Soy protein and isoflavones: their effects on blood lipids and bone density in postmenopausal women[1–3]

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ABSTRACT The effects of soy protein (40 g/d) containing moderate and higher concentrations of isoflavones on blood lipid profiles, mononuclear cell LDL receptor messenger RNA, and bone mineral density and content were investigated in 66 free-living, hypercholesterolemic, postmenopausal women during a 6-mo, parallel-group, double-blind trial with 3 interventions. After a control period of 14 d, during which subjects followed a National Cholesterol Education Program Step I low-fat, low-cholesterol diet, all subjects were randomly assigned to 1 of 3 dietary groups: Step I diet with 40 g protein/d obtained from casein and nonfat dry milk (CNFDM), Step I diet with 40 g protein/d from isolated soy protein containing 1.39 mg isoflavones/g protein (ISP56), or Step I diet with 40 g protein/d from isolated soy protein containing 2.25 mg isoflavones/g protein (ISP90). Total and regional bone mineral content and density were assessed. Non-HDL cholesterol for both ISP56 and ISP90 groups was reduced compared with the CNFDM group ($P < 0.05$). HDL cholesterol increased in both ISP56 and ISP90 groups ($P < 0.05$). Mononuclear cell LDL receptor mRNA was increased in subjects consuming ISP56 or ISP90 compared with those consuming CNFDM ($P < 0.05$). Significant increases occurred in both bone mineral content and density in the lumbar spine but not elsewhere for the ISP90 group compared with the control group ($P < 0.05$). Intake of soy protein at both isoflavone concentrations for 6 mo may decrease the risk factors associated with cardiovascular disease in postmenopausal women. However, only the higher isoflavone-containing product protected against spinal bone loss. *Am J Clin Nutr* 1998; 68(suppl):1375S–9S.

KEY WORDS Soy, isoflavones, genistein, cholesterol, bone density, postmenopausal women, HDL cholesterol, LDL receptor mRNA, bone mineral content

INTRODUCTION

The reduction of blood total and LDL cholesterol concentrations with the consumption of products containing soy protein has been shown repeatedly in humans and various animal models (1–3). The component of the soy-protein products responsible for these changes has yet to be defined. Recently, some have postulated that isoflavones may be responsible.

Isoflavones are present in relatively large amounts in virtually all products containing soy protein with the exception of soy-protein concentrates and isolates that have undergone alcohol extractions during processing (4). Isoflavones are known to be estrogen analogues and bind to estrogen receptors, eliciting an affinity of 7×10^{-6} to 8×10^{-4} that of estradiol (5). Thus, it is logical that if the isoflavones possess actions similar to estrogens, they could influence several biological processes including lipid and bone metabolism. Because postmenopausal women are at risk for health problems related to estrogen deficiency, such as cardiovascular disease and osteoporosis, consumption of soy products containing isoflavones might affect risk factors for these diseases. We designed the present study to examine the effect of soy protein containing different concentrations of isoflavones on blood lipid profiles, mononuclear cell LDL receptor messenger RNA (mRNA), and bone density in postmenopausal women.

SUBJECTS AND METHODS

We are reporting observations from a human intervention trial in which a number of variables were assessed (6). A brief description of methods will be given here.

Subjects

Sixty-six hypercholesterolemic, postmenopausal women completed the study and were included in the statistical analyses. Subjects were screened for initial total cholesterol concentrations (between 6.21 and 7.76 mmol/L), were interviewed, and completed health surveys to assess their appropriateness for inclusion as subjects in the study. Subject selection excluded those receiving any medications known to alter lipid, bone, or calcium metabolism, including hormone replacement therapy within the past 6 mo; those who had a menstrual period <12 mo before initiation of the study protocol; and those with any systemic or endocrine disease known to affect lipid, mineral, or bone metabolism. Subjects were asked not to take any vitamin or

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mineral supplements for the duration of the investigation. This study was approved by the Institutional Review Board of the University of Illinois at Urbana-Champaign.

Study design and diet

Before initiation of the study protocol, subjects were placed on a low-fat (<30% of energy), low-cholesterol (<300 mg/d) diet (National Cholesterol Education Program Step I diet) as instructed by a registered dietitian (7). All subjects followed this basal diet for ≥2 wk, at which time they were randomly assigned to 1 of the 3 dietary treatment groups that provided 40 g protein/d from one of the following: isolated soy protein containing moderate concentrations of isoflavones (ISP56; Supro 675, Protein Technologies International, St Louis), isolated soy protein containing higher concentrations of isoflavones (ISP90; Protein Technologies International), or casein and nonfat dry milk (CNFDM; New Zealand Milk Products, Wellington, New Zealand). Both isolated soy proteins were fortified with calcium (calcium phosphate) to amounts comparable with those found in casein. The study lasted 26 wk: 2 wk for the basal lead-in period and 24 wk for the intervention period.

Test proteins were incorporated into a variety of food items including breads, muffins, drinks, milks, and soups. Breakfast was provided 3 d/wk and subjects were instructed to consume food items totaling 40 g test protein/d. Food intake and activity diaries were obtained every 4 wk and consumption diaries for food items containing test proteins were obtained daily. Body weight was measured weekly throughout the study and nutrient intake was analyzed by using a nutrient database (NUTRITIONIST IV, version 3.0; N-Squared Computing, Salem Park, OR).

Blood lipid analyses

On 2 separate days at the end of the 2-wk adaptation period (baseline) and every 6 wk for the duration of the 24-wk study, fasting blood samples were collected into tubes containing either heparin or EDTA. HDL was separated immediately by heparin-manganese precipitation (8) and plasma samples were stored at −70°C in separate portions for subsequent analyses. Total plasma cholesterol, HDL, and total triacylglycerols were quantified by using automated techniques (Hitachi 704 Auto Analyzer; Boehringer Mannheim Corporation, Indianapolis) and commercially available kits (Boehringer Mannheim; Sigma Diagnostics, St Louis; and Raichem, San Diego). The accuracy of plasma measurements was verified by use of either Centers for Disease Control and Prevention or International Federation of Cereal Chemists quality-control plasma samples of known concentrations (Northwest Lipid Research Laboratories, Seattle). Non-HDL concentrations were calculated by subtracting HDL from total cholesterol.

LDL receptor mRNA analysis

Mononuclear cells were isolated as described by Lovati et al (9). Because of the labor required for the isolation process, mononuclear cell LDL receptor mRNA was analyzed for 38 of the 66 subjects (n = 15 for ISP56, n = 12 for ISP90, and n = 11 for CNFDM) at baseline and after 12 and 24 wk of the study. Selection of subjects for this analysis was random and based on subjects' willingness to provide additional blood. Total cellular RNA was prepared from mononuclear cells by using the single-step acid guanidinium thiocyanate-phenol-chloroform method (10). Reverse transcription–polymerase chain reaction was used to quantitate concentrations of LDL receptor mRNA (11). Specific details are given by Baum et al (6).

TABLE 1
Characteristics of subjects[1]

Variable	Baseline
Age (y)	
ISP56	59.8 ± 9.1 (49–73)
ISP90	61.2 ± 10.3 (39–83)
CNFDM	61.3 ± 6.3 (51–74)
Body mass index (in kg/m^2)	
ISP56	28.2 ± 6.0 (16.6–40.4)
ISP90	26.2 ± 4.6 (20.5–40.6)
CNFDM	29.1 ± 5.2 (22.6–40.8)
Body weight (kg)	
ISP56	74.1 ± 15.6 (43.5–109)
ISP90	68.5 ± 14.4 (49.4–110)
CNFDM	78.0 ± 13.2 (59.6–111)
Years since menopause (y)	
ISP56	12.2 ± 9.7 (0.6–32.6)
ISP90	13.7 ± 8.3 (1.9–31.0)
CNFDM	12.6 ± 8.5 (1.3–30.1)
Total cholesterol (mmol/L)	
ISP56	6.6 ± 0.9 (5.5–8.6)
ISP90	6.5 ± 0.9 (5.6–9.1)
CNFDM	6.3 ± 0.7 (5.4–7.5)
HDL cholesterol (mmol/L)	
ISP56	1.3 ± 0.3 (0.7–2.0)
ISP90	1.4 ± 0.3 (0.9–2.0)
CNFDM	1.4 ± 0.3 (0.8–2.2)
Non-HDL cholesterol (mmol/L)	
ISP56	5.2 ± 0.9 (4.1–7.5)
ISP90	5.1 ± 1.0 (3.9–7.7)
CNFDM	4.9 ± 0.8 (3.2–6.4)
Triacylglycerol (mmol/L)	
ISP56	1.9 ± 1.0 (0.8–4.6)
ISP90	1.7 ± 0.7 (0.8–3.4)
CNFDM	1.8 ± 1.1 (0.7–5.7)

[1] $\bar{x}$ ± SD; range in parentheses. n = 66. ISP56, isolated soy protein with moderate isoflavones; ISP90, isolated soy protein with higher isoflavones; CNFDM, casein and nonfat dry milk.

Bone measurements

The Bone mineral content and density of the lumbar spine (L1–L4), the proximal femur (including the femoral neck and Ward's triangle), and the total body was measured by dual-energy X-ray absorptiometry (DXA; Hologic QDR-2000, Waltham, MA) at the end of the lead-in period and then again after the 24-wk intervention period. Regions of interest for the spine and femur were defined according to the manufacturer's guidelines. All measurements were made and analyzed by the same 2 experienced operators; the in vivo precision error in this laboratory for bone mineral density was 1% for the total body, 1.3% for the spine, and 1.8% for the hip.

Statistical analysis

The effects of dietary intervention on various outcomes were evaluated by using multiple linear regression analyses. Treatment effects were indicated by using 2 dummy-coded variables, one comparing the ISP90 and CNFDM diets and the other comparing the ISP56 and CNFDM diets. Treatment by covariate

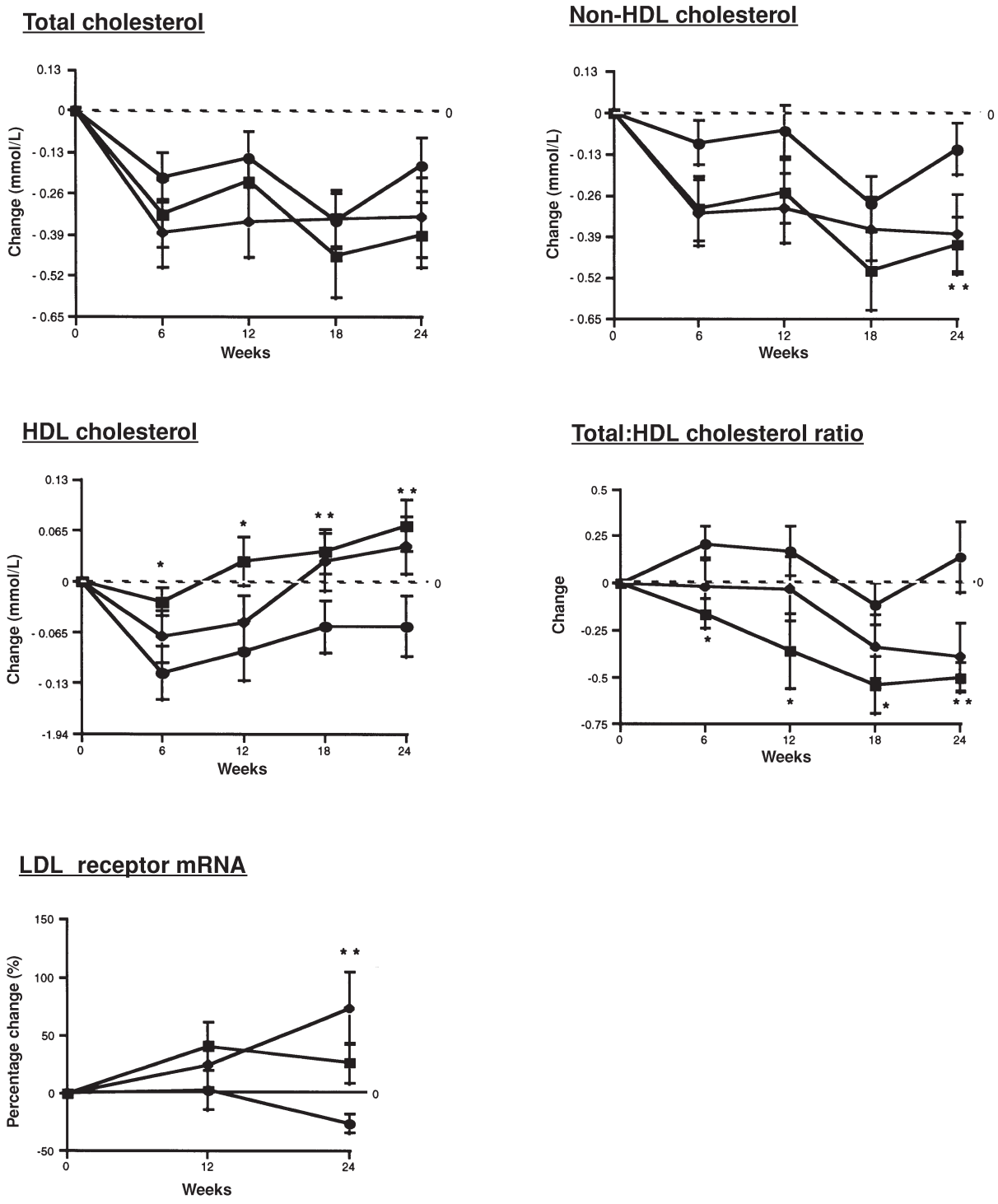

FIGURE 1. Mean (±SD) changes in blood lipids and LDL receptor messenger RNA (mRNA) expression over the 24-wk treatment period in subjects consuming isolated soy protein with moderate isoflavones (ISP56, ■), isolated soy protein with higher isoflavones (ISP90, ◆), and casein and nonfat dry milk (CNFDM, ●) (n = 22). ** ISP56 and ISP90 significantly different from CNFDM, $P < 0.05$. * ISP56 significantly different from CNFDM ($P < 0.05$).

interaction effects were tested as described by Weigel and Narvaez (12). Covariates for the bone analysis were used as baseline values of the outcome variables: body weight, age, body fat, and years since menopause. If no significant interaction effects were detected, the interaction terms were removed from the model. The temporal onset of effects for blood lipids was detected sequentially by testing for the presence of significant treatments effects first at 24 wk posttreatment and then proceeding backwards to test for significant effects at 18, 12, and 6 wk, proceeding to the earlier time in sequence only when significant effects had been identified at each later time. Changes from baseline within each group were evaluated by using paired t tests. All sta-

TABLE 2
Lumbar spine (L1–L4) bone mineral content and density of subjects completing the study[1]

Treatment group	Content	Density
	g/cm	*g/cm²*
ISP56		
Week 0	55.5 ± 11.8	0.971 ± 0.145
Week 24	55.1 ± 11.7	0.969 ± 0.143
ISP90		
Week 0	49.6 ± 8.3	0.892 ± 0.114
Week 24	50.8 ± 8.7[2]	0.912 ± 0.119[2]
CNFDM		
Week 0	56.0 ± 11.0	0.940 ± 0.159
Week 24	55.4 ± 10.3	0.934 ± 0.153

[1] $\bar{x}$ ± SD. n = 66. ISP56, isolated soy protein with moderate isoflavones; ISP90, isolated soy protein with higher isoflavones; CNFDM, casein and nonfat dry milk.
[2] Significantly different from CNFDM subjects, $P < 0.05$.

tistical procedures were conducted with the STATISTICAL ANALYSIS SYSTEM (version 2.1; SAS Institute, Cary, NC). An α level of 0.05 was used in all statistical tests.

RESULTS

Baseline characteristics of the subjects are shown in **Table 1**. Subjects consumed the products containing test proteins without difficulty. Mean changes in blood lipid as well as LDL receptor mRNA concentrations are shown in **Figure 1**. Although total cholesterol was not altered by dietary treatment, HDL cholesterol increased starting at week 6 in the ISP56 group and at week 18 in the ISP90 group ($P < 0.05$), and non-HDL cholesterol decreased at week 24 ($P < 0.05$) in both soy-protein groups compared with the CNFDM group. The ratio of total to HDL cholesterol was improved with respect to cardiovascular risk in both soy-protein groups at 18 and 24 weeks (Figure 1; $P < 0.05$). Dietary treatment did not influence total triacylglycerol concentrations (data not shown). In subjects consuming either ISP56 or ISP90, LDL receptor mRNA concentrations increased significantly at 24 wk compared with concentrations in control subjects (Figure 1; $P < 0.05$). Of the skeletal sites tested, lumbar-spine bone mineral content and density increased significantly at the end of the 24-wk treatment period in the ISP90 group (**Table 2**; $P < 0.05$). No significant changes were noted in bone mineral density or content in total-body or other skeletal sites.

DISCUSSION

Results from this study indicate that soy protein is effective in modulating the risks of both cardiovascular disease and osteoporosis in postmenopausal women. Interestingly, the amount of isoflavone consumed had little effect on blood lipid variables but was a factor in bone measurements. In fact, the ISP56 diet group, with the moderate concentration of isoflavones, had significantly improved blood lipid profiles before 24 wk whereas the ISP90 group, receiving higher concentrations of isoflavones, did not show significant improvement until later in the study (18–24 wk). The reason for this may be that the dietary concentration of isoflavones needed to affect lipid metabolism is different from that needed to influence bone metabolism. The possibility also exists that the cholesterol-lowering component of soy is not or is only partially related to isoflavones.

The cholesterol-lowering effect of soy protein was not as pronounced in women in our current study (≈6% reduction in total cholesterol and ≈7% reduction in non-HDL cholesterol) as was reported previously. We (13) reported an 11–12% reduction in total- and LDL-cholesterol concentrations in mildly hypercholesterolemic men consuming 50 g soy protein/d. In a subsequent study, we found that 25 g soy protein/d produced a 5–6% reduction in total cholesterol in men (14). Our prior work is consistent with the findings from the meta-analysis performed by Anderson et al (2) of 38 clinical trials, in which most subjects were men.

Differences between our current findings in postmenopausal women and previous findings in men could be due to differences in responsiveness to soy between the sexes. The fact that we did observe a significant increase in HDL cholesterol, a finding typically not present in men consuming soy protein, may indicate that part of the response to soy protein in women is related to isoflavones and their interaction with estrogen receptors. However, we did not observe significant decreases in total cholesterol or significant increases in total triacylglycerols, which are common responses to estrogens given to postmenopausal women (15).

Our findings that ISP90 produced significant increases in bone mineral content and density in the spine was of interest for 2 reasons. First, of all skeletal sites measured, the spine is the area that is thought to be the most sensitive to estrogen because of its higher content of trabecular bone. The spine is remodeled more rapidly than is the hip, which contains a higher proportion of cortical bone (16, 17). Second, although we had hypothesized that the isoflavone-containing soy-protein diets would delay the decrease in bone density compared with that for the control diet, we found that there was a slight increase in bone density and mineral content (2%), an intriguing finding. However, this is a short study with respect to bone and these findings need to be confirmed by longer studies (eg, 2–3 y).

In conclusion, our data suggest that isolated soy protein at either concentration of isoflavones used in this study may be protective against cardiovascular diseases by altering lipoprotein profiles in postmenopausal women. Furthermore, there may be a possible protective role of isoflavones on bone maintenance. Unfortunately, many women in the United States either cannot or will not comply with standard hormone replacement therapy, which is the therapy of choice for prevention and treatment of cardiovascular disease and osteoporosis in this population. Thus, it is possible that the addition of soy products containing isoflavones to the diet may provide a viable alternative mode of therapy in improvement of health in postmenopausal women.

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Nutrition Column

Soy Protein

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Abstract

Soy protein comes from soybeans and offers multiple health benefits, some of which are just beginning to be discovered. This column reviews the health benefits of soy products with a special focus on women and children's health. To date, little has been written or researched that is directly related to perinatal health. Thus, the column has a more broad focus so that childbirth educators have a general resource to gain knowledge related to the use of soy-based foods.

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Soy protein has received increased attention in recent years among consumers, researchers, and the media. A report released in 1995 estimated that over 12,000 food products were available that contained soy protein (Anderson, Johnstone, & Cook-Newell, 1995), and sales of soy beverages rose more than 82% in 1999 (Nestle, 2002). A recent study from Europe found that individuals with a habitually health-conscious lifestyle (e.g., individuals who did not eat meat, but did eat fish, or were vegetarians or vegans) were more likely to consume soy foods than the average person (Keinan-Boker et al., 2002). The sample included 35,955 persons, from ages 35–74 years, who completed a 24-hour dietary recall interview. From this sample, 195 men and 486 women reported consuming soy products in the last 24 hours.

The purpose of this column is to review the benefits of soy protein and to discuss what populations are likely to benefit from an intake of soy protein. Very little information is available regarding the use of soy protein foods during pregnancy, postpartum, or infancy. Therefore,

this column offers a more broad nutritional focus on soy protein with relevant information related to perinatal health interspersed throughout.

Soy Basics

Soy protein refers to the protein that is found in soybeans that is often used to replace animal proteins in an individual's diet. The soybean is a legume that contains no cholesterol and is low in saturated fat (Lindsay & Claywell, 1998). Soybeans are the only vegetable food that contains all eight essential amino acids (Dudek, 2001; Morrison & Hark, 1999). Soybeans are also a good source of fiber, iron, calcium, zinc, and B vitamins (Lindsay & Claywell, 1998).

Benefits of Soy for Health Promotion

Pregnancy

Use of soy products during pregnancy can be encouraged because expectant women are likely to receive the same health benefits as other women. Fortified milk and fortified soymilk are the only reliable dietary sources of vitamin D (Somer, 2002). All other dairy products contain little or no vitamin D. While many women will obtain enough vitamin D from exposure to sunlight, soymilk may be an alternative for those who are overly sensitive to the sun or for those who simply are not able to be or do not enjoy being outdoors. Soymilk may also be an alternative for women who do not like regular milk.

Cardiac

Consumption of soy protein in place of animal protein has been found to reduce serum concentrations of total cholesterol, low-density lipoproteins (LDLs), and trigylcerides (Arliss & Biermann, 2002; Morrison & Hark, 1999). The precise mechanism of action is not known, though several theories exist (Dudek, 2001). One theory proposes that cholesterol absorption is impaired or altered (Dudek, 2001). Another theory postulates that phytoestrogens (plant compounds that have hormone-like effects; isoflavones are the phytoestrogens found in soy products; see Table) bind to estrogen receptors and produce similar effects including lowering LDLs and increasing high-density lipoproteins, vasomotor tone changes, and arterial wall function (Dudek, 2001). Individuals with elevated cholesterol seem to receive the greatest benefit (Hasler, 2002).

Table Definitions of Soy-Related Terms

Isoflavones—Phytoestrogens found in soy products.

Lignan—Phytoestrogens from grains (flax seed).

Phytochemical—A chemical that is found in plants.

Phytoestrogens—Plant compounds that have hormone-like effects in the body.

Individuals need to consume about 25 grams of soy protein or more each day to obtain results (Wardlaw, 2000). Twenty-five grams of soy protein equals 1¼ cups of tofu, 1–2 cups of soymilk, or an ounce of soy flour. Individuals are encouraged to read food labels in order to verify a particular food's soy content. The U.S. Food and Drug Administration (FDA) approved the health claim for the relationship between soy product consumption and reduced risk of coronary heart disease in 1999, based on the result of human clinical intervention trials (Hasler, 2002). While the FDA has approved the claim of health benefits, Munro and colleagues (2003) conducted a meta-analysis of the current literature and found that the literature supports the safety of isoflavones because they are typically consumed in soy or soy products.

Obesity and Diabetes

In recent studies, soy protein contributed to the control of hyperglycemia and reduced body weight, hyperlipidemia, and hyperinsulinemia (Bhathena & Velasquez, 2002). These characteristics may be useful to both nondiabetic and diabetic persons in the control of obesity and blood sugar.

Cancer Prevention

Genistein, one of the phytochemicals found in soy, can reduce the risk of cancer (Wardlaw, 2000). To date, prevention of breast cancer has received the most attention, and more recent attention has focused on prostate cancer (Whitney & Rolfes, 2002). Genistein blocks cancer development by preventing tumors from creating blood vessels that would provide nourishment for growth (Arliss & Biermann, 2002; Wardlaw, 2000). One serving a day (e.g., 1 cup of soymilk, ½ cup of tofu or soybeans) is effective for cancer prevention (Wardlaw, 2000).

Menopausal Symptoms

Phytoestrogens are currently being researched to determine their usefulness in acting like synthetic estrogen to protect women from bone loss and maintain a healthy heart (Wardlaw, 2000). Soy protein has been found to positively influence bone and calcium balance in postmenopausal women (Arjmandi et al., 2003). Results were especially significant for women who were not receiving hormone replacement therapy. These same results were not seen in young, healthy women who were still menstruating (Anderson et al., 2002).

Benefits of Soy for Special Populations

Vegetarians and Vegans

Vegetarians are individuals who, for various reasons, do not eat meat. Vegans, in comparison, are individuals who do not eat any products from animals, including eggs, milk, and cheese. Vitamin B_{12} is only found in animal products and, therefore, may be lacking in the diet of vegans. Use of soymilk is one way to obtain this essential vitamin (Dudek, 2001). Cereals and meat substitutes are other options.

Infants with Special Conditions

Infants born with lactase deficiency or galactosemia benefit from the use of soy-based formulas (Dudek, 2001). Parents who wish to put their newborn on a vegetarian diet may choose to use a soy-based formula. In addition, infants who are recovering from episodes of diarrhea (and are normally given breast-milk substitutes) may have soy formula recommended to facilitate their recovery. Soy-based breast milk substitutes (formulas) include Prosobee (Mead Johnson) and Isomil (Ross). While soy-based formulas meet an infant's growth and development needs, they do not offer any advantage over milk-based formulas (Whitney & Rolfes, 2002).

Infants who are not able to tolerate lactose formulas (those based on cow's milk, casein/whey-based formulas; e.g., Similac, Enfamil, Carnation) may be prescribed soy-based formulas if they are not breastfed (Wardlaw, 2000). Each year, about 20%–25% of infants are converted to soy protein formulas (American Dietetic Association and Dieticians of Canada [ADA], 2000). The development of lactose-free cow's milk protein-based formulas has made it unnecessary to switch infants to soy-based formula (ADA, 2000), though the practice is still common. The use of soy-based formula is effective in only about 20%–50% of infants because the soy protein eventually triggers a reaction in susceptible infants (Wardlaw, 2000). In this instance, predigested protein formulas can be used (e.g., Nutramigen, Alimentum). According to the ADA (2000), soy-based formulas are not recommended for preterm infants weighing less than 1,000 grams and for infants with low birth weight as a means for preventing or managing colic or gastroenteritis.

Preschool Children

In a recent study, ingesting soy-based formula or soymilk was associated with peanut allergy in a geographically diverse sample of 13,971 preschool children (Lack, Fox, Northstone, Golding, & the Avon Longitudinal Study of Parents and Children Study Team, 2003). The authors proposed that the association of peanut allergy with the intake of soy products could be related to cross-sensitization through a common substance (Lack et al., 2003). More research is needed in this area.

Summary

Soy protein products offer benefits to women in various life stages. Benefits include improved diet and cardiovascular status, prevention of certain types of cancer, improved health following menopause, obesity prevention/control, and more options for food variety. The area of soy protein research has increased in popularity in recent years among multiple health disciplines. Future research efforts are likely to include more scientific advances in the use of soy in the diet of Americans. As more is learned about the health benefits of soy, additional foodstuffs will likely be available to meet the community's needs for soy products.

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Soy Protein Has a Greater Effect on Bone in Postmenopausal Women Not on Hormone Replacement Therapy, as Evidenced by Reducing Bone Resorption and Urinary Calcium Excretion

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Recent reports suggest that soy protein may reduce the risk of osteoporosis in peri- and postmenopausal women. The objective of this study was to examine whether soy supplementation exerts beneficial effects on serum and urinary biomarkers of bone metabolism in postmenopausal women, regardless of whether or not they are on hormone replacement therapy (HRT). A total of 71 women were randomly assigned to either soy protein (SP) or milk-based protein (MBP), 40 g daily for 3 months, in a double-blind parallel design. Forty-two women completed the study (20 on SP and 22 on MBP). Overall, both protein supplements positively influenced serum IGF-I, known to correlate with bone formation. However, SP had a more pronounced effect on IGF-I than MBP. Urinary deoxypyridinoline (Dpd) excretion, a specific biomarker of bone resorption, was significantly reduced by SP, but not by MBP when all women were included. Furthermore, women on MBP experienced a 33% increase in urinary calcium excretion, whereas SP did not have such an effect. To evaluate whether SP affects women differently on the basis of their HRT status, data from women on HRT (n = 22) and those not on HRT (n = 20) were analyzed separately. The subanalysis of the data indicated that SP had the greatest impact on serum IGF-I (an increase of 97%) in the women not on HRT. The changes in urinary Dpd due to SP were only observed in women not on HRT, indicating that the overall decrease in Dpd occurred with SP in the absence of HRT. These results indicate that soy protein may positively influence bone and calcium homeostasis in postmenopausal women, particularly those not on HRT. (*J Clin Endocrinol Metab* 88: 1048–1054, 2003)

THE POSTMENOPAUSAL PERIOD typically occupies one third of a woman's life (1) and places her at increased risk of osteoporosis (2). Although it is well documented that hormone replacement therapy (HRT) slows down the rate of bone turnover, especially bone resorption (3), on the basis of recent findings (4) its long-term use may not decrease fracture incidence. Other agents such as calcitonin and the bisphosphonate family are also effective antiresorptive agents (5). Despite the availability of drug therapies, there are a considerable number of women who would prefer dietary supplements as an alternative/adjunctive to conventional therapeutic options (6). Examples of these alternative therapies include the use of natural or plant-based substances such as soy isoflavones and other rich sources of phenolic compounds (5–7). Soy isoflavones, recently referred to as naturally occurring selective estrogen receptor modulators (SERMs; Refs. 8 and 9), may exert estrogen-like effects on selected tissues, *e.g.* bone, but not all tissues (8, 10). However, to what extent isoflavones behave like SERMs, *e.g.* raloxifene, remains to be illustrated.

A number of animal studies indicate that soy protein or its isoflavones, although maintaining the ovariectomy-induced elevated rate of bone formation, may simultaneously suppress the rate of bone resorption (11–13). Recently published human studies (14–16) have demonstrated the antiresorptive properties of soy or its isoflavones. These studies include both cross-sectional (16) and prospective (14) trials in which soy protein intake in postmenopausal women was associated with lower urinary specific markers of bone resorption, *e.g.* N-terminal cross-linked peptide (14) and deoxypyridinoline (Dpd; Ref. 16). Furthermore, clinical trials examining the effects of soy protein have found that daily intake of approximately 88 mg isoflavones in conjunction with 40 g soy protein for 6 months increased bone mineral density and bone mineral content in perimenopausal (17) and postmenopausal women who were not on HRT (14, 18).

The purpose of the present study was to examine the effects of soy protein on serum and urinary markers of bone turnover in postmenopausal women, with further analysis of data for women on HRT and women not on HRT.

Subjects and Methods

Subjects

A total of 71 postmenopausal women were recruited for this study irrespective of their HRT status, ethnic, and racial backgrounds. Subjects were excluded if they had gastrointestinal disorders, cancer, diabetes,

Abbreviations: BSAP, Bone-specific alkaline phosphatase; Dpd, deoxypyridinoline; E_2, 17β-estradiol; HRT, hormone replacement therapy; MBP, milk-based protein; NTx, N-telopeptide; SERM, selective estrogen receptor modulator; SP, soy protein.

hypo- or hyperthyroidism, liver or kidney problems, pelvic inflammatory disease, or endometrial polyps, and if they were heavy smokers (>1 pack/d). The mean age of study participants was 62.4 ± 2.4 yr for the soy protein (SP) group and 61.8 ± 2.4 yr for the milk-based protein (MBP) group. The study participants were asked to sign a consent form after receiving oral and written descriptions of the study. A complete medical and diet history was obtained from all subjects before initiating the treatments. Subjects were recruited by advertisement at-large in the city of Stillwater, Oklahoma, and the surrounding communities. The study protocol was approved by the Institutional Review Board at Oklahoma State University.

Study design

Study participants were randomly assigned to consume 40 g of either supplemental SP or MBP (control) daily for 3 months in a double-blind, controlled parallel design. Randomization was performed for all subjects regardless of HRT use. The protein supplements were provided to study participants in two packages, each containing 29 g of a powdered-unflavored drink-mix (Protein Technologies International, St. Louis, MO) to be consumed daily. The composition of the protein supplements is presented in Table 1. The supplements were distributed to the subjects in monthly rations. Subjects were asked to return any unused supplement and mark their calendar daily as a part of monitoring compliance. The free-living study participants were informed of the additional amount of dietary protein they were receiving and were advised to make reasonable substitutions, otherwise continue to consume their habitual diet, and maintain their usual physical activity.

Dietary assessment and anthropometric measurements

For each subject, medical and nutrition history was obtained at the beginning of the study. Anthropometric data were assessed at baseline and at the end of the study and are presented in Table 2. One-week food frequency questionnaires were obtained via interview by a registered dietitian at the beginning and at the end of the 3-month treatment period (Table 3). Nutrient analysis was performed using food analysis software (Food Processor version 7.50, ESHA Research, Salem, OR).

Blood and urine collection

A venous blood sample was obtained after an overnight fast from each subject before and after the treatment period for various analyses. Samples were centrifuged at 2000 × *g* for 15 min at 4 C, and serum was separated and stored at −80 C until analyzed. Each subject collected a 24-h urine specimen, excluding the first morning void, before and after the treatment period. Urine volume was recorded, and aliquots were stored at −20 C for later analysis.

