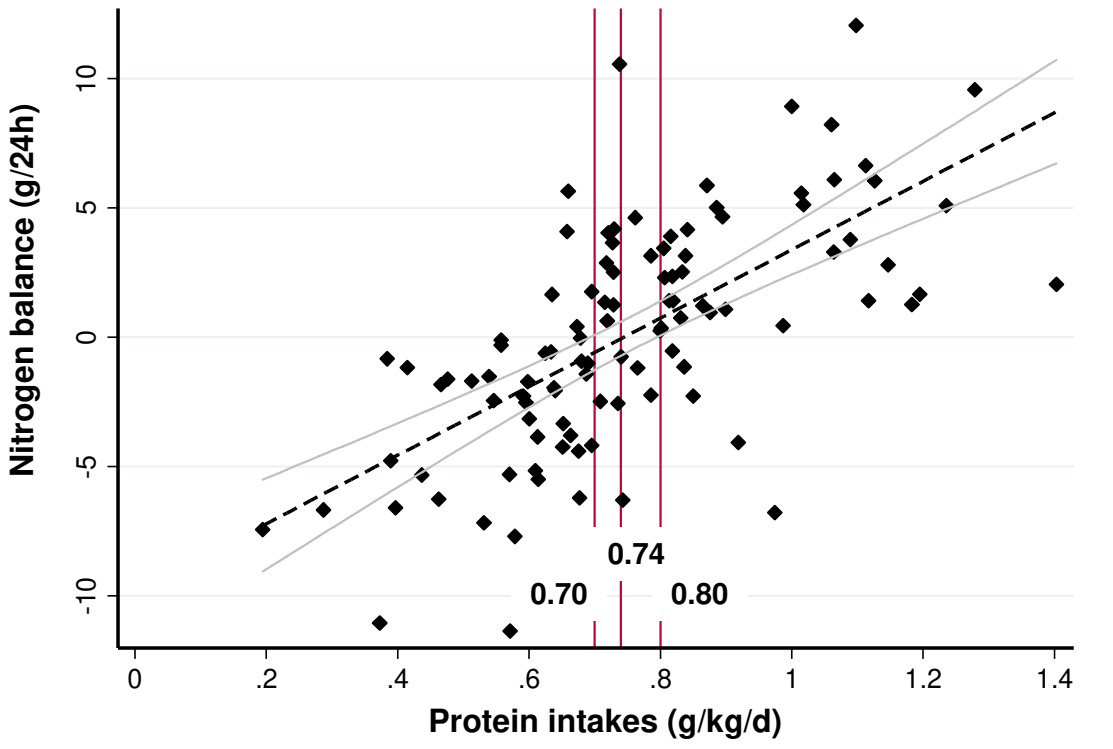

Nitrogen balance (g/24h)
10
5
0
-5
-10
0.75
0.87
0.98
0
.2
.4
.6
.8
1
1.2
1.4
1.6
Protein intakes (g/kg/d)

Figure 4.


Nitrogen balance (g/24h)
10
5
0
-5
-10
0.74
0.70
0.80
0
.2
.4
.6
.8
1
1.2
1.4
Protein intakes (g/kg/d)