Analytical methods

Blood analyses. Serum bone-specific alkaline phosphatase (BSAP) activity, a specific marker of bone formation (19), was quantified by immunoassay in a microtiter format (Metra Biosystems, Mountain View, CA). Serum 17β-estradiol (E_2) was determined using RIA kits from Diagnostic Systems Laboratories Inc. (Webster, TX). IGF-I was extracted from serum using the acid-ethanol extraction procedure and kits from Nichols Institute Diagnostics (San Juan Capistrano, CA), following the manufacturer's procedures. The intra- and interassay coefficients of variation were 9.7% and 3.9% for BSAP, 6.5% and 7.6% for E_2, and 3.0% and 8.4% for IGF-I.

Urinary analyses. Urinary creatinine was measured colorimetrically with a commercially available kit from Roche Diagnostic Systems, Inc. (Montclair, NJ) using a Cobas Fara II clinical analyzer. Urinary Dpd excretion, a specific marker of bone resorption, was measured by competitive enzyme immunoassay in a microassay stripwell format (Quidel Corporation, Mountain View, CA; Ref. 20). Urinary calcium excretion was measured using a kit from Sigma (St. Louis, MO). The intra- and interassay coefficients of variation were 1.7% and 6.3% for creatinine and 4.3% and 4.6% for Dpd.

TABLE 1. Analytical composition of SP and MBP used in the study

Component	SP	MBP
Protein (g)	40	40
Carbohydrates (g)	6	6
Total fat (g)	2	<1
Vitamins		
Vitamin A (IU)	1000	1000
Vitamin C (mg)	4.8	4.8
Vitamin D (IU)	200	200
Minerals		
Calcium (mg)	1400	1400
Iron (mg)	7.2	0
Magnesium (mg)	80	80
Phosphorus (mg)	1000	1000
Zinc (mg)	1.8	1.8
Total isoflavones (mg)	88.4	0.0

Statistical analyses

All data were analyzed using ANOVA methods with PROC MIXED in PC SAS (version 8.2, SAS Institute, Inc., Cary, NC). A three-way ANOVA model was fit, using HRT, soy treatment, and time as factors. Each factor has two levels. Because each subject was measured before and after treatment, a repeated measures analysis was used, with HRT and soy treatment as the main unit factors and time as the within-subject factor. The primary objective was to assess the effects of treatment over time, so the interaction of soy treatment with time was tested (interaction will measure the consistency of treatment differences over time). This interaction is calculated for both cases of HRT and averaged over HRT. A SLICE option in PROC MIXED was used to test soy treatment by time interaction for both levels of HRT. If the interaction was significant due to a soy treatment improvement, then that indicated an improvement over time relative to the nonsoy group. Data are reported as least squares mean ± SE. Unless otherwise indicated, a P value less than 0.05 was regarded as significant.

Results

Baseline characteristics, anthropometric measurements, and dietary intakes

Forty-two of 71 women completed the study, an attrition rate of approximately 41%. Reasons for attrition included lack of palatability of the powdered protein supplements (seven and six women in MBP and SP treatment groups, respectively), time constraints preventing adherence with study protocol (five and seven women in MBP and SP treatment groups, respectively), gastrointestinal disturbances (one and two women in MBP and SP treatment groups, respectively), and personal reasons preventing compliance with study protocol (one woman in SP treatment group). Baseline characteristic data for women who completed the study are presented in Table 2. Baseline characteristics did not differ for women receiving the soy protein regimen and those receiving the control regimen.

Those who finished the study adhered to their regimens as indicated by self-monitoring checklists provided to them and by returning any unconsumed supplement packets on a monthly basis. Daily nutrient intake as assessed by 1-wk food frequency questionnaires for subjects in both treatment groups were similar before and after the 3-month treatment period (Table 3).

Serum and urinary parameters

In assessing the effect of treatment with SP or MBP, we first analyzed the differences between baseline and final in each

TABLE 2. Subject characteristics at baseline and after a 3-month supplementation

	SP (n = 20)			MBP (n = 22)		
	Baseline	Final	*P*	Baseline	Final	*P*
Age (yr)	62.4 ± 2.4			61.8 ± 2.4		
Height (m)	1.63 ± 0.02			1.64 ± 0.02		
Weight (kg)	84.5 ± 4.6	84.7 ± 4.6	0.7385	87.3 ± 4.4	88.3 ± 4.4	0.0718
BMI (kg/m^2)	31.8 ± 1.8	31.9 ± 1.8	0.6134	32.6 ± 1.7	32.9 ± 1.7	0.0910
Waist/hip ratio	0.85 ± 0.02	0.86 ± 0.02	0.2164	0.86 ± 0.02	0.87 ± 0.02	0.3866
Body fat (%)	40.7 ± 1.7	39.4 ± 1.7	0.0532	41.9 ± 1.5	40.9 ± 1.5	0.0783

Values are least square means ± SE.

TABLE 3. Daily nutrient dietary intake calculated from 7-d food frequency questionnaires obtained from study participants before and after the 3-month dietary intervention[a]

	SP (n = 20)		MBP (n = 22)	
	Baseline	Final	Baseline	Final
Total energy (kcal)	1,343 ± 119	1,432 ± 79	1,598 ± 103	1,626 ± 169
Macronutrients				
Protein (g)	60 ± 6	61 ± 4	75 ± 9	68 ± 5
Carbohydrates (g)	181 ± 18	200 ± 10	196 ± 16	213 ± 22
Total fat (g)	46 ± 4	46 ± 4	60 ± 6	59 ± 8
Vitamins				
Vitamin A (IU)	13,512 ± 2,382	16,945 ± 2,826	10,108 ± 2,033	11,650 ± 1,549
Vitamin C (mg)	104 ± 14	131 ± 18	93 ± 11	127 ± 16
Vitamin D (IU)[b]	168 ± 28	152 ± 23	164 ± 33	173 ± 26
Vitamin E (IU)	7.8 ± 0.7	8.3 ± 0.7	21.2 ± 12.9	9.9 ± 2.3
Vitamin K (μg)	119 ± 24	139 ± 28	81 ± 15	95 ± 17
Minerals				
Calcium (mg)[b]	842 ± 112	786 ± 97	752 ± 96	826 ± 134
Iron (mg)	11.5 ± 1.3	10.8 ± 0.9	11.6 ± 1.0	11.3 ± 0.7
Magnesium (mg)	280 ± 31	246 ± 16	254 ± 14	281 ± 30
Phosphorus (mg)	1,201 ± 152	1,097 ± 94	1,201 ± 99	1,157 ± 103
Potassium (mg)	2,796 ± 308	2,788 ± 168	2,661 ± 191	3,064 ± 362
Zinc (mg)	8.58 ± 0.95	8.24 ± 0.57	9.48 ± 0.75	9.04 ± 0.62

[a] Values are least square means ± SE; analyses do not include nutrients and calcium from the protein supplements provided to the study participants. There were no statistically significant differences observed between baseline values of the two treatment groups and between baseline and corresponding final values.

[b] Each protein supplement provided 1400 mg calcium and 200 IU vitamin D.

TABLE 4. Effect of a 3-month SP or MBP daily supplementation on serum and urine parameters in all postmenopausal women

	SP (n = 20)			MBP (n = 22)			Time × treat
	Baseline	Final	*P*	Baseline	Final	*P*	*P*
Serum							
BSAP (μkat/liter)	0.438 ± 0.032	0.412 ± 0.032	0.1394	0.381 ± 0.031	0.348 ± 0.031	0.0493	0.7601
E_2 (pg/ml)	51.5 ± 22.4	66.6 ± 23.1	0.3428	84.8 ± 21.5	95.0 ± 22.2	0.5135	0.8292
IGF-I (nmol/liter)	12.42 ± 1.91	20.94 ± 1.98	<0.0001	14.02 ± 1.87	19.12 ± 1.87	0.0045	0.1718
Urine							
Dpd (nmol/mol creatinine)	9.62 ± 0.76	7.19 ± 0.74	0.0115	7.66 ± 0.69	6.79 ± 0.69	0.3055	0.2164
Calcium (mmol/mmol creatinine)	0.52 ± 0.06	0.55 ± 0.06	0.5456	0.42 ± 0.06	0.56 ± 0.06	0.0018	0.0694
Phosphorous (mmol/mmol creatinine)	2.83 ± 0.22	2.92 ± 0.22	0.6761	2.72 ± 0.20	3.09 ± 0.20	0.0794	0.3542
Magnesium (mmol/mmol creatinine)	0.53 ± 0.05	0.53 ± 0.05	0.9305	0.47 ± 0.05	0.58 ± 0.05	0.0630	0.2205

Values are least square means ± SE.

group. As expected, both protein supplements significantly increased serum levels of IGF-I (Table 4). Although SP supplementation did not alter serum levels of BSAP, MBP significantly ($P < 0.05$) suppressed its levels. Urinary Dpd levels were only significantly lowered in those who consumed SP. Subjects in the MBP group experienced a 33% increase ($P < 0.002$) in urinary calcium excretion with similar trends for phosphorus and magnesium, whereas SP had no such effect. Soy protein supplementation had no effect on serum levels of E_2, indicating its lack of apparent estrogenicity.

To evaluate the possible influence of HRT, subanalysis of the data were performed within each treatment group for those who were on HRT (Table 5) or those who were not on HRT (Table 6). When comparisons were made for

TABLE 5. Effect of a 3-month SP or MBP daily supplementation on serum and urine parameters in postmenopausal women on HRT

	SP (n = 20)			MBP (n = 22)			Time × treat
	Baseline	Final	*P*	Baseline	Final	*P*	*P*
Serum							
BSAP (μkat/liter)	0.354 ± 0.024	0.324 ± 0.032	0.2444	0.363 ± 0.033	0.338 ± 0.027	0.2872	0.4481
E_2 (pg/ml)	93.4 ± 16.8	116.9 ± 28.1	0.3335	122.84 ± 28.7	158.3 ± 48.38	0.6299	0.3990
IGF-I (nmol/liter)	13.43 ± 2.50	19.85 ± 3.72	0.0221	15.49 ± 1.85	21.21 ± 2.56	0.0147	0.0101
Urine							
Dpd (nmol/mol creatinine)	7.60 ± 1.11	6.84 ± 0.94	0.5719	6.68 ± 0.55	6.46 ± 0.55	0.8489	0.8738
Calcium (mmol/mmol creatinine)	0.50 ± 0.09	0.47 ± 0.09	0.6285	0.42 ± 0.05	0.57 ± 0.06	0.0119	0.0806
Phosphorous (mmol/mmol creatinine)	2.44 ± 0.36	2.33 ± 0.17	0.7213	2.97 ± 0.21	3.32 ± 0.23	0.2157	0.0715
Magnesium (mmol/mmol creatinine)	0.61 ± 0.11	0.49 ± 0.04	0.0682	0.41 ± 0.04	0.57 ± 0.05	0.0285	0.0306

Values are least square means ± SE.

TABLE 6. Effect of a 3-month SP or MBP daily supplementation on serum and urine parameters in postmenopausal women not on HRT

	SP (n = 20)			MBP (n = 22)			Time × treat
	Baseline	Final	*P*	Baseline	Final	*P*	*P*
Serum							
BSAP (μkat/liter)	0.523 ± 0.042	0.499 ± 0.045	0.3501	0.404 ± 0.06	0.360 ± 0.044	0.0910	0.0427
E_2 (pg/ml)	35.6 ± 6.4	61.7 ± 16.9	0.7793	45.9 ± 13.9	93.2 ± 44.4	0.6596	0.9610
IGF-I (nmol/liter)	11.40 ± 2.15	22.45 ± 4.16	0.0001	12.06 ± 1.25	16.33 ± 3.18	0.1062	0.0005
Urine							
Dpd (nmol/mol creatinine)	11.24 ± 1.89	7.49 ± 0.38	0.0041	8.85 ± 1.14	7.19 ± 0.69	0.1829	0.009
Calcium (mmol/mmol creatinine)	0.55 ± 0.14	0.63 ± 0.14	0.1859	0.42 ± 0.05	0.55 ± 0.07	0.0472	0.0974
Phosphorous (mmol/mmol creatinine)	3.23 ± 0.27	3.52 ± 0.45	0.3502	2.41 ± 0.24	2.81 ± 0.18	0.2183	0.0644
Magnesium (mmol/mmol creatinine)	0.43 ± 0.03	0.57 ± 0.08	0.0581	0.54 ± 0.07	0.59 ± 0.04	0.5665	0.1952

Values are least square means ± SE.

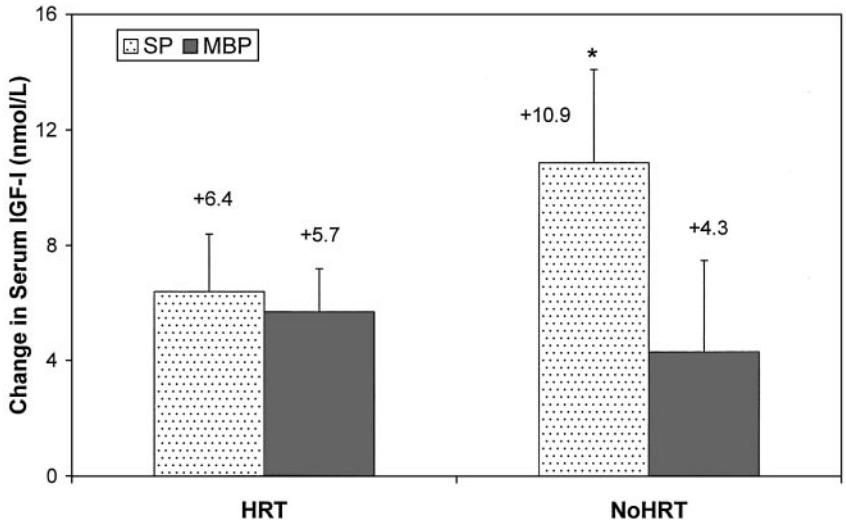

FIG. 1. Mean changes from baseline values in serum IGF-I concentrations after 3 months of SP or MBP supplementation in women on HRT and not on HRT (noHRT). *, In the noHRT group, the change in IGF-I concentration was significantly ($P < 0.05$) higher in women on SP compared with those on MBP.

women within the HRT and no-HRT groups, baseline values did not differ for the SP and MBP treatments. Similar to the overall findings, serum IGF-I levels were increased by both protein supplements; however, soy protein had a more pronounced effect in increasing serum IGF-I levels in women who were not on HRT (Fig. 1). Despite the increase in IGF-I concentrations, serum BSAP levels were not significantly influenced by any of the treatments.

In terms of the antiresorptive properties of soy, the subanalysis of the data clearly indicated that soy with its isoflavones significantly ($P < 0.01$) suppressed urinary Dpd in women who were not on HRT (Fig. 2). Additionally, MBP increased ($P < 0.05$) urinary excretion of calcium irrespective of hormonal status, whereas soy had no such negative effect. The data show that there may be an interaction between soy protein, urinary magnesium loss, and HRT. In women on HRT, urinary magnesium excretion was significantly elevated in those consuming MBP, whereas SP reduced ($P = 0.068$) its loss. In contrast, in women not on HRT, SP not only failed to lower magnesium excretion, but also tended ($P = 0.058$) to increase its urinary loss, whereas MBP had no such effect.

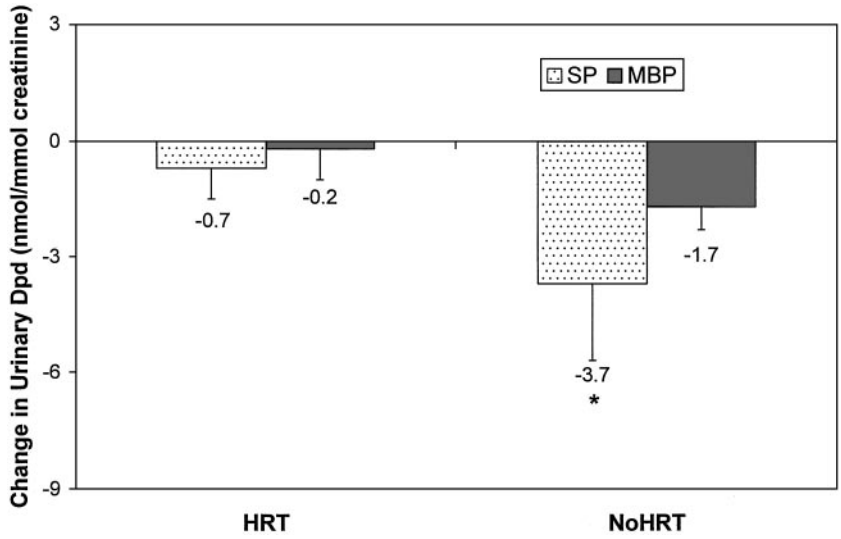

FIG. 2. Mean changes from baseline values in urinary Dpd concentrations after 3 months of SP or MBP supplementation in women on HRT and not on HRT (noHRT). *, In the noHRT group, the change in Dpd concentration was significantly ($P < 0.05$) lower in women on SP compared with those on MBP.

Discussion

This study intended to elucidate whether soy protein positively influences postmenopausal women's bone health as assessed by bone biochemical markers, regardless of HRT status. To answer this question, the data were first analyzed for all women on soy for the overall effect and subsequently were examined for the differences in the effectiveness for women on HRT or not on HRT. Based on the findings of this study, soy protein appeared to exert its bone protective effects mainly by suppressing the rate of bone resorption, while at the same time maintaining or enhancing the rate of bone formation.

When all women in the present study were considered, the overall findings indicated that soy protein was effective in reducing the rate of bone resorption as evidenced by suppressed urinary Dpd excretion. These findings are in agreement with those from a limited number of human trials (14–16) and animal studies (11–13). In a cross-sectional study by Horiuchi *et al.* (16), soy protein intake in Japanese postmenopausal women was associated with significantly lower urinary Dpd excretion. In another cross-sectional study by Kritz-Silverstein and Goodman-Gruen (15), postmenopausal women in southern California with the highest daily intake of dietary genistin had 18% lower N-telopeptide (NTx) excretion than women who did not consume genistin. The results of a prospective study by Scheiber *et al.* (14) also indicated a significant reduction of urinary NTx excretion in postmenopausal women who consumed soy foods providing 60 mg isoflavones for 3 months.

In contrast to the findings of the present study and those of other investigators (14–16), the results of a clinical trial by Wangen *et al.* (21) showed that soy isoflavones in the context of soy protein had no effect on biomarkers of bone turnover in postmenopausal women. However, in their study (21), all subjects were consuming soy protein so that conclusions were based on comparing soy protein with normal and added isoflavones to that of soy protein with minimal isoflavone content rather than a nonsoy protein control. To date, the existing clinical trials have exclusively looked at soy protein or whole soy consumption on bone; hence, it is too early to credit the positive effects of soy protein on bone solely to its isoflavones. If isoflavones act similarly to synthetic SERMs (8, 9), it is reasonable to assume that the isoflavones in soy protein, in part, are responsible for the observed reduction in markers of bone resorption.

Recent reports have indicated that soy protein supplementation may exert positive effects on calcium homeostasis as indicated by significantly reduced urinary calcium excretion in postmenopausal women (22, 23). Similarly, in the present study, subjects who consumed soy protein did not experience a significant increase in urinary calcium loss, whereas urinary calcium excretion was higher in those who received MBP. This calcium-conserving property of soy has been attributed to the lower levels of sulfur-containing amino acids in soy protein (24–26). Additionally, intestinal calcium absorption declines in ovarian hormone deficiency (27, 28), which may contribute to accelerated bone loss (27–30). In this respect, similar to estrogen (31, 32), soy isoflavones may enhance intestinal calcium absorption and further improve calcium homeostasis (7, 26, 33). Furthermore, in the present study conservatory trends were also observed with magnesium and phosphorus homeostasis, both of which are important in the maintenance of skeletal health.

Soy protein, with its nonprotein constituents, when given in conjunction with calcium not only suppresses bone resorption, but simultaneously may have the ability to enhance the rate of bone formation. In this study, although both protein sources elevated serum IGF-I levels, soy protein had a more pronounced effect in increasing serum IGF-I by 69%, compared with a 36% increase with MBP. Although the role of circulating IGF-I in bone is unclear, IGF-I has been reported to directly stimulate collagen synthesis *in vitro* by osteoblastic cells (34) and may mediate the anabolic action of parathyroid hormone on bone (35). Serum IGF-I concentrations have also been reported to correlate positively with bone mass in premenopausal (36), perimenopausal (37), and postmenopausal

(38) women. In support of the notion that soy protein with its isoflavones may have an anabolic effect, isoflavones have been shown to stimulate osteoblastic activity through activation of estrogen receptors and increase bone alkaline phosphatase activity (39). However, our findings that soy protein has the ability to increase serum IGF-I but not circulating levels of BSAP is paradoxical, making the bone-forming ability of soy protein questionable at the present time.

The subanalysis of the data in this study, however, revealed that soy protein is only effective in reducing the bone resorption marker, Dpd, in the absence of HRT. These findings seem logical because HRT has already substantially suppressed bone resorption and further reduction should not be expected. The ineffectiveness of soy protein to reduce bone resorption in the presence of estrogen has also been observed by Alekel *et al.* (17) in perimenopausal women. In that study (17), soy protein did not alter urinary NTx excretion, which is similar to our observation in postmenopausal women on HRT. Therefore, the efficacy of soy protein or its isoflavones in preventing bone loss or improving bone health may vary, depending on estrogen status of women.

In summary, our findings suggest that women who are not on HRT may greatly benefit from consuming soy products. This conclusion is based on our observations that soy supplementation not only reduced bone resorption, as assessed by urinary Dpd, but also did not exert a negative effect on calcium, magnesium, and phosphorus homeostasis. Although the aforementioned findings plus the stimulatory effect of soy protein on IGF-I are suggestive of the bone protective effects of soy protein, it is noteworthy that the conclusions derived from this study are based on biochemical parameters associated with bone metabolism. Consequently, these conclusions need to be substantiated by longer-term clinical studies in which the effects of soy protein supplementation on bone mineral density, bone mineral content, and fracture risk can be evaluated.

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Review article

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PROTEIN – WHICH IS BEST?

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ABSTRACT

Protein intake that exceeds the recommended daily allowance is widely accepted for both endurance and power athletes. However, considering the variety of proteins that are available much less is known concerning the benefits of consuming one protein versus another. The purpose of this paper is to identify and analyze key factors in order to make responsible recommendations to both the general and athletic populations. Evaluation of a protein is fundamental in determining its appropriateness in the human diet. Proteins that are of inferior content and digestibility are important to recognize and restrict or limit in the diet. Similarly, such knowledge will provide an ability to identify proteins that provide the greatest benefit and should be consumed. The various techniques utilized to rate protein will be discussed. Traditionally, sources of dietary protein are seen as either being of animal or vegetable origin. Animal sources provide a complete source of protein (i.e. containing all essential amino acids), whereas vegetable sources generally lack one or more of the essential amino acids. Animal sources of dietary protein, despite providing a complete protein and numerous vitamins and minerals, have some health professionals concerned about the amount of saturated fat common in these foods compared to vegetable sources. The advent of processing techniques has shifted some of this attention and ignited the sports supplement marketplace with derivative products such as whey, casein and soy. Individually, these products vary in quality and applicability to certain populations. The benefits that these particular proteins possess are discussed. In addition, the impact that elevated protein consumption has on health and safety issues (i.e. bone health, renal function) are also reviewed.

KEY WORDS: Sport supplementation, ergogenic aid, animal protein, vegetable protein.

INTRODUCTION

The protein requirements for athletic populations have been the subject of much scientific debate. Only recently has the notion that both strength/power and endurance athletes require a greater protein consumption than the general population become generally accepted. In addition, high protein diets have also become quite popular in the general population as part of many weight reduction programs. Despite the prevalence of high protein diets in athletic and sedentary populations, information available concerning the type of protein (e.g. animal or vegetable) to consume is limited. The purpose of this paper is to examine and analyze key factors responsible for making appropriate choices on the type of protein to consume in both athletic and general populations.

Role of Protein

Proteins are nitrogen-containing substances that are formed by amino acids. They serve as the major structural component of muscle and other tissues in

the body. In addition, they are used to produce hormones, enzymes and hemoglobin. Proteins can also be used as energy; however, they are not the primary choice as an energy source. For proteins to be used by the body they need to be metabolized into their simplest form, amino acids. There have been 20 amino acids identified that are needed for human growth and metabolism. Twelve of these amino acids (eleven in children) are termed nonessential, meaning that they can be synthesized by our body and do not need to be consumed in the diet. The remaining amino acids cannot be synthesized in the body and are described as essential meaning that they need to be consumed in our diets. The absence of any of these amino acids will compromise the ability of tissue to grow, be repaired or be maintained.

Protein and Athletic Performance

The primary role of dietary proteins is for use in the various anabolic processes of the body. As a result, many athletes and coaches are under the belief that high intensity training creates a greater protein requirement. This stems from the notion that if more protein or amino acids were available to the exercising muscle it would enhance protein synthesis. Research has tended to support this hypothesis. Within four weeks of protein supplementation (3.3 versus 1.3 $g \cdot kg^{-1} \cdot day^{-1}$) in subjects' resistance training, significantly greater gains were seen in protein synthesis and body mass in the group of subjects with the greater protein intake (Fern et al., 1991). Similarly, Lemon et al. (1992) also reported a greater protein synthesis in novice resistance trained individuals with protein intakes of 2.62 versus 0.99 $g \cdot kg^{-1} \cdot day^{-1}$. In studies examining strength-trained individuals, higher protein intakes have generally been shown to have a positive effect on muscle protein synthesis and size gains (Lemon, 1995; Walberg et al., 1988). Tarnapolsky and colleagues (1992) have shown that for strength trained individuals to maintain a positive nitrogen balance they need to consume a protein intake equivalent to 1.8 $g \cdot kg^{-1} \cdot day^{-1}$. This is consistent with other studies showing that protein intakes between 1.4 – 2.4 $g \cdot kg^{-1} \cdot day^{-1}$ will maintain a positive nitrogen balance in resistance trained athletes (Lemon, 1995). As a result, recommendations for strength/power athletes' protein intake are generally suggested to be between 1.4 - 1.8 $g \cdot kg^{-1} \cdot day^{-1}$.

Similarly, to prevent significant losses in lean tissue endurance athletes also appear to require a greater protein consumption (Lemon, 1995). Although the goal for endurance athletes is not necessarily to maximize muscle size and strength, loss of lean tissue can have a significant detrimental effect on endurance performance. Therefore, these athletes need to maintain muscle mass to ensure adequate performance. Several studies have determined that protein intake for endurance athletes should be between 1.2 – 1.4 $g \cdot kg^{-1} \cdot day^{-1}$ to ensure a positive nitrogen balance (Freidman and Lemon, 1989; Lemon, 1995; Meredith et al., 1989; Tarnopolsky et al., 1988). Evidence is clear that athletes do benefit from increased protein intake. The focus then becomes on what type of protein to take.

Protein Assessment

The composition of various proteins may be so unique that their influence on physiological function in the human body could be quite different. The quality of a protein is vital when considering the nutritional benefits that it can provide. Determining the quality of a protein is determined by assessing its essential amino acid composition, digestibility and bioavailability of amino acids (FAO/WHO, 1990). There are several measurement scales and techniques that are used to evaluate the quality of protein.

Protein Rating Scales

Numerous methods exist to determine protein quality. These methods have been identified as protein efficiency ratio, biological value, net protein utilization, and protein digestibility corrected amino acid score.

Protein Efficiency Ratio

The protein efficiency ratio (PER) determines the effectiveness of a protein through the measurement of animal growth. This technique requires feeding rats a test protein and then measuring the weight gain in grams per gram of protein consumed. The computed value is then compared to a standard value of 2.7, which is the standard value of casein protein. Any value that exceeds 2.7 is considered to be an excellent protein source. However, this calculation provides a measure of growth in rats and does not provide a strong correlation to the growth needs of humans.

Biological Value

Biological value measures protein quality by calculating the nitrogen used for tissue formation divided by the nitrogen absorbed from food. This product is multiplied by 100 and expressed as a percentage of nitrogen utilized. The biological value provides a measurement of how efficient the body utilizes protein consumed in the diet. A food with a high value correlates to a high supply of the essential

Table 1. Protein quality rankings.

Protein Type	Protein Efficiency Ratio	Biological Value	Net Protein Utilization	Protein Digestibility Corrected Amino Acid Score
Beef	2.9	80	73	0.92
Black Beans	0		0	0.75
Casein	2.5	77	76	1.00
Egg	3.9	100	94	1.00
Milk	2.5	91	82	1.00
Peanuts	1.8			0.52
Soy protein	2.2	74	61	1.00
Wheat gluten	0.8	64	67	0.25
Whey protein	3.2	104	92	1.00

Adapted from: U.S Dairy Export Council, Reference Manual for U.S. Whey Products 2nd Edition, 1999 and Sarwar, 1997.

amino acids. Animal sources typically possess a higher biological value than vegetable sources due to the vegetable source's lack of one or more of the essential amino acids. There are, however, some inherent problems with this rating system. The biological value does not take into consideration several key factors that influence the digestion of protein and interaction with other foods before absorption. The biological value also measures a protein's maximal potential quality and not its estimate at requirement levels.

Net Protein Utilization

Net protein utilization is similar to the biological value except that it involves a direct measure of retention of absorbed nitrogen. Net protein utilization and biological value both measure the same parameter of nitrogen retention, however, the difference lies in that the biological value is calculated from nitrogen absorbed whereas net protein utilization is from nitrogen ingested.

Protein Digestibility Corrected Amino Acid Score

In 1989, the Food & Agriculture Organization and World Health Organization (FAO/WHO) in a joint position stand stated that protein quality could be determined by expressing the content of the first limiting essential amino acid of the test protein as a percentage of the content of the same amino acid content in a reference pattern of essential amino acids (FAO/WHO, 1990). The reference values used were based upon the essential amino acids requirements of preschool-age children. The recommendation of the joint FAO/WHO statement was to take this reference value and correct it for true fecal digestibility of the test protein. The value obtained was referred to as the protein digestibility corrected amino acid score (PDCAAS). This method has been adopted as the preferred method for measurement of the protein value in human nutrition (Schaafsma, 2000). Table 1 provides a measure of the quantity of various proteins using these protein rating scales.

Although the PDCAAS is currently the most accepted and widely used method, limitations still exist relating to overestimation in the elderly (likely related to references values based on young individuals), influence of ileal digestibility, and antinutritional factors (Sarwar, 1997).

Amino acids that move past the terminal ileum may be an important route for bacterial consumption of amino acids, and any amino acids that reach the colon would not likely be utilized for protein synthesis, even though they do not appear in the feces (Schaarfsma, 2000). Thus, to get truly valid measure of fecal digestibility the location at which protein synthesis is determined is important in making a more accurate determination. Thus, ileal digestibility would provide a more accurate measure of digestibility. PDCAAS, however, does not factor ileal digestibility into its equation. This is considered to be one of the shortcomings of the PDCAAS (Schaafsma 2000).

Antinutritional factors such as trypsin inhibitors, lectins, and tannins present in certain protein sources such as soybean meal, peas and fava beans have been reported to increase losses of endogenous proteins at the terminal ileum (Salgado et al., 2002). These antinutritional factors may cause reduced protein hydrolysis and amino acid absorption. This may also be more effected by age, as the ability of the gut to adapt to dietary nutritional insults may be reduced as part of the aging process (Sarwar, 1997).

Protein Sources

Protein is available in a variety of dietary sources. These include foods of animal and plant origins as well as the highly marketed sport supplement industry. In the following section proteins from both vegetable and animal sources, including whey,

casein, and soy will be explored. Determining the effectiveness of a protein is accomplished by determining its quality and digestibility. Quality refers to the availability of amino acids that it supplies, and digestibility considers how the protein is best utilized. Typically, all dietary animal protein sources are considered to be complete proteins. That is, a protein that contains all of the essential amino acids. Proteins from vegetable sources are incomplete in that they are generally lacking one or two essential amino acids. Thus, someone who desires to get their protein from vegetable sources (i.e. vegetarian) will need to consume a variety of vegetables, fruits, grains, and legumes to ensure consumption of all essential amino acids. As such, individuals are able to achieve necessary protein requirements without consuming beef, poultry, or dairy. Protein digestibility ratings usually involve measuring how the body can efficiently utilize dietary sources of protein. Typically, vegetable protein sources do not score as high in ratings of biological value, net protein utilization, PDCAAS, and protein efficiency ratio as animal proteins.

Animal Protein

Proteins from animal sources (i.e. eggs, milk, meat, fish and poultry) provide the highest quality rating of food sources. This is primarily due to the 'completeness' of proteins from these sources. Although protein from these sources are also associated with high intakes of saturated fats and cholesterol, there have been a number of studies that have demonstrated positive benefits of animal proteins in various population groups (Campbell et al., 1999; Godfrey et al., 1996; Pannemans et al., 1998).

Protein from animal sources during late pregnancy is believed to have an important role in infants born with normal body weights. Godfrey et al. (1996) examined the nutrition behavior of more than 500 pregnant women to determine the effect of nutritional intake on placental and fetal growth. They reported that a low intake of protein from dairy and meat sources during late pregnancy was associated with low birth weights.

In addition to the benefits from total protein consumption, elderly subjects have also benefited from consuming animal sources of protein. Diets consisting of meat resulted in greater gains in lean body mass compared to subjects on a lactoovovegetarian diet (Campbell et al., 1999). High animal protein diets have also been shown to cause a significantly greater net protein synthesis than a high vegetable protein diet (Pannemans et al., 1998). This was suggested to be a function of reduced protein breakdown occurring during the high animal protein diet.

There have been a number of health concerns raised concerning the risks associated with protein emanating primarily from animal sources. Primarily, these health risks have focused on cardiovascular disease (due to the high saturated fat and cholesterol consumption), bone health (from bone resorption due to sulfur-containing amino acids associated with animal protein) and other physiological system disease that will be addressed in the section on high protein diets.

Whey

Whey is a general term that typically denotes the translucent liquid part of milk that remains following the process (coagulation and curd removal) of cheese manufacturing. From this liquid, whey proteins are separated and purified using various techniques yielding different concentrations of whey proteins. Whey is one of the two major protein groups of bovine milk, accounting for 20% of the milk while casein accounts for the remainder. All of the constituents of whey protein provide high levels of the essential and branched chain amino acids. The bioactivities of these proteins possess many beneficial properties as well. Additionally, whey is also rich in vitamins and minerals. Whey protein is most recognized for its applicability in sports nutrition. Additionally, whey products are also evident in baked goods, salad dressings, emulsifiers, infant formulas, and medical nutritional formulas.

Varieties of Whey Protein

There are three main forms of whey protein that result from various processing techniques used to separate whey protein. They are whey powder, whey concentrate, and whey isolate. Table 2 provides the composition of Whey Proteins.

Whey Protein Powder

Whey protein powder has many applications throughout the food industry. As an additive it is seen in food products for beef, dairy, bakery, confectionery, and snack products. Whey powder itself has several different varieties including sweet whey, acid whey (seen in salad dressings), demineralized (seen primarily as a food additive including infant formulas), and reduced forms. The demineralized and reduced forms are used in products other than sports supplements.

Whey Protein Concentrate

The processing of whey concentrate removes the water, lactose, ash, and some minerals. In addition, compared to whey isolates whey concentrate typically contains more biologically active components and proteins that make them a very attractive supplement for the athlete.

Whey Protein Isolate (WPI)
Isolates are the purest protein source available. Whey protein isolates contain protein concentrations of 90% or higher. During the processing of whey protein isolate there is a significant removal of fat and lactose. As a result, individuals who are lactose-intolerant can often safely take these products (Geiser, 2003). Although the concentration of protein in this form of whey protein is the highest, it often contain proteins that have become denatured due to the manufacturing process. The denaturation of proteins involves breaking down their structure and losing peptide bonds and reducing the effectiveness of the protein.

Table 2. Composition (%) of whey protein forms.

Component	Whey Powder	Whey Concentrate	Whey Isolate
Protein	11 – 14.5	25 – 89	90 +
Lactose	63 – 75	10 – 55	0.5
Milk Fat	1 – 1.5	2 – 10	0.5

Adapted from Geiser, 2003.

Whey is a complete protein whose biologically active components provide additional benefits to enhance human function. Whey protein contains an ample supply of the amino acid cysteine. Cysteine appears to enhance glutathione levels, which has been shown to have strong antioxidant properties that can assist the body in combating various diseases (Counous, 2000). In addition, whey protein contains a number of other proteins that positively effect immune function such as antimicrobial activity (Ha and Zemel, 2003). Whey protein also contains a high concentration of branched chain amino acids (BCAA) that are important for their role in the maintenance of tissue and prevention of catabolic actions during exercise. (MacLean et al., 1994).

Casein
Casein is the major component of protein found in bovine milk accounting for nearly 70-80% of its total protein and is responsible for the white color of milk. It is the most commonly used milk protein in the industry today. Milk proteins are of significant physiological importance to the body for functions relating to the uptake of nutrients and vitamins and they are a source of biologically active peptides. Similar to whey, casein is a complete protein and also contains the minerals calcium and phosphorous. Casein has a PDCAAS rating of 1.23 (generally reported as a truncated value of 1.0) (Deutz et al. 1998).

Casein exists in milk in the form of a micelle, which is a large colloidal particle. An attractive property of the casein micelle is its ability to form a gel or clot in the stomach. The ability to form this clot makes it very efficient in nutrient supply. The clot is able to provide a sustained slow release of amino acids into the blood stream, sometimes lasting for several hours (Boirie et al. 1997). This provides better nitrogen retention and utilization by the body.

Bovine Colostrum
Bovine colostrum is the "pre" milk liquid secreted by female mammals the first few days following birth. This nutrient-dense fluid is important for the newborn for its ability to provide immunities and assist in the growth of developing tissues in the initial stages of life. Evidence exists that bovine colostrum contains growth factors that stimulate cellular growth and DNA synthesis (Kishikawa et al., 1996), and as might be expected with such properties, it makes for interesting choice as a potential sports supplement.

Although bovine colostrum is not typically thought of as a food supplement, the use by strength/power athletes of this protein supplement as an ergogenic aid has become common. Oral supplementation of bovine colostrum has been demonstrated to significantly elevate insulin-like-growth factor 1 (IGF-1) (Mero et al., 1997) and enhance lean tissue accruement (Antonio et al., 2001; Brinkworth et al., 2004). However, the results on athletic performance improvement are less conclusive. Mero and colleagues (1997) reported no changes in vertical jump performance following 2-weeks of supplementation, and Brinkworth and colleagues (2004) saw no significant differences in strength following 8-weeks of training and supplementation in both trained and untrained subjects. In contrast, following 8-weeks of supplementation significant improvements in sprint performance were seen in elite hockey players (Hofman et al., 2002). Further research concerning bovine colostrum supplementation is still warranted.

Vegetable Protein
Vegetable proteins, when combined to provide for all of the essential amino acids, provide an excellent source for protein considering that they will likely result in a reduction in the intake of saturated fat and cholesterol. Popular sources include legumes, nuts and soy. Aside from these products, vegetable protein can also be found in a fibrous form called textured vegetable protein (TVP). TVP is produced from soy flour in which proteins are isolated. TVP is mainly a meat alternative and functions as a meat analog in vegetarian hot dogs, hamburgers, chicken patties, etc. It is also a low-calorie and low-fat source of vegetable protein. Vegetable sources of protein also provide numerous other nutrients such as phytochemicals and fiber that are also highly regarded in the diet diet.

Soy

Soy is the most widely used vegetable protein source. The soybean, from the legume family, was first chronicled in China in the year 2838 B.C. and was considered to be as valuable as wheat, barley, and rice as a nutritional staple. Soy's popularity spanned several other countries, but did not gain notoriety for its nutritional value in The United States until the 1920s. The American population consumes a relatively low intake of soy protein (5g · day^{-1}) compared to Asian countries (Hasler, 2002). Although cultural differences may be partly responsible, the low protein quality rating from the PER scale may also have influenced protein consumption tendencies. However, when the more accurate PDCAAS scale is used, soy protein was reported to be equivalent to animal protein with a score of 1.0, the highest possible rating (Hasler, 2002). Soy's quality makes it a very attractive alternative for those seeking non-animal sources of protein in their diet and those who are lactose intolerant. Soy is a complete protein with a high concentration of BCAA's. There have been many reported benefits related to soy proteins relating to health and performance (including reducing plasma lipid profiles, increasing LDL-cholesterol oxidation and reducing blood pressure), however further research still needs to be performed on these claims.

Soy Protein Types

The soybean can be separated into three distinct categories; flour, concentrates, and isolates. Soy flour can be further divided into natural or full-fat (contains natural oils), defatted (oils removed), and lecithinated (lecithin added) forms (Hasler, 2002). Of the three different categories of soy protein products, soy flour is the least refined form. It is commonly found in baked goods. Another product of soy flour is called *textured soy flour*. This is primarily used for processing as a meat extender. See Table 3 for protein composition of soy flour, concentrates, and isolates.

Table 3. Protein composition of soy protein forms.

Soy Protein Form	Protein Composition
Soy Flour	50%
Soy Concentrate	70%
Soy Isolate	90%

Soy concentrate was developed in the late 1960s and early 1970s and is made from defatted soybeans. While retaining most of the bean's protein content, concentrates do not contain as much soluble carbohydrates as flour, making it more palatable. Soy concentrate has a high digestibility and is found in nutrition bars, cereals, and yogurts.

Isolates are the most refined soy protein product containing the greatest concentration of protein, but unlike flour and concentrates, contain no dietary fiber. Isolates originated around the 1950s in The United States. They are very digestible and easily introduced into foods such as sports drinks and health beverages as well as infant formulas.

Nutritional Benefits

For centuries, soy has been part of a human diet. Epidemiologists were most likely the first to recognize soy's benefits to overall health when considering populations with a high intake of soy. These populations shared lower incidences in certain cancers, decreased cardiac conditions, and improvements in menopausal symptoms and osteoporosis in women (Hasler, 2002). Based upon a multitude of studies examining the health benefits of soy protein the American Heart Association issued a statement that recommended soy protein foods in a diet low in saturated fat and cholesterol to promote heart health (Erdman, 2000). The health benefits associated with soy protein are related to the physiologically active components that are part of soy, such as protease inhibitors, phytosterols, saponins, and isoflavones (Potter, 2000). These components have been noted to demonstrate lipid-lowering effects, increase LDL-cholesterol oxidation, and have beneficial effects on lowering blood pressure.

Isoflavones

Of the many active components in soy products, isoflavones have been given considerably more attention than others. Isoflavones are thought to be beneficial for cardiovascular health, possibly by lowering LDL concentrations (Crouse et al., 1999) increasing LDL oxidation (Tikkanen et al., 1998) and improving vessel elasticity (Nestel et al., 1999). However, these studies have not met without conflicting results and further research is still warranted concerning the benefits of isoflavones.

Soy Benefits for Women

An additional focus of studies investigating soy supplementation has been on women's health issues. It has been hypothesized that considering that isoflavones are considered phytoestrogens (exhibit estrogen- like effects and bind to estrogen receptors) they compete for estrogen receptor sites in breast tissue with endogenous estrogen, potentially reducing the risk for breast cancer risk (Wu et al. 1998). Still, the association between soy intake and breast cancer risk remains inconclusive. However, other studies have demonstrated positive effects of soy protein supplementation on maintaining bone mineral content (Ho et al., 2003) and reducing the severity of menopausal symptoms (Murkies et al., 1995).

High Protein Diets

Increased protein intakes and supplementation have generally been focused on athletic populations. However, over the past few years high protein diets have become a method used by the general population to enhance weight reduction. The low-carbohydrate, high protein, high fat diet promoted by Atkins may be the most popular diet used today for weight loss in the United States (Johnston et al., 2004). The basis behind this diet is that protein is associated with feelings of satiety and voluntary reductions in caloric consumption (Araya et al., 2000; Eisenstein et al., 2002). A recent study has shown that the Atkins diet can produce greater weight reduction at 3 and 6 months than a low-fat, high carbohydrate diet based upon U.S. dietary guidelines (Foster et al., 2003). However, potential health concerns have arisen concerning the safety of high protein diets. In 2001, the American Heart Association published a statement on dietary protein and weight reduction and suggested that individuals following such a diet may be at potential risk for metabolic, cardiac, renal, bone and liver diseases (St. Jeor et al., 2001).

Protein Intake and Metabolic Disease Risk

One of the major concerns for individuals on high protein, low carbohydrate diets is the potential for the development of metabolic ketosis. As carbohydrate stores are reduced the body relies more upon fat as its primary energy source. The greater amount of free fatty acids that are utilized by the liver for energy will result in a greater production and release of ketone bodies in the circulation. This will increase the risk for metabolic acidosis and can potentially lead to a coma and death. A recent multi-site clinical study (Foster et al., 2003) examined the effects of low-carbohydrate, high protein diets and reported significant elevation in ketone bodies during the first three months of the study. However, as the study duration continued the percentage of subjects with positive urinary ketone concentrations became reduced, and by six months urinary ketones were not present in any of the subjects.

Dietary Protein and Cardiovascular Disease Risk

High protein diets have also been suggested to have negative effects on blood lipid profiles and blood pressure, causing an increase risk for cardiovascular disease. This is primarily due to the higher fat intakes associated with these diets. However, this has not been proven in any scientifically controlled studies. Hu et al., (1999) have reported an inverse relationship between dietary protein (animal and vegetable) and risk of cardiovascular disease in women, and Jenkins and colleagues (2001) reported a decrease in lipid profiles in individuals consuming a high protein diet. Furthermore, protein intake has been shown to often have a negative relationship with blood pressure (Obarzanek et al., 1996). Thus, the concern for elevated risk for cardiovascular disease from high protein diets appears to be without merit. Likely, the reduced body weight associated with this type of diet is facilitating these changes.

In strength/power athletes who consume high protein diets, a major concern was the amount of food being consumed that was high in saturated fats. However, through better awareness and nutritional education many of these athletes are able to obtain their protein from sources that minimizes the amount of fat consumed. For instance, removing the skin from chicken breast, consuming fish and lean beef, and egg whites. In addition, many protein supplements are available that contain little to no fat. It should be acknowledged though that if elevated protein does come primarily from meats, dairy products and eggs, without regard to fat intake, there likely would be an increase in the consumption of saturated fat and cholesterol.

Dietary Protein and Renal Function

The major concern associated with renal function was the role that the kidneys have in nitrogen excretion and the potential for a high protein diet to over-stress the kidneys. In healthy individuals there does not appear to be any adverse effects of a high protein diet. In a study on bodybuilders consuming a high protein (2.8 $g \cdot kg^{-1}$) diet no negative changes were seen in any kidney function tests (Poortsman and Dellalieux, 2000). However, in individuals with existing kidney disease it is recommended that they limit their protein intake to approximately half of the normal RDA level for daily protein intake (0.8 $g \cdot kg^{-1} \cdot day^{-1}$). Lowering protein intake is thought to reduce the progression of renal disease by decreasing hyperfiltration (Brenner et al., 1996).

Dietary Protein and Bone

High protein diets are associated with an increase in calcium excretion. This is apparently due to a consumption of animal protein, which is higher in sulfur-based amino acids than vegetable proteins (Remer and Manz, 1994; Barzel and Massey, 1998). Sulfur-based amino acids are thought to be the primary cause of calciuria (calcium loss). The mechanism behind this is likely related to the increase in acid secretion due to the elevated protein consumption. If the kidneys are unable to buffer the high endogenous acid levels, other physiological systems will need to compensate, such as bone. Bone acts as a reservoir of alkali, and as a result calcium is liberated from bone to buffer high acidic levels and restore acid-base balance. The calcium released by bone is accomplished through

osteoclast-mediated bone resorption (Arnett and Spowage, 1996). Bone resorption (loss or removal of bone) will cause a decline in bone mineral content and bone mass (Barzel, 1976), increasing the risk for bone fracture and osteoporosis.

The effect of the type of protein consumed on bone resorption has been examined in a number of studies. Sellmeyer and colleagues (2001) examined the effects of various animal-to- vegetable protein ratio intakes in elderly women (> 65 y). They showed that the women consuming the highest animal to vegetable protein ratio had nearly a 4-fold greater risk of hip fractures compared with women consuming a lower animal to vegetable protein ratio. Interestingly, they did not report any significant association between the animal to vegetable protein ratio and bone mineral density. Similar results were shown by Feskanich et al (1996), but in a younger female population (age range = 35 – 59 mean 46). In contrast, other studies examining older female populations have shown that elevated animal protein will increase bone mineral density, while increases in vegetable protein will have a lowering effect on bone mineral density (Munger et al., 1999; Promislow et al., 2002). Munger and colleagues (1999) also reported a 69% lower risk of hip fracture as animal protein intake increased in a large (32,000) postmenopausal population. Other large epidemiological studies have also confirmed elevated bone density following high protein diets in both elderly men and women (Dawson-Hughes et al., 2002; Hannan et al., 2000). Hannon and colleagues (2000) demonstrated that animal protein intake in an older population, several times greater than the RDA requirement, results in a bone density accruement and significant decrease in fracture risk. Dawson-Hughes et al (2002), not only showed that animal protein will not increase urinary calcium excretion, but was also associated with higher levels of IGF-I and lower concentrations of the bone resorption marker N-telopeptide.

These conflicting results have contributed to the confusion regarding protein intake and bone. It is likely that other factors play an important role in further understanding the influence that dietary proteins have on bone loss or gain. For instance, the intake of calcium may have an essential function in maintaining bone. A higher calcium intake results in more absorbed calcium and may offset the losses induced by dietary protein and reduce the adverse effect of the endogenous acidosis on bone resorption (Dawson-Hughes, 2003). Furthermore, it is commonly assumed that animal proteins have a higher content of sulfur-containing amino acids per g of protein. However, examination of Table 4 shows that this may not entirely correct. If protein came from wheat sources it would have a mEq of 0.69 per g of protein, while protein from milk contains 0.55 mEq per g of protein. Thus, some plant proteins may have a greater potential to produce more mEq of sulfuric acid per g of protein than some animal proteins (Massey, 2003). Finally, bone resorption may be related to the presence or absence of a vitamin D receptor allele. In subjects that had this specific allele a significant elevation in bone resorption markers were present in the urine following 4-weeks of protein supplementation, while in subjects without this specific allele had no increase in N-telopeptide (Harrington et al., 2004). The effect of protein on bone health is still unclear, but it does appear to be prudent to monitor the amount of animal protein in the diet for susceptible individuals. This may be more pronounced in individuals that may have a genetic endowment for this. However, if animal protein consumption is modified by other nutrients (e.g. calcium) the effects on bone health may be lessened.

Table 4. Potential acid as sulfate from sulfur-containing amino acids.

Food	mEq per g of protein
Oatmeal	.82
Egg	.80
Walnuts	.74
Pork	.73
Wheat (whole)	.69
White Rice	.68
Barley	.68
Tuna	.65
Chicken	.65
Corn	.61
Beef	.59
Milk	.55
Cheddar	.46
Soy	.40
Peanuts	.40
Millet	.31
Almonds	.23
Potato	.23

Adapted from Massey, 2003.

Protein Intake and Liver Disease Risk

The American Heart Association has suggested that high protein diets may have detrimental effects on liver function (St. Jeor et al., 2001). This is primarily the result of a concern that the liver will be stressed through metabolizing the greater protein intakes. However, there is no scientific evidence to support this contention. Jorda and colleagues (1988) did show that high protein intakes in rats produce morphological changes in liver mitochondria. However, they also suggested that these changes were not pathological, but represented a positive hepatocyte adaptation to a metabolic stress.

Protein is important for the liver not only in promoting tissue repair, but to provide lipotropic

agents such as methionine and choline for the conversion of fats to lipoprotein for removal form the liver (Navder and Leiber, 2003a). The importance of high protein diets has also been acknowledged for individuals with liver disease and who are alcoholics. High protein diets may offset the elevated protein catabolism seen with liver disease (Navder and Leiber, 2003b), while a high protein diet has been shown to improve hepatic function in individuals suffering from alcoholic liver disease (Mendellhall et al., 1993).

Comparisons between Different Protein Sources on Human Performance

Earlier discussions on protein supplementation and athletic performance have shown positive effects from proteins of various sources. However, only limited research is available on comparisons between various protein sources and changes in human performance. Recently, there have been a number of comparisons between bovine colostrum and whey protein. The primary reason for this comparison is the use by these investigators of whey protein as the placebo group in many of the studies examining bovine colostrum (Antonio et al., 2001; Brinkworth et al., 2004; Brinkworth and Buckley, 2004; Coombes et al., 2002; Hofman et al., 2002). The reason being that whey protein is similar in taste and texture as bovine colostrum protein.

Studies performed in non-elite athletes have been inconclusive concerning the benefits of bovine colostrum compared to whey protein. Several studies have demonstrated greater gains in lean body mass in individuals supplementing with bovine colostrum than whey, but no changes in endurance or strength performance (Antonio et al., 2001; Brinkworth et al., 2004). However, when performance was measured following prolonged exercise (time to complete 2.8 $kJ \cdot kg^{-1}$ of work following a 2-hour ride) supplement dosages of 20 $g \cdot day^{-1}$ and 60 $g \cdot day^{-1}$ were shown to significantly improve time trial performance in competitive cyclists (Coombes et al., 2002). These results may be related to an improved buffering capacity following colostrum supplementation. Brinkworth and colleagues (2002) reported that although no performance changes were seen in rowing performance, the elite rowers that were studied did demonstrate an improved buffering capacity following 9-weeks of supplementation with 60 $g \cdot day^{-1}$ of bovine colostrum when compared to supplementing with whey protein. The improved buffering capacity subsequent to colostrum supplementation may have also influenced the results reported by Hofman et al., (2002). In that study elite field hockey players supplemented with either 60 $g \cdot day^{-1}$ of either colostrum or whey protein for 8-weeks. A significantly greater improvement was seen in repeated sprint performance in the group supplementing with colostrum compared to the group supplementing with whey protein. However, a recent study has suggested that the improved buffering system seen following colostrum supplementation is not related to an improved plasma buffering system, and that any improved buffering capacity occurs within the tissue (Brinkworth et al., 2004).

In a comparison between casein and whey protein supplementation, Boirie and colleagues (1997) showed that a 30-g feeding of casein versus whey had significantly different effects on postprandial protein gain. They showed that following whey protein ingestion the plasma appearance of amino acids is fast, high and transient. In contrast, casein is absorbed more slowly producing a much less dramatic rise in plasma amino acid concentrations. Whey protein ingestion stimulated protein synthesis by 68%, while casein ingestion stimulated protein synthesis by 31%. When the investigators compared postprandial leucine balance after 7-hours post ingestion, casein consumption resulted in a significantly higher leucine balance, whereas no change from baseline was seen 7-hours following whey consumption. These results suggest that whey protein stimulates a rapid synthesis of protein, but a large part of this protein is oxidized (used as fuel), while casein may result in a greater protein accretion over a longer duration of time. A subsequent study showed that repeated ingestions of whey protein (an equal amount of protein but consumed over a prolonged period of time [4 hours] compared to a single ingestion) produced a greater net leucine oxidation than either a single meal of casein or whey (Dangin et al., 2001). Interestingly, both casein and whey are complete proteins but their amino acid composition is different. Glutamine and leucine have important roles in muscle protein metabolism, yet casein contains 11.6 and 8.9 g of these amino acids, respectively while whey contains 21.9 and 11.1 g of these amino acids, respectively. Thus, the digestion rate of the protein may be more important than the amino acid composition of the protein.

In a study examining the effects of casein and whey on body composition and strength measures, 12 weeks of supplementation on overweight police officers showed significantly greater strength and lean tissue accruement in the subjects ingesting casein compared to whey (Demling and DeSanti, 2000). Protein supplementation provided a relative protein consumption of 1.5 $g \cdot kg \cdot day^{-1}$. Subjects supplemented twice per day approximately 8–10 hours apart.

Only one study known has compared colostrum, whey and casein supplementation (Fry et

al., 2003). Following 12-weeks of supplementation the authors reported no significant differences in lean body mass, strength or power performances between the groups. However, the results of this study should be examined with care. The subjects were comprised of both males and females who were resistance training for recreational purposes. In addition, the subject number for each group ranged from 4–6 subjects per group. With a heterogeneous subject population and a low subject number, the statistical power of this study was quite low. However, the authors did analyze effect sizes to account for the low statistical power. This analysis though did not change any of the observations. Clearly, further research is needed in comparisons of various types of protein on performance improvements. However, it is likely that a combination of different proteins from various sources may provide optimal benefits for performance.

CONCLUSIONS

It does appear that protein from animal sources is an important source of protein for humans from infancy until mature adulthood. However, the potential health concerns associated with a diet of protein consumed primarily from animal sources should be acknowledged. With a proper combination of sources, vegetable proteins may provide similar benefits as protein from animal sources. Maintenance of lean body mass though may become a concern. However, interesting data does exist concerning health benefits associated with soy protein consumption.

In athletes supplementing their diets with additional protein, casein has been shown to provide the greatest benefit for increases in protein synthesis for a prolonged duration. However, whey protein has a greater initial benefit for protein synthesis. These differences are related to their rates of absorption. It is likely a combination of the two could be beneficial, or smaller but more frequent ingestion of whey protein could prove to be of more value. Considering the paucity of research examining various sources of protein in sport supplementation studies, further research appears warranted on examining the benefits of these various protein sources.

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KEY POINTS

- Higher protein needs are seen in athletic populations.
- Animal proteins is an important source of protein, however potential health concerns do exist from a diet of protein consumed from primarily animal sources.
- With a proper combination of sources, vegetable proteins may provide similar benefits as protein from animal sources.
- Casein protein supplementation may provide the greatest benefit for increases in protein synthesis for a prolonged duration.

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Soy Protein, Isoflavones, and Cardiovascular Health: An American Heart Association Science Advisory for Professionals From the Nutrition Committee

Frank M. Sacks, Alice Lichtenstein, Linda Van Horn, William Harris, Penny Kris-Etherton, Mary Winston and for the American Heart Association Nutrition Committee

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AHA Science Advisory

Soy Protein, Isoflavones, and Cardiovascular Health

An American Heart Association Science Advisory for Professionals From the Nutrition Committee

Frank M. Sacks, MD; Alice Lichtenstein, DSc; Linda Van Horn, PhD, RD; William Harris, PhD; Penny Kris-Etherton, PhD; Mary Winston, EdD; for the American Heart Association Nutrition Committee

Abstract*—**Soy protein and isoflavones (phytoestrogens) have gained considerable attention for their potential role in improving risk factors for cardiovascular disease. This scientific advisory assesses the more recent work published on soy protein and its component isoflavones. In the majority of 22 randomized trials, isolated soy protein with isoflavones, as compared with milk or other proteins, decreased LDL cholesterol concentrations; the average effect was ≈3%. This reduction is very small relative to the large amount of soy protein tested in these studies, averaging 50 g, about half the usual total daily protein intake. No significant effects on HDL cholesterol, triglycerides, lipoprotein(a), or blood pressure were evident. Among 19 studies of soy isoflavones, the average effect on LDL cholesterol and other lipid risk factors was nil. Soy protein and isoflavones have not been shown to lessen vasomotor symptoms of menopause, and results are mixed with regard to soy's ability to slow postmenopausal bone loss. The efficacy and safety of soy isoflavones for preventing or treating cancer of the breast, endometrium, and prostate are not established; evidence from clinical trials is meager and cautionary with regard to a possible adverse effect. For this reason, use of isoflavone supplements in food or pills is not recommended. Thus, earlier research indicating that soy protein has clinically important favorable effects as compared with other proteins has not been confirmed. In contrast, many soy products should be beneficial to cardiovascular and overall health because of their high content of polyunsaturated fats, fiber, vitamins, and minerals and low content of saturated fat. (Circulation*. 2006;113:1034-1044.**)

Key Words: AHA Scientific Statements ■ cardiovascular diseases ■ soybean proteins ■ isoflavones ■ cholesterol

Soy protein has gained considerable attention for its potential role in improving risk factors for cardiovascular disease (CVD). In October 1999, the US Food and Drug Administration (FDA) approved labeling for foods containing soy protein as protective against coronary heart disease.[1] The FDA based this decision on clinical studies showing that at least 25 g of soy protein per day lowered total and LDL cholesterol. The FDA requires for the claim that a serving contain at least 6.25 g of soy protein, 25% of the necessary daily amount (25 g), with the expectation that foods containing soy protein would be eaten at least 4 times per day. The FDA also stated that "the evidence did not support a significant role for soy isoflavones in cholesterol-lowering effects of soy protein."[1]

In 2000, the American Heart Association (AHA) Nutrition Committee released a scientific advisory on soy protein and CVD.[2] At that time, the conclusion was that "it is prudent to recommend including soy protein foods in a diet low in saturated fat and cholesterol." Since then, many well-controlled studies on soy protein and soy-derived isoflavones substantially added to the knowledge base. For this reason, the AHA Nutrition Committee decided to reevaluate the evidence on soy protein and CVD and update its scientific advisory. Thus, this scientific advisory assesses the more recent work published on soy protein and its component isoflavones. The focus is on blood LDL cholesterol because it is by far the most studied risk factor for CVD, is the primary criterion on which the National Cholesterol Education Program estimates risk and recommends therapy,[3] and forms the basis for the FDA-approved health claim. In this advisory, we also consider the effects of soy protein and isoflavones on several other CVD risk factors: HDL cholesterol, triglycer-

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ides, lipoprotein(a), and blood pressure. The medical literature was searched comprehensively for original research publications on the effects of soy protein or isoflavones on CVD risk factors, and all controlled trials that separately listed soy protein and isoflavone content were used. In addition, this advisory reviews the evidence on soy products in other health conditions, including menopausal symptoms, osteoporosis, and cancer.

Soy protein, like any other dietary protein, contains calories and could be used in the diet to replace animal protein or other vegetable proteins. Soy protein also could replace other sources of calories such as carbohydrate or fat, raising the total amount of protein eaten and reducing carbohydrate or fat intake. Most studies exchanged soy protein for other dietary proteins, and this evidence is evaluated in the present advisory. Much less is known about the potential impact on risk factors for CVD of increasing total protein intake by adding soy or other plant protein in place of carbohydrate or fat; this important dietary change is currently being studied.

The Soy Protein Hypothesis on LDL Cholesterol

An Overview

Animal proteins raise blood cholesterol concentrations in several animal species fed cholesterol-free semisynthetic diets.[4,5] Casein, the most prevalent protein in milk, has been used most often, although other animal proteins such as pork and beef protein do the same. This is a useful established nutritional model for studying diet-induced hypercholesterolemia and atherosclerosis and an alternative to feeding animals large amounts of cholesterol. In contrast, when soy protein is substituted for the animal protein, hypercholesterolemia does not occur. Thus, either some animal proteins have a direct hypercholesterolemic action, or soy protein has a cholesterol-lowering action. This latter possibility led to intensive work in the late 1970s and 1980s to test the hypothesis that soy protein can be a nutritional approach to reducing blood cholesterol. This concept gained support from epidemiological observations on diet and CVD in Japan and other Asian countries where large amounts of soy products were eaten and blood cholesterol concentration and CVD incidence were low.[6] However, many differences in diet and lifestyle between Asian and Western countries could explain the differences in the prevalence of CVD.

Early indications that soy protein had much less effect in humans than in animals came from direct application of the animal model to humans. Diets similar to those eaten by humans, based on either soy protein or casein, were fed to rabbits, and, as expected, casein produced hypercholesterolemia.[7] However, when the same diets were fed to healthy people, the protein source did not affect blood cholesterol.[7,8] Others studied the effect of casein in strict vegetarians who ate no dairy or animal proteins to provide a human counterpart to the mainly vegetarian animal (eg, rabbit) models. Compared with soy protein, no effect of casein on blood cholesterol was found.[9]

In the late 1970s and early 1980s, the soy protein hypothesis was greatly strengthened as a result of studies by Sirtori et al[10] and Descovich et al,[11] who found that diets high in soy protein, replacing nearly all the animal protein, substantially reduced blood LDL cholesterol by 20% to 30% in severe hypercholesterolemia. Because the soy protein diets were also reduced in saturated fat and cholesterol and increased in polyunsaturated fat and because the patients also often lost weight on the dietary protocols, the results were often confounded. The authors reported that textured soy protein (50% soy flour, 50% soy protein concentrate) but not soy protein isolate (90% soy protein) was effective. This raised the possibilities that, rather than the soy protein itself, the nonprotein components of the soy protein preparation or the effect of soy displacing cholesterol-raising fats in the diet could have had a blood cholesterol–lowering action. Results of other early studies of soy protein in hypercholesterolemic subjects showed either cholesterol reduction[12] or no effect.[13,14]

A meta-analysis published in 1995 attempted to reconcile the many divergent findings among studies of soy protein.[15] In 29 controlled studies, a trend emerged that soy protein selectively reduced blood cholesterol in direct proportion to the degree of hypercholesterolemia. For example, in those with severely elevated blood cholesterol (>335 mg/dL), soy protein reduced blood cholesterol by 20%. Only a 7% reduction occurred in those with cholesterol levels between 259 and 333 mg/dL; if the initial blood cholesterol was <255 mg/dL, there was no significant effect. Thus, the response to soy protein was determined more by the initial blood cholesterol level and, surprisingly, not by the amount of soy protein eaten, which ranged widely from 18 to 124 g/d. When the control group was not included in the statistical analysis, there was a significant correlation between the dose of soy protein and the degree of cholesterol reduction. However, an analysis without a control group introduces the effects of confounding and drift in serum cholesterol that often occur in experimental situations. This meta-analysis also was limited by the quality of the studies; studies were less well controlled in people with hypercholesterolemia than in those with average cholesterol levels. It is difficult to determine how much effect this had on the overall results of the meta-analysis. Thus, the available literature provided some support, albeit with limitations, for the concept that soy protein is an effective treatment for severe hypercholesterolemia, that it produces a mild benefit in people with moderate elevations of cholesterol, but that it has no effect in those with mildly elevated or average cholesterol levels. The soy protein hypothesis culminated in FDA approval of a health claim for soy protein in foods.

Soy Isoflavones

Subsequent to the meta-analysis by Anderson et al,[15] many well-controlled studies explored the soy protein hypothesis with greater specificity. In addition, recognition that soy protein products contain bioactive molecules called phytoestrogens or isoflavones added a fascinating new aspect to the soy protein hypothesis.[16,17,18] Isoflavones remain in soy protein preparations that are not extracted with alcohol. During the preparation of soy protein isolate, the soy is washed with alcohol, removing a substantial amount of the

isoflavones. The soy isoflavones have strong biological properties in animals, causing arterial vasodilation, lowering serum cholesterol,[18] and inhibiting atherosclerosis in postmenopausal monkeys.[19] This led to the intriguing idea that the presence and amount of isoflavones explain the variable results of soy studies; only those that used high-isoflavone preparations produced favorable results.[18,20] Isoflavone content was not known in many of the earlier studies. Several subsequent studies tested the effects of soy protein and isoflavones separately.

The 3 major isoflavones found in soybeans are genistin, daidzin, and glycitin. Their abundance in soy protein preparations varies widely and depends on the processing techniques used during production.[21,22] These compounds have both estrogenic and antiestrogenic activity[23,24] and effects that are unrelated to estrogen activity.[25] Dehulling, flaking, and defatting soybeans produces a relatively pure preparation of protein that is low in isoflavones,[26,27] whereas methods used to produce textured soy protein result in a preparation that retains the isoflavones.[21] Isoflavone concentrations range from ≈2 mg/g protein in textured soy protein, soy flour, and soy granules to 0.6 to 1.0 mg/g protein in isolated soy protein. Intakes of 45 g soy flour have resulted in a 20- to 40-fold increase and a 50- to 100-fold increase in blood and urinary isoflavones, respectively,[28] and there is a dose-dependent relationship at more moderate intakes.[29]

Effect of Soy Protein on LDL Cholesterol and Other Lipoproteins

Soy Protein With Isoflavones

First, we summarize studies that tested soy protein that contained a substantial amount of isoflavones. Because it was recognized that isoflavones could be the bioactive component attributed to soy protein, studies published in the late 1990s and beyond generally stated the amount and type of isoflavones in the soy protein. In 22 randomized trials, isolated soy protein with isoflavones was compared with casein or milk protein,[20,30–46] wheat protein,[47] or mixed animal proteins.[48–50] The range of soy protein was 25 to 135 g/d; the range for isoflavones was 40 to 318 mg. LDL or non-HDL cholesterol concentrations decreased in most studies, statistically significantly in 8, with an overall effect of ≈3% (weighted average). A recent meta-analysis that included 10 studies published from 1995 to 2002 found a similar percentage reduction in LDL cholesterol with no dose effect.[51] Over all studies in Table 1, there is no apparent dose effect; the 8 studies with 50 g of soy protein showed a drop in LDL cholesterol concentration similar to those using a smaller amount of soy, ≈3% overall (Table 1). This cutpoint for daily soy protein intake, 50 g, defines a large amount, half or more of the daily average total protein intake in the United States. No significant effects were evident for HDL cholesterol or triglycerides in most of the studies; the weighted average effects were very small: 1.5% for HDL cholesterol and −5% for triglycerides.

Soy Protein Without Isoflavones

In 7 trials, soy protein, washed with alcohol to remove isoflavones, was compared with casein or milk protein[20,33,39,43,52] or various animal proteins (Table 2).[49,50] Two studies showed small significant decreases in LDL cholesterol.[49,50] These studies were very carefully controlled feeding studies, with all meals formulated according to strict nutritional specifications, and complete meals were provided to the participants.[49,50] Specifically designed to sort out the effects of the protein from the effects of the isoflavones, the studies showed an effect of protein but not isoflavones on LDL cholesterol. The declines in LDL cholesterol were small, 2% to 7%, relative to the large amounts of soy protein eaten daily, 50 to 55 g. However, other well-controlled studies did not find significant effects of soy protein on LDL cholesterol,[20,33,39,43,52] and the average change across all 7 studies was only a 1% to 2% decrease. Changes in HDL cholesterol and triglycerides were generally small and were nonsignificant in 6 of the 7 trials. No dose effect was evident.

Effect of Isoflavones

Some studies compared soy protein that did or did not contain isoflavones (Table 3),[20,30,33,39,43,49,50,52–57] whereas other studies tested isoflavones in pill form as compared with placebo.[58–63] A wide range of isoflavone amounts was studied. One study compared the effect of isoflavones provided with either soy or animal proteins.[49] Among these 19 studies,[20,30,33,39,43,49,50,52–63] only 3 showed significant reductions in LDL cholesterol concentration,[52,55,56] and the effect among all studies (weighted average) was nil, 0%. Changes in HDL cholesterol and triglycerides were not significant and showed no trend toward an effect of isoflavones. Despite large increases in blood isoflavone concentrations, there is no indication of a dose effect on blood lipids. A recent meta-analysis concluded that isoflavones do not affect blood lipid concentrations.[51]

Influence of Initial Blood LDL Cholesterol Level

In the Anderson et al[15] meta-analysis, a strong gradient of LDL cholesterol reduction was found among studies according to initial cholesterol level. Lichtenstein et al[49] and Crouse et al[20] found slightly more LDL cholesterol reduction in people with LDL cholesterol >160 to 164 mg/dL than in those with lower levels, although Dent et al[33] did not find an effect in women with hypercholesterolemia as compared with women with average cholesterol levels. However, a larger percentage reduction in LDL cholesterol in hypercholesterolemia is not evident among the 22 recent trials (Table 1). Among studies of isoflavones, no relation is evident between initial cholesterol and cholesterol lowering (Table 3).

Influence of Serum Cholesterol–Lowering Diet

In their meta-analysis, Anderson et al[15] reported that soy protein tended to have less effect on LDL cholesterol in trials in which the participants were eating a low-fat and low-cholesterol diet as compared with a more usual higher-fat and higher-cholesterol diet. In 11 of the studies listed in Tables 1 through 3, soy protein or isoflavones were tested in combination with a serum cholesterol–lowering diet.[20,30,31,34,42,45,47,48,50,58,60] The average reduction in LDL in these studies was 2%, similar to that in the full group. Thus, the effect on LDL of soy protein or isoflavones does not appear to be modulated by the saturated fat and cholesterol content of the diet.

TABLE 1. Soy Protein and Blood Lipid Risk Factors: Effects of Soy Protein With Isoflavones

Study and Year	Reference	n	Type	Age, y	Design	Dose	Duration	Base TC, mg/DL	TC, %	LDL, %	HDL, %	TG, %	Comments
West et al 2005	45	32	M, F, HC	58	X, DB	ISP 25 g IF 90 vs milk protein	6 wk	250	↑ 1 (NS)	0	↑ 1 (NS)	↑ 5 (NS)	
Kreijkamp-Kaspers et al 2004	44	88	F, HC	67	Para, DB	ISP 26 g + IF 99 mg vs milk protein	12 mo	240	↑ 2 (NS)	↑ 4 (NS)	↑ 3 (NS)	↓ 8 (NS)	
Steinberg et al 2003	43	28	F, NI	55	X, DB	ISP 25 g + IF 107 mg vs milk protein	6 wk	190	↓ 4 (NS)	↓ 3 (NS)	↓ 7 (NS)	↑ 6 (NS)	
Cuevas et al 2003	42	18	F, HC	59	X, DB	ISP 40 g + IF 80 mg vs casein	4 wk	285	↓ 1 (NS)	0	↑ 4 (NS)	↓ 15 (NS)	
Blum et al 2003	37	24	F, HC	55	X, DB	ISP 25 g + IF 85 mg vs milk prot	6 wk	270	↑ 1 (NS)	↑ 4 (NS)	↓ 4 (NS)	↓ 1 (NS)	
Dalais et al 2003	38	38	F, HC	60	Para, DB	ISP 40 g + IF 118 mg vs casein	3 mo	236	↓ 4 (NS)	↓ 6*	↑ 7 (NS)	↓ 26*	
Jenkins et al 2002	50	41	M, F, HC	62	X	ISP 50 g + IF 73 mg vs dairy + egg protein	1 mo	260	↓ 4*	↓ 6*	↑ 2 (NS)	↑ 2 (NS)	
Tonstad et al 2002	46	30	M, F, HC	52	Para	ISP 30–50 g + IF 111–185 mg vs casein	16 wk	270	↓ 4*	↓ 5*	↑ 3 (NS)	↓ 3 (NS)	No dose effect
Meinertz et al 2002	39	12	M, F, NI	30	X	ISP 133 g + IF 318 mg vs casein	32 d	164	↑ 8 (NS)	↓ 2 (NS)	↑ 10*	↑ 12 (NS)	Liquid diets
Lichtenstein et al 2002	49	42	M, 18; F, 24	63	X	F: ISP 55 g + IF 108 mg;	6 wk	236	↑ 1 (NS)	↑ 4 (NS)	↑ 3*	↓ 7*	
						M: ISP 71 g + IF 139 mg vs dairy and meat protein		280	↓ 7*	↓ 7*	↓ 1 (NS)	↓ 17 (NS)	
Sirtori et al 2002	41	20	M, F, HC	60	X, DB	Soy 25 g + IF 77 mg vs cow's milk	4 wk	325	↓ 3 (NS)	↓ 4 (NS)	ND	ND	
Puska et al 2002	36	30	HC	56	Para, DB	ISP 52 g + IF 192 mg vs casein	6 wk	290	↓ 3*	↓ 5*	↑ 1 (NS)	↑ 3 (NS)	
Dent et al 2001	33	24	F	50	Para	ISP 40 g + IF 80 mg vs milk protein	24 wk	220	No effect on lipids; numerical data not shown				
Van Horn et al 2001	34	62	F, HC	67	Para	ISP 29 g + IF 85 mg vs milk protein	6 wk	240	0	↓ 1 (NS)	↑ 1 (NS)	ND	
Teede et al 2001	35	90	M, F	61	Para, DB	ISP 40 g w/IF 118 mg vs casein	3 mo	225	↓ 2 (NS)	↓ 4 (NS)	↑ 5 (NS)	↓ 15*	
Hermansen et al 2001	40	20	DM	64	X, DB	ISP 50 g + IF 165 mg vs casein	6 wk	212	↓ 8	↓ 10*	0	↓ 9*	
Vigna et al 2000	32	40	F, Postmen	53	Para, DB	ISP 60 g + IF 76 mg vs casein	12 wk	240	0	↓ 1 (NS)	↑ 2 (NS)	↓ 1 (NS)	
Jenkins et al 2000	47	25	M, F, HC		X	ISP 36 g + IF 168 mg vs wheat protein	3 wk	270	↓ 2 (NS)	↓ 1 (NS)	↓ 2 (NS)	↓ 6 (NS)	
Teixeira et al 2000	31	16	M, HC	45	Para	ISP 50 g + IF 95 mg vs casein 50 g	6 wk	240	↓ 7*	Non-HDL ↓ 9*	↑ 2 (NS)	↑ 8 (NS)	No dose effect from 20–50 g
						ISP 20 g + IF 38 mg vs casein 50 g			↓ 5*	Non-HDL ↓ 7*	↑ 2 (NS)	↑ 1 (NS)	
Crouse et al 1999	20	30	M, F	52	Para, DB	ISP 25 g + IF 62 mg vs casein	9 wk	240	↓ 4*	↓ 6*	0 (NS)	↑ 9 (NS)	No effect in LDL <164 mg/dL group
High LDL group >164 mg/dL								260	↓ 9*	↓ 10*	↑ 4 (NS)	↓ 29*	
Wong et al 1998	48	26	HC, 13	38	X	ISP 50 g +IF vs mixed animal	5 wk	270	↓ 3	↓ 6*	↑ 3	↑ 6 (NS)	IF content not specified
			NI, 13					170	↓ 3	↓ 6*	0	↑ 6 (NS)	
Baum et al 1998	30	21	F, Postmen	61	Para, DB	ISP 40 g + IF 90 mg vs casein	24 wk	250	↓ 2 NS	Non-HDL ↓ 4*	↑ 4*	↑ 1 (NS)	

TC indicates total cholesterol; TG, triglycerides; M, male; F, female; HC, hypercholesterolemic; DM, diabetes mellitus; NI, normolipidemic; DB, double blind; X, crossover; Para, parallel group; ISP, isolated soy protein; IF, isoflavones; NS, not significant ($P>0.05$); and ND, not determined. Percentages are the mean change in soy protein minus the change in the control group.

*$P<0.05$ for effect of soy protein vs other protein.

Effects on Lipoprotein(a)

Lipoprotein(a), an LDL-like lipoprotein that is an independent predictor of CVD,[64] was increased by soy protein in 2 studies[35,65] and unchanged in 9 others.[20,31,32,36,40,44–46,50] Meinertz et al[39] found that alcohol-extracted soy protein, lacking isoflavones, did not raise lipoprotein(a) as found in their earlier study of intact soy protein,[65] which suggests an adverse effect of isoflavones. However, isoflavones had no effect on lipoprotein(a) in 6 other studies,[20,50,55,60,61,63] nor did soy protein that contained isoflavones.[20,31,32,36,40,44–46,50]

Effects on Blood Pressure

Several studies tested the effect of soy protein with isoflavones, as compared with casein or milk protein, on blood pressure.[32,35,40,42,47,50] Blood pressure decreased significantly in 1 study[35] but not in the other 5 studies.[32,40,42,47,50] The

TABLE 2. Effects of Soy Protein With Low or No Isoflavones

Study and Year	Reference	n	Type	Age, y	Design	Dose, g	Duration	Base TC, mg/dL	TC, %	LDL, %	HDL, %	TG, %
Steinberg et al 2003	43	28	F, NI	55	X,DB	ISP 25 vs milk protein	6 wk	190	↓ 2 (NS)	↓ 2 (NS)	↓ 4 (NS)	↑ 10 (NS)
Jenkins et al 2002	50	41	M, F, HC	62	X	ISP 50 vs dairy and egg protein	1 mo	260	↓ 6*	↓ 7 (NS)	↑ 2 (NS)	↓ 10 (NS)
Lichtenstein et al 2002	49	42	M, F	63	X	ISP 55 F; 71 M vs dairy and meat protein	6 wk	236	↑ 1 (NS)	↑ 3 (NS)	↑ 3%*	↓ 14*
								280	↓ 3*	↓ 5*	0	↓ 11 (NS)
Meinertz et al 2002	39	12	M, F, NI	30	X	ISP 133 vs casein liquid diets	32 d	164	↑ 4 (NS)	↑ 4 (NS)	↑ 6 (NS)	↑ 11 (NS)
Dent et al 2001	33	24	F	50	Para	ISP 40 vs milk protein	24 wk	220	No effect on lipids (data not shown)			
Gardner et al 2001	52	31	F, Postmen	60	Para	ISP 42 vs casein	12 wk	240	↑ 3 (NS)	↑ 5 (NS)	↑ 7 (NS)	↓ 8 (NS)
Crouse et al 1999	20	30	M, F	52	Para,DB	ISP 25 vs casein	9 wk	240	↓ 2 (NS)	↓ 2 (NS)	↓ 4 (NS)	↓ 1 (NS)
High LDL group >164 mg/dL								260	↓ 4 (NS)	↓ 5 (NS)	↑ 5 (NS)	↑ 21 (NS)

Abbreviations as in Table 1. Percentages are the mean change in the soy protein minus the change in the control group.
*$P<0.05$ for effect of soy protein vs other protein.

weighted average change is −1 mm Hg systolic blood pressure. Several studies that evaluated the effect of soy isoflavones also did not find a significant effect on blood pressure.[50,58,60,62,66]

Effects on Health Conditions Related to Estrogens

Menopausal Vasomotor Symptoms

Because of their weak estrogenic activity, soy isoflavones have been hypothesized to improve several estrogen-dependent conditions, including perimenopausal vasomotor symptoms (hot flashes) and postmenopausal bone loss. A recent review examined 11 clinical trials of soy protein or isoflavones[67] for treating hot flashes. Only 3 of 8 studies with treatment lasting >6 weeks found modest improvement in hot flashes, and most benefits disappeared after 6 weeks. Five additional studies[68–72] not included in that review showed no benefit for hot flashes of soy isoflavones. Longer studies showed no benefit of isoflavones at 24 weeks[73] or 2 years.[71] Substantial reduction in hot flashes, often 40% to 60%, occurred in the placebo or control group in these studies, similar to the reduction in the soy group. In contrast, estrogen replacement markedly reduces hot flashes, more so than placebo. Thus, it seems unlikely that soy isoflavones have enough estrogenic activity to have an important impact on vasomotor symptoms of estrogen deficiency in perimenopausal women.

Osteoporosis

Another estrogenic effect of soy isoflavones could be to reduce bone loss after menopause; this hypothesis gains strength from population studies and certain animal models of osteoporosis.[74] However, clinical trials so far have had insufficient duration and size to be conclusive, and results have varied.[44,74] The studies used either direct measurements of bone mineral content and density in the spine and hip or biochemical indices of bone resorption or formation to test the effect of soy isoflavones ranging in amount from 54 to 300 mg, but most studies used 80 to 110 mg. Soy isoflavones lessened bone loss over 6 to 24 months in some studies,[75–78] whereas other trials did not show a benefit over the same duration.[44,57,79] There is also inconsistency in the studies showing favorable effects, with one study showing benefit in the spine but not hip[75] and another showing the opposite,[77] or improvement in bone mineral content but not bone mineral density.[76,77] Diminution of bone loss, indicated by a reduction in biochemical markers of bone resorption, was found in some studies[78,80,81] but not in others.[38,44,53,55,82,83] The amounts of isoflavones were similar in studies that found favorable or no effects. The longest study in any primate species was in postmenopausal monkeys (cynomolgus macaques); after 3 years, soy isoflavones did not slow bone loss, whereas estrogen replacement increased bone mineral content and density, as expected.[84] These varied results of clinical trials suggest the need for investigations of isoflavones and bone health that have substantial sample size and long duration to provide a definitive result.

Cancer

The weak estrogenic action of soy isoflavones and other phytoestrogens suggested the possibility that they could lessen the deleterious effects of more potent endogenous estrogens on breast and endometrial cancer. This hypothesis came from the low incidence of breast and endometrial cancers in Asian countries where soy products are prevalent in the diet and from certain animal models of breast and endometrial cancer showing benefit of soy isoflavones.[85–87] In reality, a host of complexities have emerged that make it impossible to state a clinical recommendation for the use of soy isoflavones. In epidemiological studies, associations varied between intake of soy foods and isoflavones and incidence of breast cancer[85,88–90]; some showed protective associations, and others showed no association.[85,88–90] Clinical

TABLE 3. Effects of Isoflavones

Study and Year	Reference	n	Type	Age, y	Design	Dose, mg	Duration	Base TC, mg/dL	TC, %	LDL, %	HDL, %	TG, %
Nikander et al 2004	63	56	F, NI	55	X, DB	IF 117 vs 0 pills	3 mo	226	↑ 1 (NS)	↑ 9 (NS)	↓ 1 (NS)	↑ 1 (NS)
Gallagher et al 2004	57	17	F, NI	55	Para, DB	IF 96 vs 4; w/ISP	9 mo	218	↑ 1 (NS)	↑ 3 (NS)	↑ 3 (NS)	↓ 14 (NS)
						IF 52 vs 4; w/ISP			0	↑ 3 (NS)	↓ 2 (NS)	↓ 17 (NS)
Steinberg et al 2003	43	28	F, NI	55	X, DB	IF 107 vs 2; w/ISP	6 wk	190	↓ 2 (NS)	0	↓ 4 (NS)	↓ 4 (NS)
Jenkins et al 2002	50	41	M, F, HC	62	X, DB	IF 73 vs 10; w/ISP	1 mo	260	↑ 2 (NS)	↓ 1 (NS)	↓ 2 (NS)	↑ 20 (NS)
Lichtenstein et al 2002	49	42	M, F	63	X	IF 108–139 vs 0	6 wk	236	↑ 1 (NS)	↑ 3 (NS)	↑ 1 (NS)	↑ 4 (NS)
						w/ISP or animal protein		280	↓ 3*	↓ 2 (NS)	↓ 2 (NS)	↑ 2 (NS)
Squadrito et al 2002	58	30	F, Postmen	56	Para, DB	IF 54 vs 0 pills	6 mo	207	↑ 2 (NS)	↑ 5 (NS)	↓ 8 (NS)	↑ 26 (NS)
Sanders et al 2002	54	22	M, F, NI	30	X	IF 56 vs 0; w/ISP	2 wk	170	↑ 2 (NS)	0	↑ 4*	↑ 6 (NS)
Dewell et al 2002	59	20	F, Postmen	70	Para, DB	IF 150 vs 0 pills	6 mo	263	0	Non-HDL ↓ 6 (NS)	0	↓ 5 (NS)
Meinertz et al 2002	39	12	M, F, NI	30	X	IF 318 vs 0; w/ISP	32 d	164	↑ 4 (NS)	↓ 6 (NS)	↑ 4 (NS)	↑ 26*
Dent et al 2001	33	24	F	50	Para	IF 80 vs 0; w/ISP	24 wk	220	All NS (data not shown)			
								>220	All NS (data not shown)			
Gardner et al 2001	52	31	F, Postmen	60	Para	IF 80 vs 0; w/ISP	12 wk	240	↓ 3*	↓ 8*	0	0
Wangen et al 2001	55	18	F, Postmen	57	X	IF 65 vs 7; w/ISP	3 mo	215	↓ 2 (NS)	↓ 5 (NS)	↑ 2 (NS)	↑ 5 (NS)
						IF 132 vs 7; w/ISP			↓ 3 (NS)	↓ 6*	↑ 1 (NS)	↑ 5 (NS)
Mackey et al 2000	53	25	F, Postmen	56	Para, DB	IF 65 vs 4; w/ISP	12 wk	285	0	↓ 1 (NS)	↓ 2 (NS)	↑ 6 (NS)
Merz-Demlow et al 2000	56	13	F, Premen	26	X	IF129 vs 10; w/ISP	3 mo	150	NS	↓ 7*	0	0
						IF 65 vs 10; w/ISP			All NS			
Simons et al 2000	60	20	F, Postmen	59	X, DB	IF 80 vs 0 pills	8 wk	228	↓ 1 (NS)	↓ 2 (NS)	↓ 1 (NS)	↑ 5 (NS)
Crouse et al 1999	20	30	M, F	52	Para, DB	IF 62 vs 3; w/ISP	9 wk	240	↓ 4 (NS)	↓ 5 (NS)	↑ 4 (NS)	↓ 10 (NS)
High LDL group >164 mg/dL								260	↓ 6*	↓ 6 (NS)	↓ 1 (NS)	↓ 9 (NS)
Baum et al 1998	30	21	F, Postmen	61	Para, DB	IF 90 vs 56; w/ISP	24 wk	250	0	Non-HDL 0	↓ 4 (NS)	↑ 1 (NS)
Hodgson et al 1998	61	30	M,F, Postmen	56	Para, DB	IF 55 vs 0 pills	8 wk	210	↓ 1 (NS)	↓ 3 (NS)	↓ 1 (NS)	↑ 5 (NS)
Nestel et al 1997	62	21	F, Postmen	54	X	IF 80 vs 0 pills	5 wk	215	↑ 2 (NS)	↑ 2 (NS)	↓ 5 (NS)	↑ 18 (NS)

Abbreviations as in Table 1, plus Premen indicates premenopausal. Percentages are the mean change in the isoflavone minus the change in the control group. *$P<0.05$ for isoflavone effect.

studies suggested that soy phytoestrogens stimulate epithelial cell proliferation in breasts of premenopausal women, a potential precursor of cancer.[91,92] Animal and cell culture experiments also found a cancer-stimulating effect.[93–95] Phytoestrogens reduce the activity of enzymes that inactivate endogenous estrogens, potentially leading to increased active estrogen concentrations.[96] Nonlinear dose effects, unique effects of specific types of isoflavones, changes in isoflavone composition and structure during the processing of soy foods, and interperson variation in isoflavone metabolism all could affect cancer initiation and progression[22,86,87] and are virtually unexplored in the clinical arena. It has been hypothesized from animal experiments that soy isoflavones could be protective throughout adult life only if eaten in childhood or puberty.[97] Case-control studies in Shanghai[98] and in Asian Americans[99] found that high soy intake in adolescence was associated with low risk for breast cancer in adulthood. Finally, several recent expert reviews and editorials concluded that the research overall remains insufficient to know whether certain phytoestrogens are protective or harmful for breast cancer and at what dose and time period, if any, in a woman's life they are active.[87,100,101]

Concepts with regard to soy isoflavones and breast cancer are applicable to uterine endometrial cancer, an estrogen-dependent cancer, although data are much less extensive. Soy food or isoflavone intake was associated with low risk for endometrial cancer in case-control studies in Shanghai,[102] Hawaii,[103] and California.[104] This suggests that soy phytoestrogens have antiestrogenic effects on the uterus. However, a single pilot trial of soy isoflavones given together with estrogen to perimenopausal or postmenopausal women found no lessening of estrogen-mediated stimulation of the endometrium.[105] Several clinical trials found that isoflavones did not affect the uterine endometrium of perimenopausal or postmenopausal women.[105–110] However, these trials may have had insufficient duration (3 to 6 months) or sample size to identify an effect. Recently, a relatively large placebo-controlled trial in postmenopausal women found that isoflavone tablets caused endometrial hyperplasia, a precursor to cancer, after 5 years in 6 of 154 women compared with none on placebo ($P<0.05$).[111] Another 5 women in the phytoestrogen group had proliferative endometrium compared with none in the placebo group after 5 years. These effects were not found at 2½ years. Thus, some cautionary evidence indicates that soy phytoestrogens have enough estrogenic activity to stimulate the endometrium of postmenopausal women, although the evidence overall is inadequate to draw conclusions on whether soy protein or isoflavones taken by perimenopausal or postmenopausal women eventually would cause endometrial cancer.

Soy isoflavones have estrogenic, antiandrogenic, and other activities that could prevent prostate cancer or slow its

TABLE 4. Nutrient Content of Popular Soy-Containing Foods

Food Item	Quantity	Calories, kcal	Carbohydrates, g	Protein, g	Total Fat, g	Saturated Fat, g	Polyunsaturated Fat, g	n-3 Fatty Acids, g	n-6 Fatty Acids, g	Monounsaturated Fat, g	Cholesterol, mg	Sodium, mg	Dietary Fiber, g
Edamame	1/2 cup, 90 g	126	10	11	5	0.5	3	0.5	2.5	1.5	0	225	4
Miso	2 Tbsp, 34 g	71	10	4	2	0.5	1.1	0.1	1	0.5	0	1200	2
Tofu, extra firm	79 g	80	2	8	4	0.5	2.5	0.5	2	1	0	0	1
Tofu, firm	79 g	70	2	7	3	0.5	2	0.5	1.5	0.5	0	0	<1
Tofu, silken	91 g	45	2	4	2.5	0.5	1.5	0.5	1	0.5	0	5	0
Soy burger	1 patty, 57 g	60	6	13	0	0	0	0	0	0	0	270	3
Soy hot dog	1 link, 42 g	45	2	9	0	0	0	0	0	0	0	320	1
Roasted soy butter	2 Tbsp, 32 g	170	10	6	11	1.5	6	NA	NA	2.5	0	170	1
Soy milk, plain flavor	1 cup, 240 mL	100	8	7	4	0.5	2.5	0.5	2	1	0	120	1
Soy milk, chocolate	1 cup, 240 mL	140	23	5	3.5	0.5	2	0.2	1.8	1	0	100	2
Soy candy bar, chocolate	1 bar, 61.5 g	240	35	14	5	3	NA	NA	NA	NA	0	210	2
Soy nuts, roasted, unsalted	1 oz, 28 g	120	9	12	4	0	NA	NA	NA	NA	0	10	5

NA indicates not available. Values derived from Nutritionist Pro, version 2.10.13, First DataBank, Inc, 2004.

progression.[86,87,112,113] Prostate cancer incidence is relatively low in Asian countries where soy products are commonly eaten, and certain epidemiological studies have shown an inverse association between soy foods, serum phytoestrogen levels, and prostate cancer.[113,114] However, as pointed out by Messina,[113] the epidemiological findings are inconsistent, and there are important limitations in study design. Soy isoflavones prevent the development and growth of prostate cancer in animal models. In prostate cancer cells, genistein reduced the synthesis of prostate-specific antigen, a marker of prostate cancer development and progression that is in extensive clinical use.[86] However, soy isoflavones did not reduce either prostate-specific antigen or serum testosterone levels in men with early-stage prostate cancer[112,115,116] or in healthy middle-aged men.[117] Thus, the effectiveness of soy isoflavones in preventing or treating human prostate cancer is unknown.

Conclusions

Earlier research indicating that soy protein, as compared with other proteins, has clinically important favorable effects on LDL cholesterol and other CVD risk factors has not been confirmed by many studies reported during the past 10 years. A very large amount of soy protein, more than half the daily protein intake, may lower LDL cholesterol by a few percentage points when it replaces dairy protein or a mixture of animal proteins. The evidence favors soy protein rather than soy isoflavones as the responsible nutrient. However, at this time, the possibility cannot be ruled out that another component in soybeans could be the active factor. No benefit is evident on HDL cholesterol, triglycerides, lipoprotein(a), or blood pressure. Thus, the direct cardiovascular health benefit of soy protein or isoflavone supplements is minimal at best. Soy protein or isoflavones have not been shown to improve vasomotor symptoms of menopause, and results are mixed with regard to the slowing of postmenopausal bone loss. The efficacy and safety of soy isoflavones for preventing or treating cancer of the breast, endometrium, and prostate are not established; evidence from clinical trials is meager and cautionary with regard to a possible adverse effect. For this reason, use of isoflavone supplements in food or pills is not recommended. In contrast, soy products such as tofu, soy butter, soy nuts, or some soy burgers should be beneficial to cardiovascular and overall health because of their high content of polyunsaturated fats, fiber, vitamins, and minerals and low content of saturated fat[118] (Table 4). Using these and other soy foods to replace foods high in animal protein that contain saturated fat and cholesterol may confer benefits to cardiovascular health.[119] Soy protein also may be used to increase total dietary protein intake and to reduce carbohydrate or fat intake. However, much less is known about the potential impact of high-protein diets on risk factors for CVD. In the meantime, these remain dynamic areas for research. The AHA will continue to monitor the results and modify its advisory statement as needed.

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Frank Sacks	Harvard School of Public Health	None	None	None	None	None	None
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Acronyms

FAO	Food and Agriculture Organization of the United Nations
WHO	World Health Organisation
PDCAAS	Protein Digestibility Corrected Amino Acid Score
DIAAS	Digestible Indispensable Amino Acid Score
IAA	Indispensable Amino Acids
UNU	United Nations University
USDA	United States Department of Agriculture
PER	Protein Efficiency Ratio
RNPR	Relative Net Protein Ratio method
CCVP	Codex Committee on Vegetable Proteins
NPR	Net Protein Ratio
NPU	Net Protein Utilization
BV	Biological Value
INCAP	Institute of Nutrition of Central America and Panama
HPLC	High Performance Liquid Chromatography
CV	Coefficient of Variation
PITC	Phenylisothiocyanate
NSP	Non-starch polysaccharide
AOAC	Association of Official Analytical Communities
AA	Amino Acid
DIAA	Digestible Indispensable Amino Acid
IAA	Indispensable Amino Acid
WMP	Whole Milk Powder
Lys	Lysine
SAA	Sulphur Amino Acid
Thr	Threonine
Trp	Tryptophan
CVDs	Cardiovascular diseases
His	Histidine
Leu	Leucine
AAA	Aromatic Amino Acids

Val	Valine
Met	Lysine
Cys	Cystine
Phe	Phenylalanine
Met	Methionine
Tyr	Tyrosine
Ile	Isoleucine
PPU	Postprandial Protein Utilisation
NPPU	Net Postprandial Protein Utilization
MA	Metabolic Availability
IAAO	Indicator Amino Acid Oxidation
IDAA	Indispensable Dietary Amino Acid
EAR	Estimated Average Requirement
IEX	Ion Exchange chromatography
RP	Reversed-phase chromatography
GCMS	Gas Chromatography-Mass Spectrometry
CE	Capillary Electrophoresis
CEMS	Capillary Electrophoresis-Mass Spectrometry
UPLC	Ultra Performance Liquid Chromatography
LCMS	Liquid Chromatography-Mass Spectrometry
LC	Liquid Chromatography
ANFs	Antinutritional Factors
NRV	Nutrient Reference Value
RCT	Randomized Controlled Trials
JECFA	Joint Expert Committee on Food Additives

Chapter 1: Introduction

As the world's population increases rapidly and against the constraints of limiting land, water and food resources, it is more important than ever to be able to define accurately the amount and quality of protein required to meet human nutritional needs and describe appropriately the protein supplied by food ingredients, whole foods, sole-source foods and mixed diets. The match between dietary supply and human protein needs is vital to support the health and well-being of human populations.

In 1989 the joint FAO/WHO Expert Consultation on Protein Quality Evaluation recommended the use of the Protein Digestibility Corrected Amino Acid Score (PDCAAS) method for evaluating protein quality. In calculating PDCAAS the limiting amino acid score (i.e. the ratio of the first-limiting amino acid in a gram of target food protein to that in a reference protein or requirement value) is multiplied by protein digestibility, with the intention of assessing how well dietary protein can match the demand for amino acids, and allowing the prediction of dietary protein utilisation. The PDCAAS method has now been in use for some 20 years and has proved to be of considerable value in practice. Nevertheless, limitations of PDCAAS have been recognised and debated, and new research findings have accumulated, whereby it has become timely to review the adequacy of PDCAAS and its application vis-à-vis other methods of estimating dietary protein quality.

It was in this context that an FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition was held in Auckland, New Zealand, from March 31 to April 2, 2011. The Expert Consultation directly followed the 2011 International Symposium on Dietary Protein for Human Health (Auckland, New Zealand, 27-30 March 2011) where numerous topics relevant to the consultation were discussed. The Agenda adopted by the Consultation is attached as Appendix I and the membership of the Consultation is given in Appendix II.

The provisional meeting objectives were adopted. The objectives were to:

1. Review the effectiveness and use of the PDCAAS method for evaluating protein quality since its adoption by the expert group meeting in 1989 and further publication in 1991.
2. Review current concerns and limitations of the PDCAAS method as reported in the literature.

3. Review the advantages and disadvantages of alternative methods to evaluate protein quality.
4. Provide justifications and recommendations for accepting, rejecting and, or modifying the PDCAAS method.
5. Establish recommendations for protein quality assessments and applications.
6. Recommend further research activities related to protein quality assessments as needed, based on emerging needs or new scientific developments as identified by the expert group.
7. Review the method of calculation of PDCAAS and related scores and its uses in practice, consider the need for revisions or modifications based on the knowledge and experience generated over the past two decades.

The Expert Committee recognised that this report builds on and extends the comprehensive body of knowledge embedded in previous FAO/WHO reports on the subject, and on the wider more recent scientific literature. As in previous reports, the primary task of this Consultation has been to provide FAO with tools for addressing practical questions on matters such as the adequacy of food supplies, targets for food and nutrition policy and the norms to be applied in labelling and regulation of protein quality for normal populations, as well as providing a perspective on the potential role for protein with respect to health, well-being and clinical conditions at various stages of the life course.

The aim of a report of this kind is to provide an objective assessment of the current state of scientific knowledge in the area and thus advice for current best practice. Naturally, in the process, gaps in knowledge are identified and so the report becomes yet another important step in a process of continuous improvement. In this context, the report provides recommendations for future research.

In presenting this report the Expert Committee was mindful of the sentiments expressed in the work and teachings of the late Professor John C Waterlow, a pioneer in the field, that the outcomes of this work must, first and foremost, be directed towards combating hunger and malnutrition in all its forms. This has been the Committee's overall guiding principle.

The Committee records with sadness the recent death of esteemed Committee member, Dr Malcolm Fuller. The collection of scientific papers published in 2012 as a Special Supplement of the British Journal of Nutrition (Supplement: Dietary Protein for Human Health) that provided the background scientific material for the Expert Consultation, has been dedicated to the memory of Dr Malcolm F Fuller.

Paul J Moughan
Chair of Consultation
September, 2012

Chapter 2:
Summary of key findings from the 2011 FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition

In 1989 the joint FAO/WHO Expert Consultation on Protein Quality Evaluation recommended the use of the Protein Digestibility Corrected Amino Acid Score (PDCAAS) for evaluating protein quality in humans. In calculating PDCAAS the limiting amino acid score is multiplied by protein digestibility, with the intention of assessing how well dietary protein can match the demand for amino acids, and allowing the prediction of dietary protein utilisation. The PDCAAS method has now been in use for some 20 years and has proved to be of considerable value in practice. Nevertheless, limitations of PDCAAS have been recognised, and new research findings have accumulated, whereby it has become timely to review the adequacy of PDCAAS.

It was in this context that an FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition was held in Auckland, New Zealand, from 31 March to 2 April, 2011, the key findings of which are summarised here.

2.1 KEY FINDINGS

- In dietary protein quality evaluation, dietary amino acids should be treated as individual nutrients and wherever possible data for digestible or bioavailable amino acids should be given in food tables on an individual amino acid basis.

- A new protein quality measure (digestible indispensable amino acid score; DIAAS) is recommended to replace PDCAAS. DIAAS is defined as: DIAAS % = 100 x [(mg of digestible dietary indispensable amino acid in 1 g of the dietary protein) / (mg of the same dietary indispensable amino acid in 1g of the reference protein)].

  Both ileal and faecal amino acid digestibility approaches can be subject to important limitations, but it is concluded that on balance ileal protein or amino acid digestibility, i.e. determined at the terminal ileum at the end of the small intestine, is considered to better reflect the amounts of amino acids absorbed and should be used in

calculating DIAAS. Digestibility should be based on the true ileal digestibility of each amino acid preferably determined in humans, but if this is not possible, in growing pigs or in growing rats in that order.

It is recommended that for foods susceptible to damage from processing, 'reactive' rather than 'total' lysine contents and the true ileal digestibility of reactive lysine (lysine availability) rather than of total lysine, be determined and used in the calculation of DIAAS.

Recommended amino acid scoring patterns (i.e. amino acid pattern of the reference protein) to be used for calculating DIAAS are as follows:

- Infants (birth to 6 months), pattern of breast milk (as noted in Tables 4 and 5 of this report).
- Young children (6 months to 3 y), pattern for the 0.5 y old infant (as noted in Table 5 of this report).
- Older children, adolescents and adults, pattern for the 3 to 10 y old child (as noted in Table 5 of this report.

For regulatory purposes two scoring patterns are recommended: the amino acid composition of human milk for infant formulas, and for all other foods and population groups the pattern for young children (6 months to 3 y) as noted in Table 5 of this report.

In calculating DIAAS the ratio should be calculated for each dietary indispensable amino acid and the lowest value designated as the DIAAS. DIAAS can have values below or in some circumstances above 100%. Values above 100% should not be truncated except where calculating DIAAS for protein or amino acid intakes for mixed diets or sole source foods.

- A dataset of currently available information on the true ileal amino acid digestibility of foods for humans was collated and assessed, as part of the Expert Consultation, for its adequacy for practical application in the calculation of DIAAS.

  After assessment of the ileal amino acid digestibility dataset it was concluded that currently available data are insufficient to support the application in practice (though its use in principle is supported) of true ileal amino acid digestibility in the calculation of DIAAS.

  More data on the true ileal amino acid digestibility of human foods are urgently needed, determined in humans and animal models. More inter-species (human, pig, rat) true ileal amino acid digestibility comparisons are needed.

  If the data obtained from these studies convincingly support the move in practice to ileal digestibility, assessment of the potential public health impact of this recommendation needs to be undertaken.

- It is recommended that the FAO convene a Working Group, as a matter of urgency, to agree upon an experimental protocol to enable the development of a more robust data set of the true ileal amino acid digestibility of human foods and agree upon a method for assessment of the potential impact of the use of true ileal amino acid digestibility data. The protocol should include recommended best practice for a pig-based assay for true ileal amino acid digestibility determination.
- It is recommended that FAO establish a formal working party to review amino acid analysis methodologies and provide some guidance towards international standardization. It is recommended that the 1970 FAO Publication "Amino Acid Contents of Foods and Biological Data on Proteins" should be updated on a continuous basis with inclusion of values, where available, for protein (faecal and ileal) digestibility, ileal amino acid digestibility and DIAAS.
- Until such time as an agreed dataset of true ileal amino acid digestibility for human foods becomes available, the protein quality of human foods and diets should be assessed using DIAAS, but values for faecal crude protein digestibility should be used. In the interim, digestible individual dietary amino acid values should be calculated using faecal crude protein digestibility values applied to dietary amino acid contents.
- There will be a need for financial support for the research agenda described above (interspecies true ileal amino acid digestibility comparison and the development of a database of true ileal amino acid digestibility for human foods). It is anticipated that the private sector along with UN technical and normative agencies, multilateral, bilateral and national Government agencies, and public-good organisations will provide such support, as a matter of urgency. If resources are not allocated to fulfil the latter proposed research objectives in a timely manner, then the present recommendation for the application of DIAAS in practice may need to be reviewed, since DIAAS and the conclusions of this report rely upon a system of true ileal amino acid digestibility and availability.
- DIAAS is the recommended method for dietary protein quality assessment for regulatory purposes. The report discusses the use of DIAAS in relation to nutrition claims.
- The report makes recommendations for further research in the area.

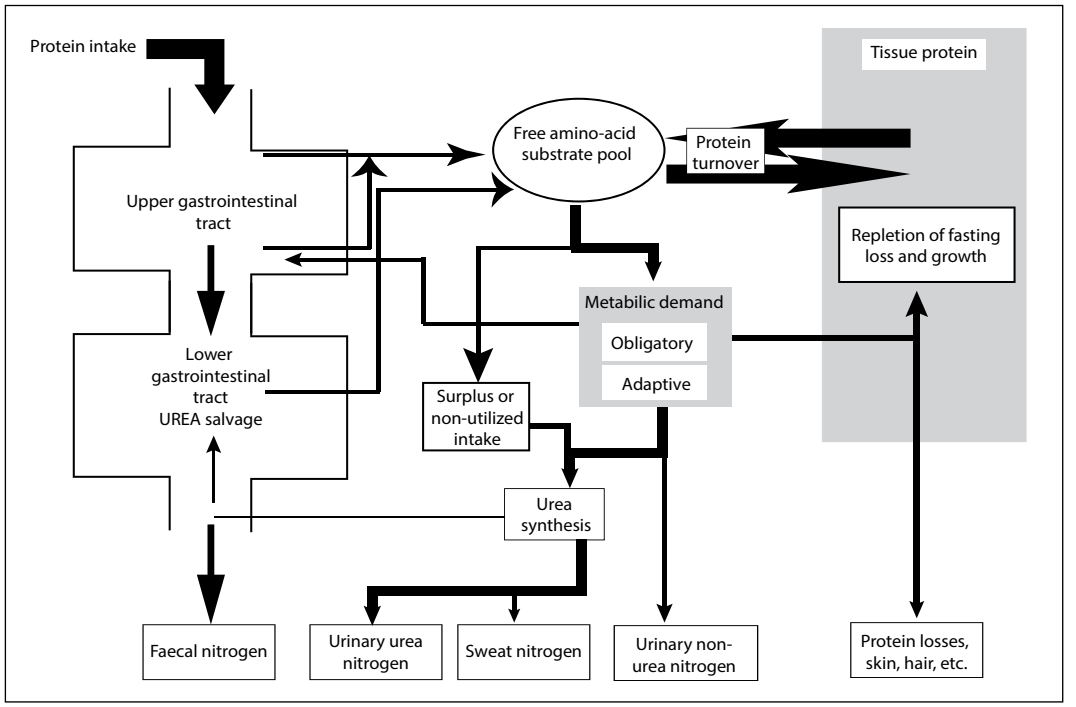
Chapter 3:
Background to the Consultation

3.1 MAJOR SCIENTIFIC REVIEWS OF PROTEIN QUALITY EVALUATION METHODOLOGY

Introduction

Protein quality evaluation aims to determine the capacity of food protein sources and diets to meet the protein and essential amino-nitrogen requirements, i.e. to satisfy the metabolic needs for amino acids and nitrogen (see Figure 1). Protein requirements are currently defined in terms of intakes required to meet metabolic needs for maintenance as indicated by nitrogen balance in the respective age group plus those associated with the protein needs for normal growth of infants and children, pregnancy and lactation

FIGURE 1.
Model of protein metabolism in humans from WHO/FAO/UNU (2007)



in women. Thus, the only truly valid measures of protein quality for humans are those that assess directly the effectiveness of different protein sources to provide for normal growth and, or other functions dependent on adequate protein nutrition in subjects that represent the target population. However, notwithstanding this definition of the ideal situation, the assessment of protein quality in human population groups over the past decades has relied on indirect approaches involving *in vitro* assays, and animal and or human metabolic studies that can be used routinely and safely to predict human protein and amino acid utilisation. To ensure accuracy and wide applicability, the routine methods must include all of the basic parameters that collectively determine the quality of a protein: absolute and relative quantities of dietary indispensable amino acids (IAA), digestibility of protein, and the bioavailability of amino acids (Harper, 1981).

3.2 AIRLEE CONFERENCE (1981)

Major reviews and evaluations of protein quality assessment methods, including those based on rat growth and nitrogen balance as well as amino acid scoring techniques were undertaken at the Airlee Conference in 1981 sponsored by Howard University, the USDA and the US National Science Foundation (Bodwell, *et al.,* 1981); by the Codex Committee on Vegetable Proteins which met between 1982 and 1989 (Codex Alimentarius Commission, 1989); by FAO/WHO (1991, 2001) and by WHO/FAO/UNU (2007). At the Airlee conference it was generally agreed that the Protein Efficiency Ratio (PER) method should be replaced by a more precise and appropriate method. Although a different rat assay procedure (the Relative Net Protein Ratio method, RNPR) was considered as an improvement over the PER method, a method based on comparison of the amino acid content of food with human amino acid requirements (amino acid scoring system) was accepted as the most suitable approach for assessing the protein quality of foods (Harper, 1981). It was also recommended that amino acid score should be corrected for incomplete digestibility of protein, and for the unavailability of individual amino acids, especially those that are susceptible to damage during food processing or cooking prior to consumption. This conference recognized the need for further research to standardize amino acid analysis methodology, to improve methods for the determination of the digestibility of protein and the bioavailability of amino acids, and to further investigate human amino acid requirements with the aim of developing an accurate amino acid scoring pattern (Bodwell, *et al.,* 1981).

3.3 DELIBERATIONS OF THE CODEX COMMITTEE ON VEGETABLE PROTEINS REGARDING PROTEIN QUALITY ASSESSMENT (1982-1989)

The recommendations of the Airlee Conference were taken up by the Codex Committee on Vegetable Proteins (CCVP) (Codex Alimentarius Commission, 1989), which was established to develop international Codex standards (including protein quality requirements) for vegetable protein products. An *Ad Hoc* Working Group on Protein Quality Measurement was formed to conduct cooperative research to identify the most

promising methods for evaluation of the protein quality of foods. In collaborative studies organized by the USDA (Bodwell, *et al.,* 1989), seventeen protein products were studied for amino acid profiles, for protein and amino acid digestibility (by *in vitro* and rat balance methods), amino acid availability (by chemical methods and rat, *Escherichia coli,* and *Streptococcus zymogenes* growth methods), and for protein quality indices based on PER, NPR (Net Protein Ratio), RNPR, Net Protein Utilization (NPU), and Biological Value (BV) obtained in the rapidly growing weanling rat. Inter-laboratory studies on protein digestibility determinations were also organized by the USDA to test the appropriateness of the *in vitro* methods (McDonough, *et al.,* 1990a), and to standardize the rat balance method (McDonough, *et al.,* 1990b). Results of these and other related studies were discussed at the Fifth Session of the CCVP (Codex Alimentarius Commission, 1989) held in 1989 in Ottawa, Canada.

Based on the recommendations of the *Ad Hoc* Working Group on Protein Quality Measurement, the CCVP at its Fifth Session agreed that, given that values for the requirements of dietary indispensable amino acids had been identified by FAO/WHO/UNU (1985) and that this report had suggested that the quality of a protein could be predicted from a comparison of the pattern of its amino acid composition to the pattern of human amino acid requirements (i.e. the amino acid score corrected for its digestibility based on the true faecal digestibility of protein as determined using the rat balance method), then this approach was the most suitable method for the routine assessment of the protein quality of vegetable protein products and other food products (Codex Alimentarius Commission, 1989). Amino acid score was based on the amount of the first limiting amino acid, and its calculation included the use of the requirement pattern suggested by the FAO/WHO/UNU (1985) for the preschool child based on human studies conducted at INCAP in the 1960s and 70s (Viteri, 2010). Because the proposed protein quality methodologies had broad implications beyond the specific purview of the CCVP, the CCVP recognized the need for the wider scientific community to address issues such as amino acid quantification, protein digestibility and amino acid bioavailability measurements, and respective correlations in humans. The CCVP accordingly recommended at its Fifth Session in 1989 that an FAO/WHO expert consultation should be held to review protein quality methodologies. Such a consultation was requested to review the results and recommendations of the research conducted by the Codex *Ad Hoc* Working Group on Protein Quality Measurement, and to evaluate the PDCAAS method for its usefulness in assessing protein quality in human nutrition.

3.4 JOINT FAO/WHO EXPERT CONSULTATION ON PROTEIN QUALITY EVALUATION (1989)

A Joint FAO/WHO Expert Consultation on Protein Quality Evaluation was held in Bethesda, MD from December 4 to 8, 1989. The objectives of the meeting were: to review present knowledge of protein quality assessment, to discuss various techniques used in assessing protein quality of foods, and to specifically evaluate amino acid score corrected for

protein digestibility (PDCAAS), the method recommended by CCVP. The report of the Joint FAO/WHO Expert Consultation was published in 1991. The Consultation concluded that PDCAAS was the most suitable regulatory method for assessing the protein quality of foods and infant formulas. It was further concluded that since this method is based on human amino acid requirements, it is inherently more appropriate than animal based assays in predicting the protein quality of foods. Therefore the Consultation recommended that PDCAAS be adopted as the preferred method for measuring the quality of proteins used in human nutrition. Other conclusions and recommendations of the Consultation (FAO/WHO, 1991) are noted below:

Amino acid analysis of foods

1. The 1989 Consultation recognized that significant advances had been made in standardizing methodologies for the determination of amino acids.
2. It noted that methods for the determination of amino acids in foods required three standardized hydrolyses including acid hydrolysis of unoxidized protein for the determination of all amino acids except tryptophan, methionine and cysteine; acid hydrolysis of oxidized protein for the determination of methionine and cysteine; and alkaline hydrolysis of unoxidised protein for the determination of tryptophan (AOAC, 2000), followed by separation and quantitation of the released amino acids by ion exchange chromatography (IEC) using cation exchange resins and post-column derivatization (by a commercial amino acid analyzer or HPLC system) or by pre-column derivatization followed by reverse phase HPLC.
3. The standardized amino acid analysis methods can provide values with a within-laboratory coefficient of variation (CV) of about 5% and between-laboratories of about 10% for most amino acids. This variability was considered acceptable for the purpose of calculating amino acid score.
4. The need for further studies to standardise the hydrolytic and oxidation procedures and to improve accuracy of the procedures for further reduction in inter-laboratory variation was noted.
5. Collaborative testing and comparative analysis of the new HPLC methods was recommended.
6. It was recommended that amino acid results should be reported as mg amino acid/g N or mg amino acid/g protein by using the nitrogen-to-protein conversion factor of 6.25. The use of other food-specific protein factors was not recommended.
7. It was recommended that FAO update their publication entitled "Amino Acid Content of Foods and Biological Data on Proteins" (FAO, 1970) and commission new amino acid analyses of local food sources for which there were insufficient data.
8. It was recommended that national tables of amino acid composition of food products, clearly defined in terms of composition and processing, be developed.

Amino acid requirements and scoring pattern

1. The 1989 Consultation recognized that the amino acid scoring pattern proposed in 1985 (FAO/WHO/UNU, 1985) for children of preschool age was the most suitable pattern for use in the evaluation of dietary protein quality for all age groups, except infants.
2. It was also noted that the amino acid profile of mature human milk should be the basis for the scoring pattern to assess protein quality in foods for infants of less than 1 year of age; considering that the growth and metabolic state of the fully breast fed infant was set as the normative standard for both growth and human nutritional needs 0-6 months.
3. It also noted that the recommendation for the two amino acid scoring patterns to be used for infants and for all other ages must be considered as temporary until the results of further research either confirmed their adequacy or demanded a revision.
4. It was recommended that further research should be carried out to confirm the currently accepted values of protein and amino acid requirements of infants and preschool children and to define the amino acid requirements of school-aged or adolescent children and of adults; and that the FAO/WHO coordinate international research programmes to determine human amino acid needs.

Digestibility considerations

1. The 1989 Consultation noted similarities in the ability of humans and rats to digest foods, and concluded that the true digestibility of crude protein is a reasonable approximation of the true digestibility of most amino acids (as determined by the rat balance method) in diets based on animal protein sources, cereals, oilseeds, legumes or mixtures of protein sources. Therefore, it was recommended that amino acid scores be corrected for the true digestibility of protein only.
2. The Consultation agreed that the rat balance method was the most suitable practical method for predicting protein digestibility for humans.
3. It further recommended that research should be undertaken to compare protein digestibility values of humans and rats for identical foods.
4. It recommended that further research be carried out to perfect and evaluate the most promising *in vitro* procedures for estimating protein digestibility; and when human balance studies cannot be used, the standardized rat faecal-balance method of Eggum (1973) or McDonough *et al.* (1990b) should be used.
5. Digestibility determinations must be carried out for novel products or processes. However, established protein digestibility values of well-defined foods may be taken from a published data base for use in the routine assessment of the protein quality of foods by the amino acid scoring procedure, provided that all safety and toxicological criteria have been met. Moreover, a data base for the protein digestibility of raw and processed products should be established.

6. Further research was encouraged to perfect and evaluate the most promising *in vitro* methods for predicting protein digestibility, such as those of Satterlee *et al.* (1979) and of Pederson and Eggum (1983).
7. It was recognized that amino acid digestibility values obtained by the faecal method, are, for most amino acids in most food products, inaccurate in comparison to those obtained by the ileal analysis method. In some studies, net synthesis of methionine and lysine has been reported to occur in the large intestine. Thus, depending on the amino acid and on the food, amino acid digestibility values obtained by the faecal analysis method are overestimated (which is usually the case) or underestimated when compared to those obtained by the ileal analysis method. While it was recognised that the measure of true faecal protein or amino acid digestibility has shortcomings, it was considered that the method was still superior in practice to the ileal analysis method. This decision was based on uncertainties concerning the contribution and variation of endogenous protein secretions at the terminal ileum.

Overall recommendation of the FAO/WHO 1989 Expert Consultation (published 1991)

Based on the above conclusions, the Consultation agreed that the protein digestibility-corrected amino acid score (PDCAAS) method was the most suitable approach for the routine evaluation of overall protein quality for humans and recommended the adoption of this method as an official method at the international level.

3.5 FAO/WHO/UNU EXPERT CONSULTATION ON PROTEIN AND AMINO ACID REQUIREMENTS IN HUMAN NUTRITION (ROME 2001, GENEVA 2002, PUBLISHED AS A WHO/FAO/UNU REPORT IN 2007)

The primary objectives of this Consultation were: "to review, advise and update protein and amino acid requirements for all age groups (infants, children, adolescents, adults, elderly), and for women during pregnancy and lactation; to review and develop recommendations on protein requirements in health and disease, including their implications for developing countries; and to develop recommendations on protein quality and labelling, with respect to new requirement levels, for use worldwide and in the Codex Alimentarius".

Since its adoption by FAO/WHO in 1991, the PDCAAS method had been widely accepted but also criticised for a number of reasons. In preparation for the Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition, experts met at a preliminary meeting in Rome in 2001 in working groups, one of which (working group 5) considered, amongst other things, analytical issues regarding protein, protein quality and food labelling.

Working group 5, in an unpublished report, assessed the validity of criticisms of the PDCAAS method. These criticisms of the PDCAAS method included:

1. The PDCAAS method does not credit extra nutritional value to high quality proteins.
2. The PDCAAS method overestimates protein quality of products containing antinutritional factors.
3. The PDCAAS method does not adequately take into account the bioavailability of amino acids.
4. The PDCAAS method overestimates the quality of poorly digestible proteins supplemented with limiting amino acids, and of proteins co-limiting in more than one amino acid.

After addressing the above-noted criticisms of the PDCAAS method, the Working Group made the following observations and recommendations:

1. There are two distinct uses of protein quality data: assessment of a diet's ability to meet human protein and amino acid requirements and assessment of the protein adequacy for regulatory purposes of foods and food products sold to consumers.
2. Amino acids should be treated as individual nutrients, and the ultimate evaluation of the nutritional value of proteins should be made from amino acid data in comparison to requirements. This would require the use of adjustments for the digestibility of protein and/or amino acids, and their availability.
3. There are sufficient data on the digestibility of proteins in foods and these data should be compiled. However, there is insufficient information on the digestibility and bioavailability of amino acids. Until sufficient data on digestible amino acids in foods become available, inclusion of correction for protein digestibility would serve a useful nutritional purpose in predicting information on the levels of digestible amino acids. This would indicate the capacity of individual protein sources to complement protein sources that are deficient in specific dietary indispensable amino acids.
4. Until data on digestible amino acids in foods become available, the digestibility of protein should be considered as a good approximation of the bioavailability of amino acids in mixed human diets based on properly processed (containing minimal amounts of residual antinutritional factors) foods. In such cases, the PDCAAS method would be the preferred method for the routine prediction of protein quality.
5. The PDCAAS method may be inappropriate for the routine prediction of the protein quality of sole-source foods such as infant formulas and enteral nutritionals and novel protein sources that contain high levels of known antinutritional factors, both those occurring naturally and those formed during processing. Because high levels of antinutritional factors (substances present in foods other than nutrients that can perturb digestion or metabolism) may have an adverse impact on the digestibility of amino acids and the utilisation of protein the use of the PDCAAS method would overestimate the protein quality of products containing these factors. There is a need to establish safe upper limits of antinutritional factors.
6. For regulatory uses, the PDCAAS method is also inappropriate for the prediction of the protein quality of high quality protein food ingredients because it fails to recognize their nutritional value as supplements to low quality proteins; therefore,

the PDCAAS method should be revised to permit values of more than 100 for food ingredients.

7. To improve accuracy and to further reduce inter-laboratory variation in amino acid analysis, additional studies should be undertaken to standardize the hydrolytic and oxidation procedures. Collaborative studies should be undertaken of the extensively used HPLC methods for the determination of amino acids such as the pre-column derivatization with PITC (phenylisothiocyanate). Moreover, an official standardised method for the determination of amino acids in foods and faeces and ileal digesta should be developed.
8. Research should be undertaken to compare ileal amino acid digestibility values derived using human-based assays and animal models for identical foods. In addition, standardised ileal digestibility procedures should be developed and sufficient data on foods should be generated to facilitate replacement of the faecal method by the ileal method. Ileal digestibility is defined as the disappearance of a nutrient between the mouth and the end of the small intestine (terminal ileum) whereas faecal digestibility is the disappearance of a nutrient between the mouth and the end of the digestive tract.
9. The 1970 FAO Publication, "Amino Acid Contents of Foods and Biological Data on Proteins" should be revised with new data and additional information on nitrogen-to-protein conversion factors and amino acid digestibility values where applicable.
10. The above-noted recommendations for revision, further compilation of data and further research, would improve the usefulness of the PDCAAS method and suggest new suitable *in vitro* or biological assays for the routine prediction of protein quality of foods that would be applicable to the entire range of foods used in human nutrition.

Overall recommendation

In view of the perceived shortcomings of the PDCAAS method noted above, it was recommended that a new FAO/WHO expert consultation on protein quality evaluation be convened to re-examine the validity of the PDCAAS method for the routine protein quality assessment of foods, and to suggest appropriate revisions and, or adoption of a biological assay that would be applicable to the entire range of foods used in human diets.

In the final report of the Consultation, published in 2007, the PDCAAS method was endorsed with minor modifications to the calculation method but the following concerns were also raised about the method:

In previous reports, scoring patterns were calculated by dividing amino acid requirement values by the safe level of protein intake. However, more recent scoring patterns had been based on amino acid requirement values, which generally reflected best estimates of average requirements. This approach is supported by the values derived

by Hegsted (1963) from his regression analysis of nitrogen balance data. Therefore, in the WHO/FAO/UNU (2007) report, scoring patterns were based on amino acid requirement values divided by the mean protein requirement.

New scoring patterns were proposed for four age groups including infants, preschool children (1-2 y), older children and adolescents (4-18 y), and adults (> 18 y).

A second concern identified, related to correction for faecal as opposed to ileal protein digestibility in the calculation of PDCAAS. In the introduction to the final report digestibility of dietary proteins had been reviewed in terms of both ileal and faecal digestibility. It was argued that because of the considerable exchange of nitrogen in terms of protein, amino acids and urea between systemic pools and the gut lumen, digestibility is more complex than usually assumed, a principle captured in the overall model for human nitrogen metabolism shown in Figure 1. In this context two important issues were raised.

Firstly because of the considerable magnitude of flow of endogenous nitrogen-containing compounds into the lumen of the small intestine (possibly as much as 70 to 100 g protein each day) which mixes with dietary amino acids, and which are both substantially absorbed by the time they reach the terminal ileum, "ileal digestibility" (the difference between dietary amino acids and those appearing in the terminal ileum) is at best a crude approximation of the handling of nitrogen-containing materials in the small intestine. It was noted, however, that there are methodologies to allow the determination of the ileal endogenous amino acids, and the correction of amino acid digestibility values for this component.

Secondly tracer studies show that faecal nitrogen derives from a pool of nitrogen that includes not only ileal effluent and any residue from the dietary consumption, but also sloughed away cells and mucins derived within the colon, and nitrogen-containing compounds sourced from the systemic circulation of the host, especially urea and possibly uric acid and creatine. This nitrogen is present in faeces mainly as microbial protein in quantities that have been shown in some cases to be much less than estimates of total nitrogen inflo into the colon, because of considerable reuptake of nitrogen from the colon. Furthermore, human studies have shown that faecal nitrogen is to some extent a function of bacterial biomass in the colon, itself related to dietary resistant starch and non-starch polysaccharide (NSP) intake which serve as energy sources for colonic bacterial synthesis using nitrogen largely from urea salvage. Because reuptake of nitrogen from the colon is mainly in the form of ammonia which re-enters the metabolic pool as shown in Figure 1, its ultimate excretory fate can include urinary urea, and evidence exists to show that with human diets with a high proportion of plant foods and NSP there can be an inverse relationship between faecal and urinary nitrogen excretion. Taken together this means that for human diets containing large amounts of non-digestible carbohydrate, faecal nitrogen cannot be used as a reliable measure of digestibility. It was concluded that

the concepts of both ileal digestibility and faecal digestibility can be subject to important limitations especially where there is a need to determine the critical nutritional value of foods at the margins of satisfying dietary requirements. It was concluded that methods of assessing the digestibility of dietary protein in human nutrition cannot be used with any confidence in the development of policy options, unless the limitations of the underlying assumptions have been taken into account adequately.

Against this background the question of the use of ileal as opposed to faecal digestibility was examined noting especially literature reports (Darragh and Hodgkinson, 2000; Moughan, 2003) about practically important ileo-faecal differences in non-ruminant animals such as pigs and rats and the general applicability of these observations to humans, and ileo-faecal differences observed in humans (Rowan *et al.,* 1994; Gaudichon *et al.,* 2002; Moughan, 2003). It was recommended that while faecal digestibility may remain the appropriate measure of overall nitrogen digestibility, it is unlikely to be an accurate measure of amino acid digestibility.

A third concern related to the reduced bioavailability of some amino acids, such as lysine, that may be chemically transformed during the processing of foods. It was noted that the correction for protein digestibility in the calculation of PDCAAS values may not account for this reduction in bioavailability. Therefore, the need to have a specific assay to accurately measure lysine digestibility in such cases was recognized. A specific assay (Moughan and Rutherfurd, 1996; Rutherfurd *et al.,* 1997a; Rutherfurd and Moughan, 1998; Moughan, 2003) for "reactive" lysine, which distinguishes it from biologically unavailable lysine that has undergone Maillard reactions, was considered suitable in such cases.

A fourth important and controversial concern related to truncation of the amino acid score and consequent PDCAAS value. It was argued that truncation removes any nutritional differences between high protein foods such as milk and soya, although actual concentrations of important dietary indispensable amino acids, which may be limiting in some diets, are higher in milk than in soya. This could be recognized by giving individual protein sources an amino acid score of > 1 (or > 100). In the FAO/WHO 1991 report, truncation was not used for calculating amino acid scores but was applied to the calculation of the PDCAAS value, and this created considerable confusion.

The PDCAAS value should predict the overall efficiency of protein utilization based on its two components, digestibility and biological value (BV; nitrogen retained divided by digestible nitrogen). The principle behind this approach is that the utilization of any protein will be first limited by digestibility, which determines the overall amount of dietary amino acid nitrogen absorbed, and BV describes the ability of the absorbed amino acids to meet the metabolic demand. For any amount of absorbed nitrogen the best that can be achieved is that the amino acid pattern exactly matches the requirements, so that all amino acids are utilized. Furthermore it was noted that while score is determined

only from indispensable amino acid content, the metabolic demand is for both dietary indispensable amino acids and dietary non-essential nitrogen. This means that when any or all indispensable amino acids are present in excess of the demand, the absorbed mixture could become unbalanced and limited by dispensable amino acids. Therefore, BV can never exceed 1 or 100. In this respect, and for mixed diets or whole foods, PDCAAS values of > 1 or 100 should never be used.

Calculation of the amino acid score for a dietary protein mixture especially when the digestibility of individual proteins varies was also considered to require clarification. In this case, amino acid score is calculated for the mixture from its overall amino acid profile without identifying the score of component proteins. Based on the principle that protein digestibility is first limiting, the amino acid score for a protein mixture should be calculated from the weighted average digestible amino acid content. This is in contrast to the recommendation given in the FAO/WHO 1991 report.

The final report (WHO/FAO/UNU, 2007) concluded that there were several aspects of protein quality evaluation that required further consideration. Thus it was recommended that a complete listing of the digestibility and amino acid scores of food proteins based on updated data on amino acid composition, and on the new scoring patterns (derived in the WHO/FAO/UNU 2007 report), should be the subject of a new technical report. However it was suggested that the principles discussed in the report should be applied. That is, protein quality should be assessed in terms of PDCAAS calculated from the best estimate of protein digestibility and the amino acid score, based on a comparison of the amino acid composition of digestible protein with the scoring pattern appropriate for the age group. Also when such PDCAAS values are used to adjust the intakes of the dietary mixture to meet the safe level, the score of the mixture should not be > 1 or 100. However, the case for giving non-truncated amino acid scores >1 or 100 for individual protein sources was considered to require further evaluation.

Since the FAO/WHO (1991) report, significant advances have been made in methods for amino acid analysis of foods and for determining amino acid digestibility. Moreover, working group 5 of the 2001 Rome consultation recommended that protein should be measured as the sum of individual amino acid residues (the molecular weight of each amino acid less the molecular weight of water) plus free amino acids. Since there is no official AOAC (Association of Official Analytical Chemists) international method for the amino acid analysis of foods, collaborative research and scientific consensus would be required to achieve this objective.

The 2011 FAO Consultation

Based on the deliberations of the FAO/WHO (2001) Working Group and the WHO/FAO/UNU Expert Consultation on Protein and Amino Acid Requirements held in 2002, with findings published in 2007, it was decided to hold a further FAO Expert Consultation

on dietary protein evaluation, specifically addressing key issues raised in the earlier consultations, but remaining unresolved. To this end an FAO Consultation was held in Auckland, New Zealand in 2011 immediately following the International Symposium on Dietary Protein for Human Health organized by the Riddet Institute, Massey University, New Zealand, FAO, Rome and Health Canada, Ottawa.

Chapter 4:
Findings and recommendations of the 2011 FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition

4.1 SIGNIFICANCE AND APPROPRIATENESS OF PDCAAS IN PRACTICE AND TRUNCATION OF PDCAAS

Increasingly there is interest in the metabolic effects of specific individual dietary amino acids, and for this reason it is important to have accurate information on the amounts of digestible or preferably bioavailable amino acids in foods and proteins. **It is thus recommended that dietary amino acids be treated as individual nutrients and that wherever possible data for digestible or bioavailable amino acids be given in food tables on an individual amino acid basis.**

In the context of whole diets and the nutritional adequacy of a food protein or a mixture of food proteins, the assessment of the nutritional value of a protein should reflect its ability to satisfy the metabolic needs for individual amino acids and nitrogen. Once again dietary protein should be considered as a source of amino acids as individual nutrients. The Amino Acid Score is intended to predict protein quality in terms of the potential capacity of the food protein to provide the appropriate pattern of dietary indispensable amino acids. The actual capacity of the protein to satisfy the amino acid needs will require the use of corrections for amino acid digestibility and availability. Although the general principles inherent in the calculation of PDCAAS values are not disputed, the use of a single value of crude protein digestibility to correct the dietary amounts of each individual dietary indispensable amino acid for its digestibility is considered to be a short-coming, when there are practically important quantitative differences in digestibility between crude protein and individual dietary indispensable and dispensable amino acids. In this case the accuracy of a calculated Amino Acid Score can be enhanced by using appropriate digestibility or bioavailability data for each individual dietary indispensable amino acid. This also makes full use of the information currently available. A further inherent shortcoming of the PDCAAS approach is that correction for digestibility is based on an estimate of crude protein digestibility determined over the total digestive tract (i.e. faecal digestibility). Although, as discussed earlier (Section III), both the ileal and faecal digestibility approaches can be subject to important limitations,

the consultation concluded that on balance protein or amino acid digestibility determined at the end of the small intestine (i.e. terminal ileum, ileal digestibility) is considered to better reflect the amount of amino acid absorbed. Based on both these considerations, a new protein quality measure, (digestible indispensable amino acid score; DIAAS) is recommended to replace PDCAAS.

The digestible indispensable amino acid score (DIAAS)

As protein digestibility does not always reflect the digestibility of individual dietary indispensable amino acids, using a score based on individual dietary indispensable amino acid digestibility is preferable.

It is recommended that a revised score called the Digestible Indispensable Amino Acid Score (DIAAS) be used and be defined as follows:

DIAAS % = 100 x [(mg of digestible dietary indispensable amino acid in 1 g of the dietary protein) / (mg of the same dietary indispensable amino acid in 1g of the reference protein)].

Digestibility should be based on the true ileal digestibility (i.e., determined at the end of the small intestine) of each amino acid preferably determined in humans (Gaudichon *et al.,* 2002; Moughan, 2003; Fuller and Tomé, 2005), but if this is not possible, in the growing pig (Stein *et al.,* 2007) or in the growing rat, (Moughan *et al.,* 1984), in that order. When amino acid digestibility data are not available amino acid digestibility is assumed to be equivalent to crude protein digestibility. In this case, true ileal crude protein digestibility data are preferable, but where unavailable, true faecal crude protein digestibility may be used. It is recognised that amino acid digestibility may vary quite greatly between batches of food or food ingredients. It is impractical, however, to submit all batches of a food to bioassay and thus the use of tabulated mean data is permitted. However, where a new cultivar, food by-product or food appears, it should be subject to an *in vivo* assay for true ileal amino acid digestibility.

Recommended amino acid scoring patterns (i.e. amino acid pattern of the reference protein) to be used for calculating protein quality for dietary assessment are as follows:

- I**nfants (birth to 6 months), the pattern of breast milk (as noted in Tables 4 and 5).**
- **Young children (6 months to 3 y), the pattern for the 0.5 y old infant (as noted in Table 5).**
- **Older children, adolescents and adults, the pattern for 3 to 10 y old children (as noted in Table 5).**

For regulatory purposes two scoring patterns are recommended: the amino acid composition of human milk for infant formulas, and for all other foods and population groups the pattern for young children (6 months to 3 y) as noted in Table 5.

The ratio should be calculated for each dietary indispensable amino acid and the lowest value designated as the DIAAS and used as an indicator of dietary protein quality. The DIAAS can have values below or in some circumstances above 100%. Values above 100% should not be truncated as was done for the PDCAAS value, except where calculating DIAAS for protein or amino acid intakes for mixed diets or sole source foods (see below) where truncated values must be used.

Examples of calculations are shown for single food and multiple ingredient dishes and diets in Section 2 of the report.

Practical application of the DIAAS

There are three distinct uses of the DIAAS:

- Calculation of DIAAS in mixed diets for meeting the needs for quality protein, as humans consume proteins from varied protein sources in mixed diets.
- To document the additional benefit of individual protein sources with higher scores in complementing less nutritious proteins.
- For regulatory purposes to classify and monitor the protein adequacy of foods and food products sold to consumers.

When examining the quality of protein in mixed diets or in sole source foods (e.g., infant formulas) the DIAAS is used to estimate the available protein intake and the DIAAS can be used to adjust dietary protein intakes to meet requirements, (i.e. safe intake of any diet in relation to protein = safe protein requirement/DIAAS value of diet).

In this case a DIAAS value >100% should never be used, since this would mean that for "high quality" diets based on egg or milk for example, for which the DIAAS values of the proteins individually may exceed 100%, the safe intake of that diet would be lower than the safe requirement level even though the safe requirement level may have been established with egg or milk in the first place.

When examining protein intakes of mixed diets or sole source foods (e.g., infant formulas) the DIAAS and protein content can be used to estimate the available protein intake. DIAAS can be used as a means of defining protein equivalent intake (protein adequacy), when it is multiplied by the actual protein content or intake (i.e. measured protein intake times DIAAS). However, protein intake can be corrected for its quality by using DIAAS only when $\leq$100 but

not above. The DIAAS should not be used to inflate the apparent protein content of the food or diet.

DIAAS may be used to assess the quality of single ingredients or individual foods to take into consideration complementation. A DIAAS over 100 indicates potential to complement protein of lower quality provided that a suitable total N intake is maintained. For individual foods or food ingredients, not truncating the score allows ready calculation of the protein quality of mixed diets. The DIAAS for a mixed diet itself should be truncated.

4.2 EXAMPLE CALCULATIONS OF DIAAS AND THE EXPRESSION OF DIGESTIBLE AMINO ACID CONTENTS OF FOODS

Digestible amino acid contents

The true ileal digestible amino acid (AA) content of a food may be expressed in a number of ways:

mg AA per gram of food (on an 'as is' or 'as consumed' basis)
or
mg AA per gram of food dry matter (oven dry matter)
or
mg AA per gram of food protein.

The latter mode of expression is required for the calculation of DIAAS (see below).

Calculation of DIAAS

The digestible (dietary) indispensable amino acid score (DIAAS) for a food or food ingredient can be obtained from the digestible indispensable amino acid (DIAA) content in 1 g protein of food and the IAA reference ratio. These values can be calculated using the following equations:

Digestible IAA content for each IAA in 1 g protein of food

Digestible IAA content = mg of IAA in 1 g protein of food multiplied by the true ileal digestibility coefficient for the same dietary indispensable amino acid (the digestibility coefficient is the percentage value divided by 100, e.g. digestibility = 90%, coefficient = 90/100 = 0.90);

Digestible IAA reference ratio for each IAA

Digestible IAA reference ratio = *Digestible IAA content* in 1 g protein of food (mg)/mg of the same dietary indispensable amino acid in 1g of the reference protein (amino acid scoring pattern);

Digestible IAA score (DIAAS)

For a given reference protein amino acid pattern (amino acid scoring pattern), the DIAAS is the lowest calculated value for the DIAA reference ratio, expressed as a percentage (i.e., the IAA having the lowest digestible reference ratio; ratio x 100).

The DIAAS may, therefore, be expressed by the following equation:

> *DIAAS %* = 100 x *lowest value* [(mg of digestible dietary indispensable amino acid in 1 g of the dietary protein)/(mg of the same dietary indispensable amino acid in 1g of the reference protein)]

or

> DIAAS % = 100 x *lowest value* [*"Digestible IAA reference ratio"* for a given amino acid scoring pattern].

Note that the main difference between DIAAS and PDCAAS is that true ileal amino acid digestibility for the dietary indispensable amino acids is used rather than a single faecal crude protein digestibility value.

Example of calculation of DIAAS for a single food ingredient

Refer Table 1.

Example of calculation of DIAAS for a food mixture

Refer Table 2.

4.3 BACKGROUND TO THE VALIDITY OF THE AMINO ACID SCORING PATTERNS

Definition of dietary indispensable amino acid scoring patterns to be used in the calculation of DIAAS from immediately post infancy to adulthood

Consideration was given to the accuracy of current estimates of dietary indispensable amino acid scoring patterns (see Millward, 2012 a,b). Discussions were held in the context of an overall model of protein metabolism in humans (refer Figure 1) and a framework for short- and long-term protein quality related health outcomes (refer Figure 2). The Committee noted emerging knowledge on long-term transgenerational changes due to dietary protein intakes during pregnancy in the F_0 generation in rats (Hoile *et al.,* 2011) and in humans (Waterland *et al.,* 2010).

TABLE 1.
Calculation of DIAAS value for whole milk powder (WMP)

		Composition data[1]					True ileal IAA Digestibility[1]				True ileal digestible IAA content in WMP[2]				
	Weight (g)	Protein (g/100g)	Lys	SAA	Thr	Trp	Lys	SAA[5]	Thr	Trp	Lys	SAA	Thr	Trp	
			(mg/g protein)								(mg/g protein)				
	A	B	C	D	E	F	G	H	I	J	CxG	DxH	ExI	FxJ	
Milk Powder	100	28	78	35	44	13	0.95	0.94	0.90	0.90	74	33	40	12	

Age group (y)	IAA Reference pattern: mg/g protein (refer to Table 5 in this report)				[3]Digestible IAA reference ratio				[4]DIAAS for WMP (%)
	Lys	SAA	Thr	Trp	Lys	SAA	Thr	Trp	
Infant (birth to 6 mths)	69	33	44	17	1.07	1.00	0.91	0.69	69 (Trp)
Child (6 months to 3 yrs)	57	27	31	8.5	1.30	1.22	1.29	1.41	122 (SAA)
Older child, adolescent, adult	48	23	25	6.6	1.54	1.43	1.60	1.82	143 (SAA)

[1] Reference: CVB Feed Tables (2007). Chemical compositions and nutritional values of feed ingredients. Product Board Animal Feed, CVB, The Hague. True ileal indispensable amino acid (IAA) digestibility coefficients are based on predicted human values obtained from pig data.

[2] For the sake of example, calculation is shown for four amino acids, where possible all IAA should be included in the calculation.

[3] Digestible IAA reference ratio (Digestible IAA in 1 g protein of whole milk powder /mg of the same dietary indispensable amino acid in 1g of the reference protein)

[4] DIAAS for whole milk powder (Lowest value of the "digestible IAA reference ratio" expressed as % for each reference pattern; for infants WMP has a calculated DIAAS of 69; for children 122 and for older children, adolescents and adults 143).

[5] This is the weighted average of the digestibility coefficients for methionine and cysteine.

Lys=lysine, SAA=sulphur amino acids (methionine + cysteine), Thr = threonine, Trp = tryptophan).

TABLE 2.
Calculation of DIAAS value for a mixture of wheat, peas and whole milk powder

	Composition[1]						True ileal IAA Digestibility[1]					True ileal digestible IAA content in mixture[2]				
	Weight	Protein	Lys	SAA	Thr	Trp	Lys	SAA	Thr	Trp	Protein content in mixture	Lys	SAA[5]	Thr	Trp	
	(g)	(g/100g)	(mg/g protein)								(g)	(mg)				
	A	B	C	D	E	F	G	H	I	J	AxB	(AxB)xCxG	(AxB)xDxH	(AxB)xExI	(AxB) xFxJ	
Wheat	400	11	28	38	29	12	0.82	0.895	0.86	0.91	44	1 010	1488	1 097	480	
Pea	100	21	71	25	37	9	0.79	0.69	0.73	0.66	21	1 178	362	567	125	
Milk powder	35	28	78	35	44	13	0.95	0.94	0.90	0.90	10	726	322	388	115	
Totals	535										75	2 914	2 172	2052	720	
Amino acids: mg/g protein (total for each amino acid/total protein)												38.9	29.0	27.4	9.6	

Age group	**Reference pattern: mg/g protein (Refer to Table 5 in this report)**				**[3]Digestible IAA reference ratio**				**[4]DIAAS for mixture (%)**
	Lys	SAA	Thr	Trp	Lys	SAA	Thr	Trp	
Infant (birth to 6 moths)	69	33	44	17	0.56	0.88	0.62	0.56	**56 (Lys)**
Child (6 months to 3 yrs)	57	27	31	8.5	0.68	1.08	0.88	1.13	**68 (Lys)**
Older child, adolescent, adult	48	23	25	6.6	0.82	1.26	1.10	1.45	**82 (Lys)**

[1] Reference: CVB Feed Tables (2007). Chemical compositions and nutritional values of feed ingredients. Product Board Animal Feed, CVB, The Hague. True ileal indispensable amino acid (IAA) digestibility coefficients are based on the predicted human values obtained from pig data.

[2] For the sake of example, calculation is shown for four amino acids; where possible all IAA should be included in the calculation.

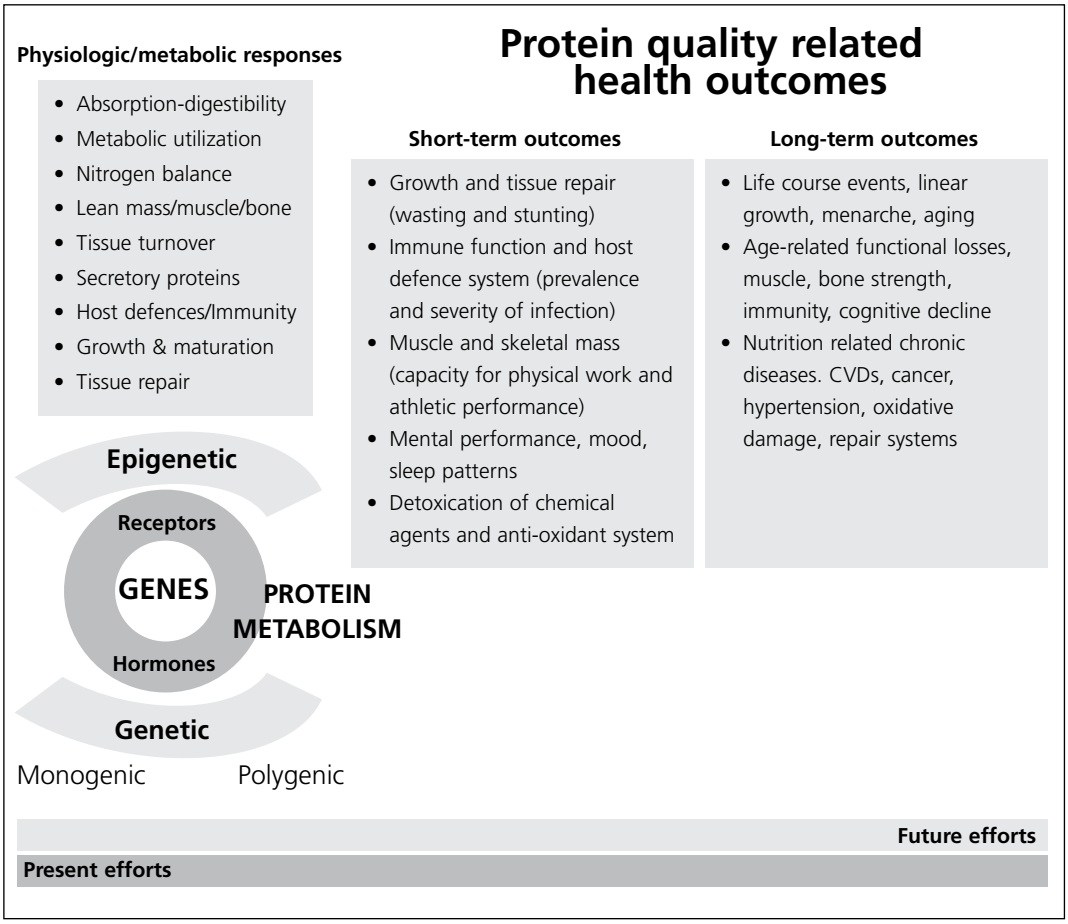
[3] Digestible IAA reference ratio (Digestible IAA in 1 g protein of mixed diet /mg of the same dietary indispensable amino acid in 1g of the reference protein)

[4] DIAAS for mixed diet (Lowest value of the "digestible IAA reference ratio" expressed as % for each reference pattern; for infants the mixed food has a calculated DIAAS of 56; for children 68 and for older children, adolescents and adults 82; NB: In this case as this is a mixed diet if the calculated DIAAS exceeded 100%, it would be truncated to 100%).

[5] These are the weighted average of the digestibility coefficients for methionine and cysteine.

Lys=lysine, SAA=sulphur amino acids (methionine + cysteine), Thr = threonine, Trp = tryptophan.

FIGURE 2.
Framework depicting short- and long-term potential protein quality related health outcomes. This indicates the need to look beyond physiological and metabolic responses in assessing health effects



The amino acid composition of human milk is recommended for predicting the protein quality of foods for infants and is discussed in the following section. Scoring patterns developed and published in the FAO/WHO/UNU (2007) report are recommended for age groups other than infants, and values for six-months-on are given in Table 3. Small calculation errors were found in the table given in the 2007 report for the three to 10 year age group and these have been corrected in the present table.

Inspection of the scoring patterns in relation to growth has led us to suggest that three scoring patterns (refer Table 5) be applied. **Recommended amino acid scoring patterns for calculating protein quality for dietary assessment are as follows:**

- **Infants (birth to 6 months), pattern of breast milk.**
- **Young children (6 months to 3 y), pattern for the 0.5 y old infant.**
- **Older children, adolescents and adults, pattern for the 3 to 10 y old child.**

For regulatory purposes, two scoring patterns are recommended, the amino acid composition of human milk for infant formulas and for all other foods and population groups the pattern for young children (6 months to 3 y); refer to Table 5 in this report.

TABLE 3.

Amino acid scoring patterns for toddlers, children, adolescents and adults (amended values from the 2007 WHO/FAO/UNU report)

			His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
Tissue amino acid pattern (mg/g protein)[1]			27	35	75	73	35	73	42	12	49
Maintenance amino acid pattern (mg/g protein)[2]			15	30	59	45	22	38	23	6	39
	Protein requirements (g/kg/d)										
Age (yr)	**Maintenance**	**Growth[3]**	*amino acid requirements (mg/kg/d)[4]*								
0.5	0.66	0.46	22	36	73	63	31	59	35	9.5	48
1-2	0.66	0.20	15	27	54	44	22	40	24	6	36
3-10	0.66	0.07	12	22	44	35	17	30	18	4.8	29
11-14	0.66	0.07	12	22	44	35	17	30	18	4.8	29
15-18	0.66	0.04	11	21	42	33	16	28	17	4.4	28
>18	0.66	0.00	10	20	39	30	15	25	15	4.0	26
			scoring pattern mg/g protein requirement[5]								
0.5			20	32	66	57	27	52	31	8.5	43
1-2			18	31	63	52	25	46	27	7	41
3-10			16	30	61	48	23	41	25	6.6	40
11-14			16	30	61	48	23	41	25	6.6	40
15-18			16	30	60	47	23	40	24	6.3	40
>18			15	30	59	45	22	38	23	6.0	39

His, histidine; Ile, isoleucine; Leu, leucine; SAA, sulphur amino acids; AAA, aromatic amino acids, Thr, threonine, Trp, tryptophan; Val, valine

1 Amino acid composition of whole-body protein.

2 Adult maintenance pattern.

3 Calculated as average values for the age range: growth adjusted for protein utilization of 58%.

4 Sum of amino acids contained in the dietary requirement for maintenance (maintenance protein x the adult scoring pattern) and growth (tissue deposition adjusted for a 58% dietary efficiency of utilization x the tissue pattern).

5 Amino acid requirements/protein requirements for the selected age groups. Note that these values, some of which are slightly amended from the 2007 report, are the correctly calculated values. In the published report, the value for the SAA requirement for children aged 3-10 is incorrect (18mg/kg/d) as are the SAA patterns for infants preschool and school children up to 10, (28, 26 and 24 mg/g protein).

Breast milk pattern

The amino acid composition of human milk has been used as a reference pattern to define the amino acid scores for infant foods (FAO/WHO/UNU, 2007). The metabolic demand for amino acids of the new born infant is not known with any certainty and the pattern of amino acids in human milk is not necessarily the same as the pattern of amino acid requirements. In fact amino acid intakes from breast milk are likely to be in excess of the actual demand for two reasons. Firstly as discussed in the FAO/WHO/UNU (2007) report, various calculations of the likely demand for amino acids by the new born infant indicate values that are lower than intakes from breast milk (Dewey *et al.,* 1996). Indeed the values for individual amino acids in the requirement pattern at 6 months, calculated by FAO/WHO/UNU (2007) on the basis of a maintenance and growth factorial model, are on average 30% lower. Secondly the true ileal digestibility of breast milk amino acids in the human infant may be less than 100%. Actual values are not known although studies using bottle-fed piglets as a model for the human infant have shown values for the digestibility of amino acids in human milk ranging from 81–100 % (Darragh and Moughan, 1998). Nevertheless, because intakes of breast milk from a healthy well-nourished mother are considered to satisfy protein requirements for the first 6 months of life, the amino acid content of breast milk is recommended as the current best estimate of amino acid requirements for this age group. The amounts of amino acids in human breast milk corrected for the true ileal digestibility of amino acids in human breast milk may provide useful information on the pattern of amino acids required by the infant.

Table 4.
Dietary indispensable amino acid profile of human milk[1]

Amino acid* (mg/g total protein)	
His	21
Ile	55
Leu	96
Lys	69
Met + Cys	33
Phe + Tyr	94
Thr	44
Trp	17
Val	55

[1] Values from FAO/WHO/UNU (2007)

* The three-letter abbreviations for amino acids (His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Cys, cystine; Phe, phenylalanine; Tyr, tyrosine; Thr, threonine; Trp, tryptophan; Val, valine) are used.

The amounts of each of the dietary indispensable amino acids in human milk, shown in table 4 are those listed by FAO/WHO/UNU (2007) (which derive from reports published by Heine *et al.,* 1991, Davis *et al.,* 1994, and Villalpando *et al.,* 1998.) These values are calculated from the amino acid content of proteins in breast milk, with protein calculated as 75% of total nitrogen given that 25% of nitrogen in breast milk is non-protein nitrogen. For weaned infants from the age of six months and for older children the scoring patterns shown in Table 5 derived for the various age groups in Table 3 are more appropriate.

Pattern for preschool and older children and adults: historical perspective

The use of an amino acid requirement pattern based on values for preschool-age children to evaluate protein quality for all age groups apart from infants derives from

Table 5.
Recommended amino acid scoring patterns for infants, children and older children, adolescents and adults

Age Group	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
	scoring pattern mg/g protein requirement								
Infant (birth to 6 months)[1]	21	55	96	69	33	94	44	17	55
Child (6 months to 3 year)[2]	20	32	66	57	27	52	31	8.5	43
Older child, adolescent, adult[3]	16	30	61	48	23	41	25	6.6	40

[1] Infant is based on the gross amino acid content of human milk from Table 4.

[2] Child group is from the 6 month (0.5 y) values from Table 3.

[3] Older child, adolescent, adult group is from the 3-10 y values from Table 3.

the joint 1991 FAO/WHO expert consultation on protein quality evaluation (see Millward, 2012b). At that time the available information on amino acid requirement patterns had been summarized in the 1985 report in which values had been reported for infants, preschool and older children and adults. In the case of both preschool children and schoolchildren, the 1985 report commented on the limited and unsatisfactory nature of the information available. The 1991 Consultation, which was asked to report on protein quality evaluation, re-examined the amino acid requirement values identified in the 1985 report. That report argued that the amino acid requirement values for adults were too low and were unsuitable for use in scoring patterns for the evaluation of protein quality in adults. Whereas the values for schoolchildren were considered flawed, the values reported for preschool children were adopted as the basis of a scoring pattern within the protein digestibility-corrected amino acid score methodology for all ages, as an interim measure until more satisfactory values could be defined.

The 2007 WHO/FAO/UNU Expert Consultation conducted a detailed critical analysis of the reported amino acid requirement values for infants, children and adults and the methodologies used in their derivation (see Millward, 2012a). This committee report endorsed the 1985 report in recommending the breast milk content of amino acids as the best estimate of infant amino acid requirements but was unable to identify reliable requirement values for any other age groups apart from adults. In relation to the values for preschool children, it argued that the reported values were difficult to interpret. They had not been peer reviewed and derived from a report that gave incomplete information about their origin. In particular, the limited details that were given (e.g. for lysine) suggested nitrogen accretion rates that were several-fold greater than expected for children of this age with values overall corresponding more closely to the needs of the 3–6-month-old infant than to those of a preschool child for whom growth has fallen to much lower rates than observed in infants. It therefore adopted a factorial approach for infants and children based on the amino acid requirements for maintenance and growth. Maintenance was assumed to exhibit the same amino acid pattern at all ages on a mg/kg body weight basis so that the adult requirement pattern was adopted, while growth

was assumed to reflect the amino acid pattern of human tissue protein. On this basis amino acid requirement patterns were derived for children aged 0.5, 1–2, 3–10, 11–14, 15–18 years and for adults.

Calculation of the scoring patterns from amino acid requirement values

A scoring pattern for protein quality evaluation is calculated on the basis of the ratio of amino acid to protein requirement (i.e. it is expressed as mg amino acid per g of protein). Thus the magnitude of the denominator, the protein requirement, influences the magnitude of each amino acid within the scoring pattern and consequently the extent to which the pattern would identify a food protein as adequate or deficient in each amino acid (Millward, 2012b). Previous reports on protein and amino acid requirements (FAO/WHO, 1973; WHO, 1985) had defined these scoring patterns from values for amino acid requirements expressed in relation to the safe protein requirement on the basis that the amino acid values represented the upper range of requirement values. Although this issue was not specifically discussed in the 2007 report in calculating a requirement pattern it identified estimates for the dietary indispensable amino acids as mean requirement values and therefore calculated the pattern with the mean total protein requirement, 0.66 g/kg for the adult.

It can be argued (Millward, 2012b) that although the values for each amino acid requirement identified in the 2007 report were selected as the best estimates from a range of different values, some higher and some lower than the selected values, they represented mean values so that the denominator in the pattern should be the mean protein requirement. An alternative argument is that in all of the experimental stable isotope studies from which amino acid requirement values have been derived the subjects have received intakes of protein or more often purified amino acids, at higher levels than the mean or even safe requirement levels (i.e. 1 g/kg/d). On the basis of an adaptive metabolic demand in which the requirement varies with the intake, the values obtained in these studies are likely to be higher than the minimum requirement and relate more closely to a protein intake that is higher than the minimum value indicated by the mean protein requirement value. In this case the safe protein intake would be a more appropriate value for the denominator of the scoring pattern.

This is an important issue in that the scoring pattern calculated with the mean protein requirement will contain values for each amino acid that are 20% higher than those calculated with the safe protein requirement. Thus dietary proteins judged inadequate by the former pattern may be judged adequate by the latter and vice versa. The 2007 report evaluated the implications of the scoring patterns derived with the mean protein requirement for the adequacy of dietary protein intakes and quality, and identified a significant prevalence of protein deficiency in several population groups in developing and developed countries and discussed the possibility that the scoring patterns may contain values for important amino acids such as lysine that are too

high. However the 2007 report also made the point that any risk assessment aimed at identifying prevalence of deficit should aspire to an acceptable balance between the numbers of false positives and false negatives. Moreover, there has been no direct experimental demonstration that the requirement for each dietary indispensable amino acid directly varies with the total intake of protein. In this context the present committee decided it was better to overestimate than underestimate risk and accepted the view that the scoring pattern should be based on the mean rather than the safe protein requirement.

Optimal amino acid requirements

Current estimates of the nutritional requirements for protein as reported by WHO/FAO/UNU (2007) are defined as: the lowest level of dietary protein intake that will balance the losses of nitrogen from the body, and thus maintain the body protein mass (assumed to be at a desirable level), in persons at energy balance with modest levels of physical activity and any special needs for growth, reproduction and lactation. That report acknowledged that such a definition does not necessarily identify the optimal intake for health, which is less quantifiable and would require more specific and validated biomarkers. After reviewing the evidence base for any relationships between protein intakes and health the report concluded: "Current knowledge of the relationship between protein intake and health is insufficient to enable clear recommendations about either optimal intakes for long-term health or to define a safe upper limit". Such research is ongoing and it may prove to be the case that there are circumstances in which benefits accrue from intakes above the minimum protein requirements, especially given that most definitions of "the healthy diet" involve overall protein intakes which are considerably higher than the minimum protein requirement derived from nitrogen balance. In circumstances in which an increased intake of protein or intake of specific amino acids may be appropriate or recommended, the optimal profile of amino acids in the protein is important in achieving the desired response. Further, the pattern of absorption of amino acids may affect the response to the ingested protein. **For these reasons, use of estimates of the amounts of individual digestible amino acids in a protein is likely to be the most successful approach to determining the optimal protein, or combinations of proteins, to be used in any circumstance.** This approach accounts for the possibility that in certain specific circumstances a particular protein may be more or less appropriate than reflected by the DIAAS value.

Examples of cases in which it has been suggested that benefit may accrue from protein intakes that are greater than the minimum include older individuals who might benefit in terms of muscle mass, strength and functional outcomes and these benefits may in turn be reflected in improved health outcomes (Wolfe, 2012). In specific circumstances younger as well as older individuals may benefit from increased intakes of protein. Fat loss in overweight individuals eating a low energy diet may be greater with a relatively high intake of protein due to both satiating effects of protein as well as the thermogenic

response to protein intake (Clifton, 2012; Te Morenga and Mann, 2012; Westerterp-Plantenga *et al.,* 2012). Gains in muscle mass and strength are greater when resistance exercise is coupled with increased intake of protein above the minimal amount necessary to maintain N-balance (Phillips, 2012). Individuals with chronic infection or inflammation may benefit from higher protein intakes, and the effects of less than optimal levels of total caloric intake may be offset to some extent by higher protein intake. It should be noted, however, that there are circumstances whereby higher protein intakes may be associated with risk. One example is pregnancy where it has been suggested that the protein requirement identified in the 2007 FAO/WHO report, which represents a three-fold increase compared with previous estimates, may be too high and represents a risk of adverse outcomes to both mother and child (Millward, 2012a).

In addition to potential beneficial effects of a protein intake greater than the amount necessary to maintain nitrogen balance in a variety of circumstances, there may be specific cases in which it is desirable to increase the intake of specific amino acids. For example, leucine is recognized as a potential regulator of protein synthesis in a variety of circumstances (McNurlan, 2012; Millward, 2012c); a high level of leucine intake may facilitate overcoming the normal resistance to the anabolic effect of protein intake in clinical situations such as cancer. A number of clinical situations, such as sepsis, are associated with an impairment of the normal rate of synthesis of arginine, and in these circumstances an increased intake of arginine may be beneficial in terms of protein synthesis as well as immune function (Jonker *et al.,* 2012).

The Committee noted current research trends towards examining dietary protein and amino acid levels that optimise certain health outcomes or organ/body functions in people of different ages and physiological states, rather than the previous focus on determining protein and amino acid requirements to meet body nitrogen balance. Research in this direction is encouraged. **The recommendation in this report of treating amino acids as separate individual nutrients by stating the amounts of each truly digestible (ileal) dietary indispensable amino acid in foods is viewed as a useful development in this respect.**

4.4 CORRECTION FOR AMINO ACID DIGESTIBILITY AND AVAILABILITY IN THE CALCULATION OF DIAAS

Bioavailability of amino acids

Since its adoption by FAO/WHO (1991), the PDCAAS has been widely accepted. However, the method has been criticized because it does not adequately account for the bioavailability of amino acids.

The term "bioavailability" encompasses three properties of foods that can alter the proportion of an amino acid that can be utilized; these are:

1. Digestibility, which describes the net absorption of an amino acid.
2. Chemical integrity, which describes the proportion of the amino acid that, if absorbed, is in a utilizable form.
3. Freedom from interference in metabolism resulting from the presence in the food of substances that limit the utilization of the amino acid.

Of these, the greatest source of variation in bioavailability is, in most cases, digestibility.

Digestibility: amino acids

It is worth emphasizing at the outset that digestibility is not a fixed attribute of a food but reflects an interaction between the food and the person eating it and so may be subject to individual variation. The term "amino acid digestibility" as used in this report is the proportion of consumed amino acids that is absorbed (i.e. has disappeared from the digestive tract).

In earlier work protein quality assessment was based on the digestibility of crude protein determined over the total digestive tract. This approach assumes that the digestibility of each amino acid is the same as that of total protein and that amino acid digestibility determined over the total digestive tract is an accurate estimate of dietary amino acid absorption. However, observations with simple-stomached animals have raised questions about the validity of these assumptions.

As reviewed by WHO/FAO/UNU (2007) (see Section III) most faecal nitrogen is in the form of microbial protein (Mason and Palmer, 1973). Mason *et al*. (1976) estimated from the faecal excretion of diaminopimelic acid (DAPA) that some 90% of faecal N was of bacterial origin. Subsequent studies using a variety of microbial markers have confirmed this observation. Consequently, the amino acid composition of faeces is closer to that of microbial protein than to that of undigested food residues, and the amino acid composition of faeces varies little with diet, although total faecal nitrogen does vary with faecal bulk and NSP intake. It was concluded that undigested food residues reaching the large intestine are largely degraded by microbial activity during their relatively long residence, when their nitrogen can be converted through microbial amino acid synthesis into microbial biomass with an amino acid profile more or less independent of their initial composition.

The second observation was that although nitrogen is absorbed from the large intestine, it is mainly in the form of ammonia with only limited evidence for absorption of intact amino acids. In pigs, infusing hydrolyzed casein into the caecum (Zebrowska, 1973; 1975; Gargallo and Zimmerman, 1981) resulted in very little increase in faecal nitrogen; most of the additional infused N was excreted in the urine with little if any improvement in N retention. Also infusion into the large intestine of a single dietary indispensable amino

acid that was deficient in the diet has been shown to be of little or no benefit (Darragh *et al*., 1994; Krawielitzki *et al*., 1984). The results of such studies suggest that most of the carbon skeletons of dietary indispensable amino acids entering the large intestine from the ileum are irreversibly lost, either through microbial metabolism or excretion in the faeces, although their nitrogen may be absorbed and used. However, as discussed earlier (Section III), and as indicated in Figure 1, human studies have shown that the hydrolysis of urea within the large intestine and the salvage of its urea nitrogen which is returned to the host amino nitrogen pool, is a quantitatively important part of nitrogen metabolism within this compartment of the digestive tract (Jackson, 1998), and the extent to which this may be a source of nutritionally important amino acids has been investigated with ^{15}N tracer studies. Clearly the appearance of ^{15}N-labelled protein in blood plasma after intracaecal instillation of labelled proteins (e.g. Heine *et al.,* 1987) is not evidence for the absorption of specific amino acids: most amino acids in the body can acquire ^{15}N by transamination, as seen in the extensive ^{15}N labelling of body protein after giving $^{15}NH_4Cl$ (Patterson *et al.,* 1995; Metges *et al.,* 1999). However, studies with human infants have identified the transfer of ^{15}N from orally administered urea to not only glycine, alanine and histidine in the circulating amino acid pool (sampled as urinary amino acids), but also to lysine which does not gain nitrogen through transamination (Millward *et al*., 2000a). Furthermore in normal healthy adults transfer of ^{15}N from oral lactose-ureide to lysine in both faecal bacterial protein and in urine has been reported (Jackson *et al.,* 2004) which is significant because lactose-ureide is resistant to digestion in the upper gastrointestinal tract but is fermented by the colonic microflora to release NH_3. Thus, bacterial amino acid biosynthesis from nitrogen released by urea salvage appears to be a source of indispensable amino acids which can enter the circulating pool. Furthermore the extent of ^{15}N transfer in these studies has been shown to indicate that this process can be nutritionally important. Clearly the evidence base for these processes is currently limited and the route by which colonic urea N is transferred to systemic lysine and other indispensable amino acids is not clear. However such studies raise important questions about nitrogen metabolism in the human large intestine, suggesting that it can not only remove indispensable amino acids but may also in some circumstances be a source of dietary indispensable amino acids. Fuller (2012) has concluded, albeit acknowledging that such a conclusion is based on limited evidence, that a large proportion of the amino acids in the protein of the upper gastrointestinal tract microbiota are incorporated directly from the diet or from endogenous materials rather than being synthesised *de novo*. Despite some remaining uncertainties with respect to microbial amino acid synthesis, it seems that the amino acid composition of ileal digesta provides the best available basis for estimating the proportion of dietary amino acids absorbed. "Ileal digestibility, while not a perfect measure of net amino acid absorption, nonetheless takes us considerably closer to that ideal than amino acid digestibility determined over the whole gut", (Fuller, 2012).

These observations show that the process of amino acid digestibility is complex and not entirely understood. Overall the consultation concluded that estimates of the amino

acids absorbed from the diet would best be derived from measurement of the flow of amino acids leaving the small intestine; that is, ileal digestibility (Moughan and Smith, 1985). However, and as discussed earlier (Section III), some of the amino acids leaving the ileum are not of immediate dietary origin but are the remnants of endogenous secretions and cellular material (Skilton *et al.,* 1988; Moughan and Rutherfurd, 2012). This loss of endogenous protein occurs even when no protein is given in the diet and therefore represents part of the requirement. This amount, termed the basal endogenous loss must be deducted from the ileal amino acid flow to estimate the contribution of unabsorbed amino acids from the diet. When apparent amino acid digestibility is corrected for the basal endogenous loss the resulting value is termed true digestibility (Donkoh and Moughan, 1994). When apparent amino acid digestibility is corrected by deduction of a constant agreed basal endogenous loss value, the resulting value is termed standardized ileal digestibility (Stein *et al.,* 2007). The basal endogenous amino acid losses can be measured using several methods (Moughan *et al.,* 1998; Boisen and Moughan, 1996; Fuller and Tomé, 2005). Endogenous and dietary amino acid losses at the terminal ileum are 0.6–1 g/day and 0.4–0.7 g/day, respectively (Chacko and Cummings, 1988; Mahé *et al.,* 1992; Rowan *et al.,* 1993; Fuller *et al.,* 1994; Gausserès *et al.,* 1996; Mariotti *et al.,* 1999; Gaudichon *et al.,* 2002; Moughan *et al.,* 2005).

For protein as a whole, however, because nitrogen absorbed in forms other than amino acids can contribute to the nitrogen economy, the absorption of nitrogen over the whole digestive tract is the more appropriate measure. This latter measure also requires correction for endogenous losses (often referred to as metabolic faecal nitrogen).

It is therefore recommended that protein quality assessment should be based on true ileal digestibility values of individual amino acids rather than the overall (faecal) digestibility of protein.

This conclusion is supported by a number of recent critical reviews on the subject (Fuller, 2012; Fuller and Tomé, 2005; Hendriks *et al.,* 2012; Levesque and Ball, 2012; Moughan, 2003). At the present time, there is a limited quantity of data on the ileal amino acid digestibility of foods as determined in humans (Rowan *et al.,* 1994; Gaudichon *et al.,* 2002; Deglaire *et al.,* 2009). Where human data are lacking **it is recommended that true ileal amino acid digestibility values from the growing pig be used, and where these data are not available from the growing laboratory rat.** For digestibility measures in infants the bottle-fed piglet has been a useful animal model (Moughan *et al.,* 1990). Although regression equations have been published (Deglaire *et al.,* 2009) to allow the prediction of human true ileal amino acid digestibility from corresponding pig values, it was concluded that more work is required to improve the robustness of these equations. When an accurate prediction equation is available, human digestibility values, predicted on the basis of pig values, should be used. For those foods for which neither human nor pig or rat ileal digestibility values yet exist, overall (faecal) protein digestibility values must serve as the best available proxy.

It is recommended that the 1970 FAO Publication "Amino Acid Contents of Foods and Biological Data on Proteins" should be updated on a continuous basis with inclusion of values, where available, for protein (faecal and ileal) digestibility, ileal amino acid digestibility and DIAAS. These tables should be available in electronic format compatible with the proposed spreadsheet for the calculation of amino acid requirements and DIAAS.

At the 2011 Expert Consultation, a sub-committee (consisting of Sarwar Gilani, chair; Daniel Tomé, Paul Moughan and Barbara Burlingame, ex officio) was constituted to collate currently available data on the true ileal amino acid digestibility of foods for humans (refer Sub-Committee report at http://www.fao.org/ag/humannutrition/nutrition/en/ and http://www.fao.org/ag/human_nutrition/nutrition/63158/en/). A separate sub-committee (consisting of Ricardo Uauy, chair; Joe Millward, Paul Pencharz, Malcolm Fuller and Barbara Burlingame, ex officio) was constituted to receive the data set from the first sub-committee and assess its suitability for practical application in the calculation of DIAAS values and to assess implications of these data for the final consultation report.

After assessment of the dataset of currently available ileal amino acid digestibility values, the sub-committee chaired by R Uauy concluded (refer Sub-Committee report at http://www.fao.org/ag/ humannutrition/nutrition/en/ and http://www.fao.org/ag/humannutrition/nutrition/63158/en/):

1. In principle, true ileal amino acid digestibility is preferable to faecal crude protein or amino acid digestibility for the purpose of defining dietary indispensable amino acid digestibility and assessing the protein quality of dietary protein sources for humans.
2. There is a fair body of evidence on ileal amino acid digestibility in rats and pigs but there are limited data on ileal amino acid digestibility determined in humans; very few studies have compared the ileal amino acid digestibility of the same protein sources in animals (rats, pigs) and humans. Studies of this kind are greatly needed to be able to support moving in practice to ileal digestibility in the assessment of dietary protein quality for humans.
3. Future studies should include comparisons of true ileal amino acid digestibility values across the different animal models (pig, rat) and humans using protein sources that are representative of those consumed by human populations.
4. If the data obtained from these studies (as specified under #3) convincingly support the move to ileal digestibility, assessment of the potential impact of this recommendation (to be used in the assessment of individual protein sources as well as mixed diets commonly consumed by humans) needs to be undertaken before the new evaluation model is implemented. This should include potential gains and or losses to public health consequent upon the implementation of the new recommendations on the assessment of protein quality for humans.

The Expert Consultation Committee accepted the above conclusions of the sub-committee and recommended: that the FAO convene a Working Group, as a matter of urgency, to agree upon an experimental protocol to enable the realisation of outcomes numbers 3 and 4 above to be expedited. The implementation of studies to determine true ileal amino acid digestibility broadly across human food types and a subsequent assessment of the potential impact of introducing such data in the context of protein quality evaluation for humans is strongly encouraged. Until such time as an agreed dataset of true ileal amino acid digestibility for human foods becomes available, the protein quality of human foods and diets should be assessed using DIAAS, but values for faecal crude protein digestibility should be used.

There will be a need for financial support for the latter research agenda (interspecies true ileal amino acid digestibility comparison and the development of a database of true ileal amino acid digestibility for human foods). It is anticipated that the private sector along with UN technical and normative agencies, multilateral, bilateral and national Government agencies, and public-good organisations will provide such support, as a matter of urgency. If resources are not allocated to fulfil the latter proposed research objectives in a timely manner, then the present recommendation for the application of DIAAS in practice may need to be reviewed, since DIAAS and the conclusions of this report rely upon a system of true ileal amino acid digestibility and availability.

Chemical availability of amino acids

Some amino acids present in foods may be in a structural form that is unavailable (i.e. the amino acid may be absorbed in a form that cannot be utilized). This is most likely to be encountered in foods that are heat-treated or subjected to other severe processes (Rutherfurd and Moughan, 1990; Rutherfurd and Moughan, 2012). The formation of Maillard reaction products, leading to a loss of lysine availability, is the most common example. **It is recommended that for foods susceptible to damage from processing, 'reactive' rather than 'total' lysine contents and the true ileal digestibility of reactive lysine (lysine availability) rather than of total lysine, be determined (Moughan and Rutherfurd, 1996; Rutherfurd *et al.*, 1997b).** Reactive lysine is lysine whereby the epsilon amino group of the molecule has not been modified chemically and is free to react with a test agent (e.g. fluorodinitrobenzene or o-methylisourea).

Other amino acids, especially the sulphur amino acids, tryptophan and threonine, may be susceptible to oxidation, with loss of bioavailability, and assays such as the reactive lysine digestibility assay (Moughan and Rutherfurd, 1996) need to be developed for these amino acids.

Loss of bioavailability due to the presence of interfering substances

Many foods contain bioactive (protein or non-protein) substances that may modify amino acid bioavailability either by affecting digestibility or postabsorptive utilisation (Gilani *et al*., 2012). Many foods, including novel protein sources, may contain high levels of known antinutritional factors, which may be naturally occurring (e.g. tannins, phytates, trypsin inhibitors, glucosinolates, isothiocyanates), formed during processing (e.g. D-amino acids, lysinoalanine), or formed during genetic modification of crops (e.g. lectins).

Many of these affect digestion and will be taken into account in the determination of true ileal amino acid digestibility but others, such as glucosinolates, isothiocyanates, etc., have more general metabolic effects and their influence on protein metabolism will only be detected in a growth-based bioassay. Where they present a potential problem, recommendations on proper processing to minimize their levels are required as well as recommendations on the safe limits for their inclusion in diets.

4.5 CONSIDERATIONS REGARDING THE USE OF BIOASSAYS TO DETERMINE PROTEIN QUALITY

The nutritive value of food protein sources is primarily dependent on the amounts of bioavailable indispensable amino acids and nitrogen in food. Bioavailability refers to the proportion of the total amount of dietary amino acids that is absorbed in a form that can be utilized for body protein synthesis and other pathways which constitute the metabolic demand. In some cases, such as with inadequate energy intake or when dietary protein is in excess, absorbed amino acids may be utilized via catabolism to provide ATP, rather than for body protein synthesis and associated anabolic pathways. This requires that amino acid bioavailability is evaluated under standardised conditions in relation to dietary protein and energy contents. Amino acid availability and utilization are not synonymous. Traditionally the methods developed to determine amino acid bioavailability have focused on intestinal absorption or digestibility, which is calculated as the proportion of amino acid intake that does not appear in digesta or faeces. While considerable progress has been made to arrive at the "true ileal digestibility of amino acids" and the "true ileal digestibility of reactive lysine", digestibility-based methods may not always fully account for all losses associated with gut endogenous amino acid losses or absorbed amino acids which are unavailable due to heat processing or the presence of anti-nutritional factors. Therefore, there is a need from time to time to apply growth-based bioassays (such as the slope-ratio assay,).In some circumstances the classical Protein Efficiency Ratio (PER) can be used when there is doubt about the protein quality of a food or diet, although it must be recognised that in human nutrition the demand for dietary amino acids for growth is a minor or even negligible component of the demand apart from during early life. This is a major limitation in the use of animal growth models to assess overall protein quality since such trials may underestimate protein quality for human nutrition. In the latter case short term nitrogen balance trials have been used but these have generally lacked

discriminatory power (Millward *et al.,* 1989) and resulted in unrealistically low efficiencies of utilisation (shallow slopes) because of an inappropriate analytical model which fails to take into account the adaptive nature of the metabolic demand (Millward, 2003; 2012a). While long term feeding trials based on body composition and maintenance of fitness have been used to assess protein quality of specific foods such as wheat (Bolourchi *et al.,* 1968; Edwards *et al.,* 1971), such feeding trials are expensive and logistically difficult to undertake and few have been reported. The diurnal nature of human feeding does involve post-prandial net protein synthesis to replace post absorptive losses and the efficiency of postprandial protein utilisation can be studied. This, to some extent, can be used as a measure of protein quality in humans. Several groups have developed stable isotope tracer studies to do this.

Postprandial protein utilization (PPU)

As discussed by Millward and Pacy (1995) postprandial protein utilisation is influenced by both dietary energy intake and by the quality of the protein in terms of its ability to meet the metabolic demand. This means that measurement of acute changes in ^{13}C-1 leucine balance during the transition from a low to high protein intake during a ^{13}C-1 leucine infusion indicates the efficiency of postprandial protein utilisation (PPU). Values obtained in this way are more realistic than those obtained from the slope of nitrogen balance studies which underestimate protein utilisation (Millward, 2003; Millward, 2012a). This approach has been used to compare milk and wheat protein utilisation in normal adults at their habitual levels of protein intake showing that the PPU of milk and wheat protein were 1.00 and 0.68 in a multiple small meal protocol (Millward *et al.,* 2000b) and 0.93 and 0.61 in a single large meal protocol (Millward *et al.,* 2002). In each case the wheat protein was better utilised than was predicted from its lysine content relative to human tissue protein lysine content possibly through reutilisation of the lysine liberated in the postabsorptive state for postprandial protein deposition. While such studies help to understand utilisation of highly digestible proteins they would be less able to entirely evaluate poorly digestible dietary protein sources.

Net postprandial protein utilization (NPPU)

[^{15}N]-labelled proteins (milk, soya protein isolate, wheat and meat) have been used to measure the metabolic fate of dietary nitrogen after its consumption in humans. NPPU is calculated using true ileal digestibility and ^{15}N-labelled protein utilization parameters (Tomé and Bos, 2000). Intrinsic labelling of dietary proteins with ^{15}N allows the investigation of postprandial N transfers into different metabolic pools. Ileal digesta, blood and urine are sampled. The kinetics of dietary N appearance in ileal effluent, plasma proteins, plasma free amino acids, body urea, urinary urea and urinary ammonia are calculated using a 13-compartment, 21 parameter model (Juillet *et al.,* 2006). NPPU values determined for milk, soya protein isolate and wheat were 81%, 78% and 66%, respectively (Bos *et al.,* 1999; Tomé and Bos, 2000; Mariotti *et al.,* 1999; Bos *et al.,* 2005). This approach also

incorporates the determination of true ileal amino acid digestibility (Gaudichon *et al.,* 2002).

This method is a major advance in the evaluation of dietary protein quality. It is restricted, however, to foods that can be intrinsically labelled with ^{15}N and the study requires that ileal digesta be collected via a naso-intestinal intubation technique, and the model calculations are fairly complex (Juillet *et al*., 2006). Therefore, this method is unlikely to be widely adopted for routine application. Furthermore the NPPU technique cannot be readily used to estimate the bioavailability of individual amino acids.

Application of the IAAO method to determine the metabolic availability (MA) of amino acids

The Indicator Amino Acid Oxidation (IAAO) technique is based on the concept that when one dietary indispensable amino acid in a diet (IDAA) is deficient for protein synthesis, then all other amino acids including the indicator amino acid (another IDAA, usually L-[1-13C]phenylalanine) will be oxidized (Pencharz and Ball, 2003). Fundamentally, this is because free amino acids cannot be stored and therefore must be partitioned between incorporation into protein or oxidation. With increasing intake of the limiting amino acid, oxidation of the indicator amino acid decreases, reflecting increasing incorporation into protein. Once the requirement for the limiting amino acid is met, there is no further change in the oxidation of the indicator amino acid. The inflection point, where the oxidation of the indicator amino acid stops decreasing and reaches a plateau is referred to as the 'breakpoint'. The breakpoint identified with the use of bi-phase linear regression analysis indicates the mean or Estimated Average Requirement (EAR) of the limiting (test) amino acid (Pencharz and Ball, 2003). This minimally invasive IAAO method has been systematically applied to determine IDAA requirements in adult humans (Pencharz and Ball, 2003; Elango *et al.,* 2008(a); Elango *et al.,* 2008(b)).

The IAAO method can also be applied to determine the bioavailability or metabolic availability (MA) of amino acids (Moehn *et al.,* 2005; Moehn *et al.,* 2007). IAAO is inversely proportional to the rate of protein synthesis (Ball and Bayley, 1986; Rafii *et al.,* 2008). Therefore, at a given amino acid intake, the relative difference in the IAAO rate between test and reference proteins will be proportional to the whole body MA of the test amino acid for protein synthesis, and thus account for all losses of dietary amino acids during digestion, absorption, and cellular metabolism. It would be expected, under controlled conditions and for the often dietary first-limiting amino acid, lysine, that the predicted uptake of reactive lysine (true ileal digestible reactive lysine) from the digestive tract would equal bioavailable lysine determined using the IAAO method and such an experimental comparison for a range of foods would be of interest. The IAAO approach has been used in pigs to determine the availability of dietary protein-bound amino acids (including lysine, threonine and methionine) and in humans for methionine and lysine. It is proving to be a practical method to determine the utilization of protein bound limiting amino acids for net protein synthesis.

4.6 AMINO ACID ANALYSIS AND TRUE AMINO ACID DIGESTIBILITY/ BIOAVAILABILITY METHODOLOGIES

Amino acid analysis methodology

Considerable progress has been made over recent years in amino acid analysis (Rutherfurd and Sarwar-Gilani, 2009; Otter, 2012) and the Committee agreed that no one method of analysis is necessarily the best, with a variety of approaches being acceptable.

Amino acids occur in foods in either the free amino acid form or as the building blocks of proteins. The analysis of amino acids in foods is composed of a number of unit operations; the release of the amino acids (if they are in protein form) from the food matrix, the separation of the individual amino acids and their quantification using calibration standards.

Each of these steps has its own idiosyncrasies, (e.g. different hydrolysis conditions are required for the optimal release of different amino acids and not all amino acids have baseline separation for some chromatographic methods) and there is a diversity of food matrices, such that most laboratories adapt methods to best suit their applications.

There is currently no official standardised method for amino acid analysis although AOAC have a number of validated methods for individual components.

The established analytical techniques of HPLC (IEX or RP) and GCMS have recently been supplemented by a number of new methods for the characterisation of amino acids. These include capillary electrophoresis (CE), CEMS and UPLC, LCMS and LC with other detectors.

The Committee agreed that it would be useful if a guide as to suitable approaches (and attendant pitfalls and shortcomings) could be developed, and supported by an international standardization of methods (including approaches to the hydrolysis, separation, detection and presentation of data). **The Committee recommended that the FAO establish a formal working party to review amino acid analysis methodologies and provide some guidance towards international standardization.**

True amino acid digestibility/availability assays

A working party should review and recommend best practice for a pig-based assay for true ileal amino acid digestibility determination. Such an assay would replace the rat true faecal crude protein digestibility assay. Ideally a rapid *in vitro* protein digestibility assay to determine amino acid digestibility in foods would be available. Many such assays have been developed, but none has been adequately fully and independently validated. There is an urgent need to develop a standardised, independently validated

in vitro protein and amino acid digestibility assay. The application of *in vivo* amino acid bioavailability assays and other assays such as the slope ratio assay is relatively laborious.

4.7 BIOACTIVE COMPONENTS INTRINSICALLY ASSOCIATED WITH FOOD PROTEINS INCLUDING THOSE OCCURRING NATURALLY OR FORMED DURING PROCESSING

Bioactive components are sometimes associated intrinsically with food proteins. Potentially, these may have either negative effects (e.g. ANFs such as trypsin inhibitors and glucosinolates) or a positive effect (e.g. antioxidant effects of polyphenolics or certain effects of bioactive peptides released during the digestion of a protein). Many of the negative effects of compounds such as plant fibre and ANFs are captured in measures of apparent ileal amino acid digestibility and true ileal amino acid digestibility (where correction has been made for basal endogenous amino acid losses), as their effects are often mediated through inducing increased ileal endogenous amino acid losses above the basal endogenous loss value. Nevertheless, there may be both positive and negative effectors, intrinsically associated with dietary proteins, the effects of which will not be reflected in true amino acid digestibility or DIAAS values, and this needs to be recognized. Where such factors may be deleterious, **it is recommended that upper limits of these compounds in diets be established and it is further recommended that the Joint Expert Committee on Food Additives (JECFA) give due consideration to these safety aspects.** Food processors need to be aware of safe upper limits and ensure quality control, so that in the finished product such compounds are below these set levels.

The role of bioactive peptides is a rapidly emerging area of science (Rutherfurd-Markwick, 2012) and the myriad of potential effects of peptides released during natural digestion cannot be, nor should be expected to be, expressed in a single value of dietary protein quality such as DIAAS. However, their potential importance does need to be recognized, and there is clearly still a need for the application of traditional methods of dietary protein quality evaluation such as PER, NPPU, biological value etc, and a need to understand physiological effects of proteins in addition to direct effects on body protein metabolism.

4.8 DIAAS – REGULATORY ISSUES

DIAAS is the recommended method for dietary protein quality assessment for regulatory purposes, and the use of true ileal digestible amino acid contents in their own right for describing foods is also encouraged.

Individual countries have their own regulations, (e.g. Canada uses protein rating: the amount of protein in a serving of reference food, multiplied by PER). **The recommendation is to use DIAAS as the measure of protein quality, rather than measures such as PER.**

For the purpose of Codex, a quality assessment needs to be applied to protein claims. DIAAS is recommended for such protein quality assessment and should be given in conjunction with the protein quantity value. Substitute foods should not have DIAAS lower than the scores for the equivalent real food. Statement: the protein content of the food should be declared as determined by an appropriate analytical method and the quality determined by the DIAAS.

For making a protein content claim the protein content should be determined analytically and evaluated for quality using DIAAS. The nutrient reference value (NRV) for protein recommended for labelling purposes in the interests of international standardization and harmonization is 50 g.

To qualify for the nutrition claim: "source" for protein, a food must meet the following criteria:

10% of NRV per 100 g (solids);
5% of NRV per 100 ml (liquids);
or 5% of NRV per 100 kcal (12% of NRV per 1 MJ);
or 10% of NRV per serving.

To qualify for: "High" for protein, the food must contain two times the values for "source".

When a food meets the criteria for protein quantity, then a quality measure should be applied.

A comparison table for foods should be prepared to establish cut off values for nutrition claims for "source" and "high".

DIAAS cut-off values are needed to distinguish between excellent/high (e.g. 100 or more), good/source (e.g. 75-99), and no claim.

It is recommended that no nutrition claim should be allowed to be made for source/high protein for proteins with DIAAS less than a certain cut-off (e.g. 75).

In assessing the quality of proteins, quality cannot be substituted for quantity. An example of how these DIAAS cut-off values may be applied is given in Table 6. The actual values for the DIAAS cut-off points in the context of making claims requires careful further consideration (e.g. in relation to national and local dietary patterns).

It is recommended that a "quality" statement related to protein (e.g., source of quality protein) be allowed.

When calculating the DIAAS of new formulations of foods supplemented with crystalline amino acids, DIAAS should be confirmed by biological testing.

Table 6.
Example of the use of DIAAS for protein quality assessment in the context of making claims.

Food	Amount	Protein content (g/100g)	DIAAS[1]	Judged quality	Eligible for claim based on quantity	Eligible for claim based on quantity and quality
Wheat	100 g	11	40	Low	Yes, high	No, none
Peas	100 g	21	64	Low	Yes, high	No, none
Whole milk powder	100 g	28	122	High	Yes, high	Yes, High

[1] DIAAS calculated using true ileal indispensable amino acid digestibility values and reference amino acid pattern for child (6 months to 3 years).

Protein sources for which there are no previous data available must be subjected to biological evaluation for protein quality.

The Committee recommends that a full published set of guidelines for industry be developed (including recommendations on methods for biological testing), along with a published set of dietary guidelines aimed at providing advice to consumers and policy-makers.

4.9 RECOMMENDATIONS FOR FURTHER RESEARCH

Human amino acid requirements

1. Determine amino acid requirements for subjects fully adapted to lower than usual protein intakes, especially the current mean protein intake of 0.66 g protein/kg/day. A recent study has provided an estimate for the mean adult protein requirement of 0.91 g protein/kg/day. The relevance of such a finding in relation to other recent experimental findings and to the overall data on the mean adult requirement needs to be carefully assessed.
2. Determine amino acid requirements in different conditions and circumstances, such as in children, pregnancy, aging and exercise, as well as gender effects.
3. Further validate existing methodologies by comparison with long-term outcomes of body composition and possibly functional outcomes.
4. Investigate the role of specific amino acids as regulators of metabolism and other functions in various physiological and clinical states, and how such actions of specific amino acids would affect the amino acid profile of the reference protein for DIAAS calculation.
5. Determine the importance of dietary dispensable amino acid intake, and determine if there are circumstances in which account should be taken of the dispensable amino acids in calculating the DIAAS value of a protein.

6. Explore new approaches for determining amino acid requirements, including the use of gene expression studies (including nutrigenomics), metabolomics and/or specific biomarkers.
7. Explore the implications of dietary protein quality on lifetime health and longevity.

Analytical

To update and expand the FAO database of amino acid contents of foods and include true ileal amino acid digestibility data.

Ileal digestibility

1. Further determine true ileal digestibility of protein and amino acids in a wider range of foods and determine the ileal digestible tryptophan content of human milk.
2. Develop non-invasive accurate methods to determine or predict true ileal dietary protein and amino acid digestibility in humans based on identified biomarkers.
3. Validate the use of animal model data (including providing more robust inter-species prediction equations for true ileal amino acid digestibility) to quantify ileal digestibility in humans, including relating digestibility to functional outcomes.
4. Determine more fully the role of the small intestinal and colonic microflora on ileal amino acid digestibility values.
5. Develop new bioavailability assays such as the reactive lysine assay, for other amino acids.
6. Develop and validate *in vitro* methods for predicting amino acid digestibility and bioavailability in humans.

Evaluation and perfection of techniques to directly measure the bioavailability of protein bound dietary amino acids in humans

While DIAAS, combining ileal amino acid digestibility with predicted bioavailability identified as the amino acid score, is a step forward it is still dependent on the score accurately predicting the biological value of the absorbed amino acid mixture and hence the overall protein quality. Because the actual metabolic demand and requirement for amino acids is complex and not fully understood, any approach to predicting protein quality will likely be imperfect to a greater or lesser extent. The stable isotope methods outlined above offer additional useful information about dietary protein quality in human nutrition, but each has limitations of one sort or another in their application. Nevertheless these or other novel approaches need to be further developed. Methods using metabolomics approaches and relating complex metabolite profiles from plasma and urine samples to protein and amino acid true ileal digestibility and availability offer a promising perspective for the evaluation of dietary protein quality in humans.

Impact of interaction between bioactive factors and protein quality and function

1. Investigate bioactive factors intrinsically associated with specific proteins [such as peptides resulting from digestion, trypsin inhibitors, lectins, isoflavones (e.g., genistein), etc.].
2. Assess nutrient interactive effects during or after digestion that may enhance or depress the bioactivity of the test protein, or may have independent effects, for example, phytic acid, plant fibre, sugars.
3. Determine the effect of the nature and amount of simultaneous non-protein energy intake on the bioactivity of the test protein.

Communication

1. FAO to prepare a manual to provide guidance to policy makers, industry and the public on dietary protein quality evaluation and the use of DIAAS in making protein related claims.
2. FAO to prepare guidance on integrating aspects of dietary protein quality evaluation into food based dietary guidelines to provide advice for consumers and policy makers.
3. Incorporation of indicators of protein quality (e.g., lysine value) into food balance sheets for national and global applications.

Animal and plant breeding, food preparation and processing effects

1. Determine effects of food preparation and processing methods to optimize dietary protein quality and protein utilization.
2. Generate data at the level of the genetic resource (i.e., biodiversity and biotechnology) on amino acid composition and digestibility related to sustainability issues and to lead to the recognition of existing and the development of new environmentally sustainable higher protein quality foods.

4.10 STRENGTH OF EVIDENCE USED IN MAKING THE RECOMMENDATIONS

Preamble

The 2011 FAO Expert Consultation focused on the current state of knowledge relating to amino acid digestibility and availability in foods, and methodologies in which these values, together with the amino acid composition of dietary protein, are used for predicting dietary protein quality in the human diet. Such prediction involves comparing the dietary amino acid supply in terms of the composition, digestibility and bioavailability of amino acids in dietary protein with estimates of protein and amino acid requirements represented by reference amino acid scoring patterns. These latter values were the subject of the 2007 FAO/WHO/UNU expert consultation report and the values *per se*

were not re-examined in this report apart from a careful consideration of the reference amino acid scoring patterns (i.e. age related amino acid requirements per gram of protein requirements), which are proposed for use in this report (see Table 5). The main work of the presently reported consultation involved an analysis of the strengths and weaknesses of the existing PDCAAS classification compared with the proposed replacement DIAAS approach. It is thus important to assess the 'strength of evidence' underlying the conclusions reached by the Committee in relation to the proposed eventual change to the new approach.

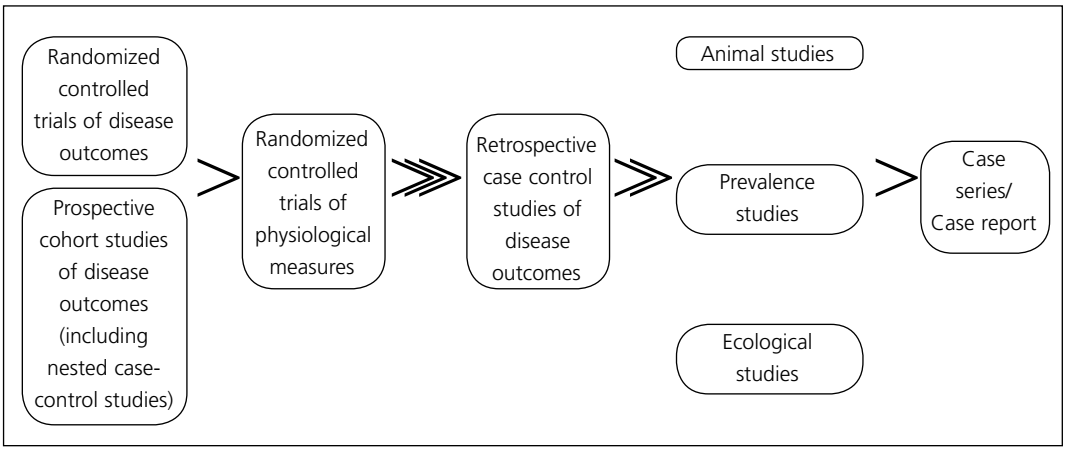
In reaching their conclusions and making recommendations after assessing the scientific evidence, the Expert Consultation Committee was mindful of discussions in previous FAO/WHO reports of the hierarchy of strength of evidence.

A hierarchy of evidence

In the most recent FAO report (Fats and Fatty Acids in Human Nutrition, FAO 2010) and in the context of defining dietary requirements for fatty acids, general criteria were identified, namely:

- To prevent clinical deficiencies.
- To provide optimal health.
- To reduce the risk of developing chronic disease.

Figure 3.
Ranking of the validity of types of evidence for establishing dietary fatty acid requirements (favourability decreasing from left to right)[1]



[1] Adapted from the 2010 FAO report on recommendations for Fats and Fatty Acids, FAO Food and Nutrition Paper (2010), (FAO, 2010).

In addition physiological measures were identified in which risk factors known to be associated with specific disease outcomes might be assessed as an indirect measure of chronic disease risk reduction. Equilibrium maintenance is another approach and is the balance of nutrient intake and loss, which can be determined directly or predicted in factorial estimates of intakes that balance losses and supply additional needs. Finally animal model studies that have evaluated disease outcomes or physiological measures have been used as supporting evidence for recommendations.

Because intakes that prevent clinical deficiency are, for almost all nutrients, much lower than intakes that reduce the risk of chronic disease, it has been argued that they can be judged as sub-optimal and lower than likely recommended intakes. Thus reducing the risk of developing chronic disease became the main criterion for setting fatty acid requirements. This was further discussed in relation to a ranking system for the evidence from relevant studies (i.e. studies of diet-disease outcomes, of physiological measures and animal studies) with randomized controlled trials, (RCT) of disease outcomes most highly rated, and case reports least important in the hierarchy (see Figure 3).

Strength of evidence pertaining to this consultation

Amino acid scoring patterns

This Consultation was only concerned with setting nutrient requirements in relation to identifying appropriate amino acid scoring patterns. These derive from the 2007 report on Protein and Amino Acid Requirements in Human Nutrition (WHO/FAO/UNU, 2007) and the current Consultation has accepted the appropriate values. In that report it was stressed that there is a paucity of long-term prospective studies examining health outcomes. In fact no evidence of relationships between protein or amino acid intakes and health and/or disease was found which was sufficient to identify intakes associated with either optimal health or to reduce the risk of developing chronic disease. Indeed for protein and amino acids, as with many individual nutrients, intake-health relationships are mainly limited to case reports with few examples of sufficient evidence to warrant a meta analysis or systematic review to establish the strength of any relationship, and virtually none which include sufficient dose-response data to identify a suitable intake level. For example dietary protein intakes have long been discussed as an influence on bone health with evidence for both adverse and beneficial influences, but to date only one meta analysis of the relationship has been published (Darling *et al.,* 2009). Although this identified some positive effects that indicate a small benefit of protein on bone health, it is insufficient evidence to alter current estimates of protein requirements. Similarly there is a large literature on the wide ranging influences of leucine on human physiology and metabolism which have made it subject to special interest, but to date none of these studies has led to revised estimates of the leucine requirement (Millward, 2012c). For this reason it was not possible to apply strictly the hierarchy of evidence as discussed in the 'Fats and Fatty Acids in Human Nutrition' report (FAO, 2010) in the evaluation of the evidence base.

In practice current estimates of protein requirements have been derived from nitrogen balance studies in adults with estimates of amino acid requirements deriving from a combination of nitrogen balance studies and various stable isotope studies in adults with physiological or metabolic endpoints, (e.g. amino acid balance or isotope oxidation). The outcomes of these studies have been used to predict requirements for children and pregnant and lactating women by means of a factorial method together with descriptive, observational data on breast milk amino acid composition used to define the amino acid requirements of infants. In the 2007 WHO/FAO/UNU report all of these approaches were deemed to be subject to limitations of one kind or another with none judged as ideal.

This Consultation recognises the inherent limitations in currently accepted values of protein and amino acid requirements identified in this report as amino acid scoring patterns. Further studies are clearly needed that include chronic disease related outcomes and functional studies as delineated in Figure 2 of this report. It is also noted that with very few exceptions, N-balance studies of the protein requirement have not included measures of specific physiological outcomes. It is recommended that future studies of the protein requirement incorporate where possible measures of specific physiological outcomes.

Examples of physiological measures and chronic disease outcomes related to setting criteria for dietary protein and amino acid recommendations might include pregnancy-induced hypertension, intrauterine infections and foetal growth retardation. For young children they would include wasting and stunting, frequency of infections, and overall mortality. For older children they would include stunting, rates of infection and cognitive performance. For adults, relevant outcomes might be undernutrition and frequency of infections, muscle strength and labour productivity and in terms of excessive dietary protein intake, bone health, hypertension, muscle strength and work capacity. For the elderly, sarcopenia, bone health, cognitive decline, immune function and infections, work capacity, hypertension, renal disease, obesity and diabetes would be considered. The primary strength of using disease outcomes as an indicator of adequacy or optimal intake is that they represent the most direct method to assess effects on health. However, an important drawback of using disease outcomes is that because they are affected by multiple nutrients, and their interaction with genotype, they are unlikely, to be specific to individual amino acids.

Protein quality evaluation by DIAAS

The proposed change from protein digestibility as indicated by faecal nitrogen excretion to ileal amino acid digestibility is based on a consideration of a current understanding of the physiology of protein digestion and amino acid and nitrogen absorption in humans. This understanding derives from experimental studies in humans over many years together with experimental studies in monogastric animals especially rodents and pigs. The nature of these studies is diverse and consequently the evaluation of the strength of the arguments that an amino acid score calculated from ileal amino acid digestibility is

a better predictor of human dietary protein quality than one adjusted by faecal nitrogen digestibility is a difficult task especially in the context of any hierarchical framework of evidence as discussed above. This is because the experimental studies that have generated the evidence base cannot be easily categorised and ranked by type of study as can be done for diet-disease relationships. The experimental studies have involved a wide range of quite different experimental approaches to the study of intestinal protein, amino acid and nitrogen metabolism and absorption. Furthermore it is the case that these processes are by no means fully understood, to the extent that legitimate differences of opinion remain especially about the amino acid and nitrogen transactions in the human colon. Because of this, the decision that the DIAAS approach is more likely to enable accurate prediction of dietary protein quality than PDCAAS was reached on the basis of a collective judgement of the members of the Consultation. Because the assessment of ileal amino acid digestibility is inherently more difficult than that of faecal nitrogen digestibility the Consultation considered the balance between the potential benefit from application of DIAAS and the difficulty of its determination compared with that of PDCAAS. The outcome of that deliberation is described in Section IV, under: "Correction for amino acid digestibility and availability in the calculation of DIAAS".

Direct evaluation of protein quality

On the basis that an evidence base relating dietary protein and amino acid intakes with measureable short and long term health outcomes (as indicated in Figure 2 of this report) will accumulate, **the Consultation identifies an urgent need to conduct appropriate research investigating the direct influence of the quality of dietary protein on such dietary protein-related health outcomes in well-controlled studies undertaken with human subjects directly.**

Appendices:
Appendix I:
FAO Expert Consultation on Protein Quality Evaluation

31 March–2 April 2011

Auckland, New Zealand, SKYCITY Auckland Convention Centre, 88 Federal Street, Auckland

DRAFT MEETING OBJECTIVES:

1. Review effectiveness and use of the PDCAAS method for evaluating protein quality since its adoption in 1991.
2. Review current concerns and limitations of the PDCAAS method as reported in the literature.
3. Review advantages and disadvantages of other methods for evaluating protein quality.
4. Provide justifications and recommendations for accepting, rejecting or modifying the PDCAAS method.
5. Provide list of recommendations for protein quality assessments and applications.
6. Recommend further research activities related to protein quality assessments.

DRAFT PROGRAMME:

DAY 1:

Morning

08:30
- Welcome and introductions
- Election of Chair
- Election of Vice-Chair and Rapporteurs
- Approval of agenda
- Overview of recommendations from the last Expert Consultation
- Presentation of objectives for the current Expert Consultation

10:00 ■ *Health Break*

10:30 ■ Presentation of Background Information

■ Human amino acid requirements

Professor Joe Millward, University of Surrey, UK

■ Advantages/limitations of the PDCAAS as a method for evaluating protein quality in human diets

Professor Gertjan Schaafsma, HAN University, The Netherlands

■ Historical overview of PDCAAS calculation

Dr Joyce Boye, Food and Agriculture Organization, Rome

11:45 ■ Presentation of specific issues to be considered by Science Experts

12:15 ■ *Lunch*

Afternoon

13:30 ■ Discussion Session 1

ISSUE 1: **Truncation of PDCAAS scores for proteins with higher than 100% scores to 100%.**

(At issue: Additional benefit of proteins with higher scores in complementing less nutritious proteins is not captured). Discussions and Recommendation.

ISSUE 2: **Validity of the use of the preschool-age child amino acid requirement values.**

(At issue: Does current knowledge support this? Also, is there a need to consider conditionally indispensable amino acids?). Discussions and Recommendation.

15:30 ■ *Health Break*

16:00 ■ Discussion Session 1 (*continued...)*

ISSUE 3: **Use of the amino acid composition of human milk in predicting protein quality of foods for infants.**
(At issue: Review of literature to assess the suitability of the FAO/WHO/UNU (1985) reference values for amino acid composition of human milk for use in predicting protein quality of foods for infants). Discussions and Recommendation.

18:00 ■ *End of Day 1*

DAY 2

Morning

08:30 ■ Welcome remarks

08:40 ■ Discussion Session 2

ISSUE 4: **Amino acid analysis methodology.**

(At issue: Review of IEC and HPLC methods for the determination of amino acids in foods and faeces/digesta with the objective of adopting a standardized method for this analysis.). Discussions and Recommendations.

ISSUE 5: **Use of (a) faecal vs ileal protein/amino acid digestibility and (b) true versus apparent digestibility in calculating PDCAAS values.**

(At issue: Faecal digestibility may overestimate digestibility due to microbial degradation in the large intestines. Also effect of age on faecal and ileal protein/amino acid digestibility not clarified. Is the rat still an acceptable model? Are there any developments in in vitro digestibility measurements?). Discussions and Recommendations.

10:00 ■ *Health Break*

10:30 ■ Discussion Session 2 *continued...*

ISSUE 6: **Bioavailability vs digestibility of proteins.**

(At issue: Is there a need to include corrections for the bioavailability of individual amino acids and not just for digestibility of protein?). Discussions and Recommendation.

12:15 ■ *Lunch*

Afternoon

13:30 ■ Discussion Session 3

ISSUE 7: **Impact of anti-nutritional factors associated with proteins, including naturally occurring and those formed during processing.**

(At issue: The effect of process modifications and the presence of anti-nutritional components in some protein sources may impact protein quality). Discussions and Recommendation.

ISSUE 8: **Significance of PDCAAS values in practical terms.**

(At issue: Humans consume proteins from varied protein sources. PDCAAS values of single protein sources may not have practical significance. Calculation of PDCAAS in mixed diets.).

15:30 ■ *Health Break*

16:00 ■ Discussion Session 3 *continued...*

ISSUE 9: **Regulatory issues (Codex vs national guidelines)**

(At issue: How can countries use recommended protein quality methodology for regulatory purposes?). Discussions and Recommendation.

Evening

17:00-19:00 ■ First meeting of drafting committee

DAY 3

Morning

8:30 ■ Welcome remarks

8:40 ■ Discussions and recommendations on further research work and data needed.

(Examples of some issues requiring consideration: (a) Human sulphur amino acid requirements (cysteine vs methionine); (b) Possible adverse effects of proteins with disproportionate levels of amino acids; (c) Update of the FAO amino acid content of foods data and need for national data; (d) Others).

10:00 ■ *Health Break*

10:30 ■ Review of Report and Recommendations

12:15 ■ *Lunch*

Afternoon

13:30 ■ Second meeting of drafting committee.

Final review and adoption of report and recommendations.

17:00 ■ Adjournment

Appendix II
Attendance at the Expert Consultation on Protein Quality in Human Nutrition

31 March–2 April 2011

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Protein is supplied by food ingredients, whole foods, sole-source foods and mixed diets and the match between dietary supply and human protein needs is vital to support the health and well-being of human populations. Since 1989 the Protein Digestibility Corrected Amino Acid Score (PDCAAS) method for evaluating protein quality has been used widely. However, limitations of PDCAAS have been recognised and new research findings led to a review of the adequacy of PDCAAS and its application vis-à-vis other methods of estimating dietary protein quality. This report of the FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition, held in Auckland, New Zealand, from March 31 to April 2, 2011, considers the effectiveness and concerns about the PDCAAS method for evaluating protein quality and provides justifications and recommendations concerning the PDCAAS method. A new method of dietary quality evaluation called DIAAS is recommended for application in practice.

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Values for digestible indispensable amino acid scores (DIAAS) for some dairy and plant proteins may better describe protein quality than values calculated using the concept for protein digestibility-corrected amino acid scores (PDCAAS)

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Abstract

An experiment was conducted to compare values for digestible indispensable amino acid scores (DIAAS) for four animal proteins and four plant proteins with values calculated as recommended for protein digestibility-corrected amino acid scores (PDCAAS), but determined in pigs instead of in rats. Values for standardised total tract digestibility (STTD) of crude protein (CP) and standardised ileal digestibility (SID) of amino acids (AA) were calculated for whey protein isolate (WPI), whey protein concentrate (WPC), milk protein concentrate (MPC), skimmed milk powder (SMP), pea protein concentrate (PPC), soya protein isolate (SPI), soya flour and whole-grain wheat. The PDCAAS-like values were calculated using the STTD of CP to estimate AA digestibility and values for DIAAS were calculated from values for SID of AA. Results indicated that values for SID of most indispensable AA in WPI, WPC and MPC were greater ($P < 0{\cdot}05$) than for SMP, PPC, SPI, soya flour and wheat. With the exception of arginine and tryptophan, the SID of all indispensable AA in SPI was greater ($P < 0{\cdot}05$) than in soya flour, and with the exception of threonine, the SID of all indispensable AA in wheat was less ($P < 0{\cdot}05$) than in all other ingredients. If the same scoring pattern for children between 6 and 36 months was used to calculate PDCAAS-like values and DIAAS, PDCAAS-like values were greater ($P < 0{\cdot}05$) than DIAAS values for SMP, PPC, SPI, soya flour and wheat indicating that PDCAAS-like values estimated in pigs may overestimate the quality of these proteins.

Key words: Amino acids: Dairy protein: Digestible indispensable amino acid scores: Protein digestibility-corrected amino acid scores: Plant protein

The protein digestibility-corrected amino acid score (PDCAAS) has been used for more than 20 years to evaluate protein quality in human foods[1], but the PDCAAS procedure has limitations because values are calculated from the total tract digestibility of crude protein (CP) and calculations for PDCAAS are based on the assumption that all amino acids (AA) have the same digestibility as CP. It is, however, recognised that digestibility of AA is most correctly determined at the end of the small intestine (the ileum), because AA are absorbed only from the small intestine and because hindgut fermentation can affect faecal AA excretion[2]. Therefore, ileal digestibility is a more accurate estimate of AA bioavailability than total tract digestibility in both humans and pigs[3,4]. In addition, the digestibility of CP is not representative of the digestibility of all AA[3], because individual AA are digested with different efficiencies[3]. Other criticisms of the PDCAAS procedure have been recently reviewed and include use of truncation to avoid having values >1, use of a scoring pattern that is based on AA requirements for children and use of metabolic faecal N to correct for endogenous losses of AA[5–7]. It was also recently concluded that PDCAAS generally underestimates the value of high-quality proteins and overestimates the value of low-quality proteins[7].

To avoid the flaws of the PDCAAS procedure, the Food and Agriculture Organization (FAO)[8] now recommends an AA evaluation procedure called digestible indispensable amino acid score (DIAAS). To calculate DIAAS, it is necessary to determine the digestibility of individual AA at the end of the small intestine (the ileum), and the pig has been recognised as an appropriate model for estimating CP and AA digestibility in foods for humans[8–10]. In contrast, PDCAAS values according to the original definition are determined in rats[1]. The apparent ileal digestibility of AA is defined as the net disappearance of ingested dietary AA from the digestive tract before the distal ileum[3]. If values for apparent ileal digestibility are corrected

Abbreviations: AA, amino acids; CP, crude protein; DIAAS, digestible indispensable amino acid score; MPC, milk protein concentrate; PDCAAS, protein digestibility-corrected amino acid score; PPC, pea protein concentrate; SID, standardised ileal digestibility; SMP, skimmed milk powder; SPI, soya protein isolate; STTD, standardised total tract digestibility; WPC, whey protein concentrate; WPI, whey protein isolate.

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for the basal endogenous losses of AA, the resulting values are described as standardised ileal digestibility (SID)[3]. Values for SID of AA are additive in mixed diets[11] and may be used to calculate DIAAS in proteins used in human nutrition[4,8].

Research in our laboratory estimated DIAAS in eight cereal grains by calculating SID values for all indispensable AA in pigs[4]. Results indicated that to meet dietary requirements for AA in humans, diets based on sorghum, wheat, rye or maize require more AA supplementation than diets based on polished rice or dehulled oats. However, in human nutrition, protein is usually supplied by either animal-based proteins or plant-based proteins. Animal proteins include a number of dairy products, and commonly used dairy proteins include whey protein concentrate (WPC), whey protein isolate (WPI), milk protein concentrate (MPC) and skimmed milk powder (SMP). Commonly used plant proteins include soya protein isolate (SPI), soya flour and pea protein concentrate (PPC). To our knowledge, there are no published values for DIAAS for these proteins that have been determined in pigs and it is not known how values for DIAAS determined in pigs compare with PDCAAS-like values determined in pigs. Therefore, the aim of this experiment was to compare PDCAAS-like values determined in pigs and values for DIAAS in eight commonly used proteins and test the hypothesis that values for DIAAS are more appropriate to quantify protein quality than values for PDCAAS.

Methods

The protocol for the experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois (protocol no. 13354). Four dairy proteins (WPI, WPC, MPC and SMP) were procured from Cereal Byproducts Company. SPI and soya flour were obtained from Archer Daniels Midland Company and PPC was obtained from AGT Foods. Wheat was obtained from Siemers (Table 1). Each ingredient was included in one diet as the only source of CP and AA with the exception that wheat was included in combination with soya flour (Tables 2 and 3). A N-free diet was also formulated to measure basal endogenous losses of CP and AA. Vitamins and minerals were included in all diets to meet or exceed current requirement estimates for growing pigs[12]. All diets also contained 0·4 % chromic oxide as an indigestible marker and all diets were provided in meal form.

Nine growing barrows (initial body weight: 26·25 (SD 1·48) kg) were equipped with a T-cannula in the distal ileum using procedures adapted from Stein *et al.*[13]. Pigs were allowed a 7-d recovery after the surgery and they were then allotted to a 9 × 9 Latin square design with nine diets and nine 9-d periods. No pig received the same diet more than once during the experiment and there was, therefore, nine replicate pigs per treatment. With nine replicates we expected to be able to detect differences in SID values among ingredients of 2·5–4 percentage units (depending on the AA). Pigs were housed in individual pens (0·9 × 1·8 m) in an environmentally controlled room. Pens had smooth sides and fully slatted concrete floors. A feeder and a nipple drinker were installed in each pen. At the conclusion of the experiment, pigs were approximately 19 weeks of age and had a body weight of 84·70 (SD 6·48) kg.

All pigs were fed their assigned diets in a daily amount of three times the estimated energy requirement for maintenance (i. e. 824 kJ metabolisable energy/$kg^{0·60}$)[12]. The daily feed allotment was provided every day at 08.00 hours. Water was available at all times. Pig weights were recorded at the beginning of each period and at the conclusion of the experiment. The amount of feed supplied each day was recorded as well. The initial 5 d of each period were considered an adaptation period to the diet. Faecal samples were collected on days 6 and 7 and immediately frozen at −20°C. Ileal digesta were collected for 8 h (from 08.00 to 16.00 hours) on days 8 and 9 using standard operating procedures[13]. In brief, cannulas were opened and cleaned, a plastic bag was attached to the cannula barrel and digesta flowing into the bag were collected. Bags were removed whenever they were filled with digesta or at least once every 30 min, and immediately frozen at −20°C to prevent bacterial degradation of the AA in the digesta. Individual pig weights recorded at the conclusion of each period were used to calculate the feed provision for the subsequent period.

At the conclusion of the experiment, faecal samples were dried in a forced air oven and finely ground through a 1-mm screen in a Wiley Mill (model 4; Thomas Scientific) before analysis. Ileal samples were thawed, mixed within animal and diet, and a sub-sample was collected for analysis. A sample of each source of protein and of each diet was collected at the time of diet mixing. Digesta samples were lyophilised and finely ground before chemical analysis. Diets, ingredients, faecal samples and ileal digesta samples were analysed for DM (method 927·05)[14] and CP by combustion (method 990·03)[14] on an Elementar Rapid N-cube protein/N apparatus (Elementar Americas Inc.). Aspartic acid was used as a calibration standard and CP was calculated as N × 6·25. Samples were analysed in duplicate, but analyses were repeated if the analysed values were >5 % apart. Diets, faecal samples and ileal digesta were also analysed in duplicate for Cr (method 990·08)[14] and all diets, ingredients and ileal digesta samples were analysed for AA on a Hitachi Amino Acid Analyzer (model L8800; Hitachi High Technologies America Inc.) using ninhydrin for post-column derivatisation and norleucine as the internal standard. Samples were hydrolysed with 6 N-HCl for 24 h at 110°C before analysis, but methionine and cysteine were analysed as methionine sulfone and cysteic acid after cold performic acid oxidation overnight before hydrolysis and tryptophan was determined after NaOH hydrolysis for 22 h at 110°C (method 982·30 E (a, b, c))[14].

Calculations

Values for apparent ileal digestibility of CP and AA, basal endogenous losses of CP and AA, and SID of CP and AA were calculated for all diets as previously explained[3]. For all ingredients except wheat, the SID for CP and AA in the diets also represented the SID of the ingredient, but for wheat, the SID of CP and AA were calculated using the difference procedure[15]. Values for the standardised total tract digestibility (STTD) of CP were calculated as explained for the calculation of SID of CP.

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Table 1. Analysed nutrient composition of ingredients (as-fed basis)*

	Ingredients							
Items	WPI	WPC	MPC	SMP	PPC	SPI	Soya flour	Wheat
DM (%)	93·22	92·93	92·83	90·59	93·70	93·79	92·23	88·22
Crude protein (%)	85·23	78·01	67·93	34·65	54·46	92·66	52·29	11·67
Ca (%)	0·36	0·36	1·77	1·15	0·08	0·05	0·28	0·04
P (%)	0·23	0·31	1·18	0·91	0·69	0·73	0·69	0·37
Indispensable amino acids (%)								
Arg	1·96	2·38	2·45	1·20	4·83	6·95	3·71	0·56
His	1·71	1·72	2·04	1·07	1·43	2·41	1·43	0·30
Ile	5·95	4·94	3·61	1·80	2·31	4·38	2·35	0·39
Leu	9·91	9·27	6·91	3·47	4·04	7·38	4·00	0·78
Lys	8·64	7·83	5·50	2·90	4·11	5·69	3·30	0·39
Met	1·94	1·77	1·83	0·83	0·49	1·18	0·73	0·21
Phe	2·85	2·87	3·42	1·70	2·70	4·86	2·60	0·52
Thr	6·58	5·39	3·02	1·50	1·95	3·35	2·00	0·34
Trp	1·83	1·57	1·01	0·54	0·48	1·30	0·79	0·12
Val	5·29	4·83	4·43	2·27	2·61	4·42	2·53	0·52
Dispensable amino acids (%)								
Ala	4·58	4·20	2·27	1·14	2·25	3·74	2·20	0·44
Asp	10·22	8·79	5·29	2·68	5·99	10·56	5·84	0·62
Cys	2·14	1·91	0·46	0·26	0·63	1·06	0·72	0·25
Glu	15·97	13·62	14·55	7·37	8·62	17·10	9·20	3·06
Gly	1·57	1·62	1·31	0·68	2·25	3·77	2·16	0·50
Pro	5·35	4·50	6·69	3·33	2·17	4·65	2·52	1·03
Ser	4·10	3·86	3·51	1·81	2·37	4·25	2·33	0·49
Tyr	2·60	2·55	3·42	1·61	1·79	3·31	1·82	0·24

WPI, whey protein isolate; WPC, whey protein concentrate; MPC, milk protein concentrate; SMP, skimmed milk powder; PPC, pea protein concentrate; SPI, soya protein isolate.
* The trypsin inhibitor units in soya flour and SPI were 8·06 and 2·75 units/mg, respectively.

Table 2. Ingredient composition of experimental diets (as-is basis)*

	Diets								
	WPI	WPC	MPC	SMP	PPC	SPI	Soya flour	Wheat	N-free
Ingredients (%)									
WPI	21·00	–	–	–	–	–	–	–	–
WPC	–	23·00	–	–	–	–	–	–	–
MPC	–	–	40·00	–	–	–	–	–	–
SMP	–	–	–	50·00	–	–	–	–	–
PPC	–	–	–	–	25·00	–	–	–	–
SPI	–	–	–	–	–	21·00	–	–	–
Soya flour	–	–	–	–	–	–	35·00	11·30	–
Wheat	–	–	–	–	–	–	–	82·50	–
Soyabean oil	3·00	3·00	3·00	3·00	3·00	3·00	3·00	3·00	4·00
Solka-Floc	–	–	–	–	–	–	–	–	4·00
Monocalcium phosphate	1·60	1·60	1·60	1·60	1·60	1·60	1·60	0·80	2·40
Limestone	0·60	0·60	0·60	0·60	1·30	1·30	1·30	1·30	0·50
Sucrose	20·00	20·00	20·00	20·00	20·00	20·00	20·00	–	20·00
Chromic oxide	0·40	0·40	0·40	0·40	0·40	0·40	0·40	0·40	0·40
Maize starch	52·70	50·70	33·70	23·70	48·00	52·00	38·00	–	67·50
Magnesium oxide	–	–	–	–	–	–	–	–	0·10
Potassium carbonate	–	–	–	–	–	–	–	–	0·40
Sodium chloride	0·40	0·40	0·40	0·40	0·40	0·40	0·40	0·40	0·40
Vitamin–micromineral premix†	0·30	0·30	0·30	0·30	0·30	0·30	0·30	0·30	0·30

WPI, whey protein isolate; WPC, whey protein concentrate; MPC, milk protein concentrate; SMP, skimmed milk powder; PPC, pea protein concentrate; SPI, soya protein isolate.
* All diets were formulated to contain approximately 17 % crude protein, 0·70 % Ca and 0·33 % standardised total tract digestible P.
† The vitamin–micromineral premix provided the following quantities of vitamins and micro minerals per kg of complete diet: vitamin A as retinyl acetate, 3·83 mg; vitamin D_3 as cholecalciferol, 0·06 mg; vitamin E as DL-α-tocopheryl acetate, 48·53 mg; vitamin K as menadione dimethylprimidinol bisulfite, 1·42 mg; thiamin as thiamine mononitrate, 0·24 mg; riboflavin, 6·59 mg; pyridoxine as pyridoxine hydrochloride, 0·24 mg; vitamin B_{12}, 0·03 mg; D-pantothenic acid as D-calcium pantothenate, 23·5 mg; niacin, 44·1 mg; folic acid, 1·59 mg; biotin, 0·44 mg; Cu, 20 mg as copper sulfate and copper chloride; Fe, 126 mg as ferrous sulfate; I, 1·26 mg as ethylenediamine dihydriodide; Mn, 60·2 mg as manganese sulfate; Se, 0·3 mg as sodium selenite and Se yeast; and Zn, 125·1 mg as zinc sulfate.

The concentration of SID AA (g/kg) in each ingredient was calculated by multiplying the SID value (%) for each AA by the concentration (g/kg) of that AA in the ingredient, and this value was then divided by the concentration of CP in the ingredient to calculate digestible indispensable AA content (mg) in 1 g protein[4]. The digestible indispensable AA reference ratios

Table 3. Analysed nutrient composition of experimental diets (as-fed basis)

	Diets								
Items	WPI	WPC	MPC	SMP	PPC	SPI	Soya flour	Wheat	N-free
DM (%)	93·22	92·93	92·83	90·59	93·70	93·79	92·23	88·22	92·41
Crude protein (%)	17·61	16·35	16·90	16·76	15·65	17·04	16·53	16·59	0·13
Indispensable amino acids (%)									
Arg	0·39	0·49	0·58	0·55	1·23	1·27	1·13	1·00	0·01
His	0·38	0·41	0·52	0·51	0·41	0·49	0·48	0·46	0·02
Ile	1·27	1·08	0·91	0·88	0·64	0·86	0·77	0·69	0·01
Leu	2·09	2·07	1·71	1·65	1·10	1·42	1·28	1·22	0·02
Lys	1·85	1·72	1·37	1·38	1·13	1·12	1·05	0·80	0·02
Met	0·40	0·39	0·46	0·42	0·13	0·23	0·22	0·26	0·00
Phe	0·59	0·62	0·84	0·80	0·72	0·92	0·82	0·79	0·01
Thr	1·39	1·17	0·73	0·70	0·52	0·64	0·63	0·56	0·01
Trp	0·37	0·38	0·26	0·29	0·17	0·22	0·25	0·18	0·02
Val	1·15	1·05	1·13	1·08	0·72	0·89	0·82	0·80	0·01
Total	9·88	9·38	8·51	8·26	6·77	8·06	7·45	6·76	0·13
Dispensable amino acids (%)									
Ala	0·99	0·95	0·57	0·55	0·62	0·73	0·71	0·68	0·01
Asp	2·17	1·94	1·30	1·27	1·64	2·02	1·85	1·37	0·02
Cys	0·43	0·42	0·11	0·12	0·16	0·20	0·22	0·31	0·00
Glu	3·41	3·04	3·49	3·40	2·38	3·29	2·92	3·68	0·05
Gly	0·34	0·37	0·31	0·32	0·62	0·72	0·69	0·70	0·01
Ser	1·10	0·94	1·62	1·55	0·57	0·85	0·77	1·12	0·01
Tyr	0·94	0·86	0·83	0·79	0·62	0·79	0·70	0·69	0·01
Ala	0·46	0·48	0·74	0·70	0·43	0·54	0·54	0·51	0·01
Total	9·84	9·00	8·97	8·70	7·04	9·14	8·40	9·06	0·12
Total amino acids (%)	19·72	18·38	17·48	16·96	13·81	17·20	15·85	15·82	0·25

WPI, whey protein isolate; WPC, whey protein concentrate; MPC, milk protein concentrate; SMP, skimmed milk powder; PPC, pea protein concentrate; SPI, soya protein isolate.

were calculated for each ingredient using the following equation[8]: digestible indispensable AA reference ratio = digestible indispensable AA content in 1 g protein of food (mg)/mg of the same dietary indispensable AA in 1 g of the reference protein. The reference proteins were based on FAO[8] and separate ratios were calculated using the reference protein for infants less than 6 months old, children from 6 to 36 months old and children older than 36 months, adolescents and adults[8]. The DIAAS values were then calculated using the following equation[8]:

$$\text{DIAAS}(\%) = 100 \times \text{lowest value of the digestible indispensable AA reference ratio}$$

Values for STTD of CP were used to calculate PDCAAS-like values using the following equation[16]:

$$\text{PDCAAS-like values}(\%) = \text{mg of limiting AA in 1 g of test protein/mg of the same AA in 1 g of reference protein} \times \text{standardised total tract digestibility}(\%) \times 100.$$

Calculation of PDCAAS-like values used the reference protein for 2–5 year-old children as recommended if values are calculated from STTD of CP in rats[1]. However, to allow for a direct comparison between PDCAAS-like values and values for DIAAS, PDCAAS-like values were also calculated using the three reference proteins that were used to calculate DIAAS values[8].

Statistical analyses

Normality of data was verified and outliers were identified using the UNIVARIATE and BOXPLOT procedures, respectively (SAS Inst. Inc.). Data were analysed by ANOVA using the MIXED procedure of SAS (SAS Institute Inc.) in a randomised complete block design with the pig as the experimental unit. The statistical model to determine differences in SID of AA values among ingredients included diet as the main effect and pig and period as random effects. The model to compare values for SID and STTD of CP within each ingredient included calculation procedure (SID or STTD) as main effect and pig and period as random effects. The model to compare values for DIAAS and PDCAAS used calculation procedure (DIAAS or PDCAAS) as main effect and pig and period as random effects. Treatment means were calculated using the LSMEANS statement, and if significant, means were separated using the PDIFF option of the MIXED procedure. Significance and tendency was considered at $P < 0{\cdot}05$ and $0{\cdot}05 \le P < 0{\cdot}10$, respectively.

Results

All pigs remained healthy throughout the experiment and readily consumed their diets. Gross chemical composition of all ingredients was generally in agreement with published values[12]. The concentration of CP in ingredients ranged from 11·67 to 92·66 %.

With the exception of tyrosine, the SID of all AA was not different between WPI and WPC (Table 4). The SID of isoleucine, cysteine and serine was less ($P < 0{\cdot}05$) in MPC than

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Table 4. Standardised ileal digestibility of amino acids in ingredients*
(Pooled standard errors)

	Ingredients									
Items	WPI	WPC	MPC	SMP	PPC	SPI	Soya flour	Wheat	Pooled SEM	*P*
Indispensable amino acids (%)										
Arg	104^{a}	101^{a,b}	102^{a,b}	98^{d}	99^{c,d}	101^{b,c}	99^{c,d}	87^{e}	1·00	<0·05
His	100^{a}	97^{a,b}	99^{a}	94^{b,c}	95^{b,c}	97^{a,b}	92^{c}	85^{d}	1·55	<0·05
Ile	98^{a}	97^{a,b}	93^{c,d}	89^{e}	91^{d}	95^{b,c}	92^{d}	86^{f}	1·00	<0·05
Leu	99^{a}	98^{a}	98^{a}	94^{b}	92^{c}	95^{b}	91^{c}	86^{d}	0·74	<0·05
Lys	98^{a}	96^{a,b}	96^{a,b}	95^{a,b}	96^{a,b}	97^{a}	93^{b}	77^{c}	1·31	<0·05
Met	98^{a}	97^{a,b}	97^{a,b}	96^{b,c}	90^{e}	96^{c}	93^{d}	88^{f}	0·58	<0·05
Phe	98^{a}	96^{a,b}	97^{a}	94^{b}	92^{c}	96^{a,b}	92^{c}	87^{d}	0·82	<0·05
Thr	94^{a}	91^{a,b,c}	93^{a}	82^{d}	88^{b,c}	92^{a,b}	87^{c}	80^{d}	1·91	<0·05
Trp	100^{a}	98^{a,b}	97^{a,b}	91^{d}	87^{e}	96^{b,c}	92^{c,d}	74^{f}	1·31	<0·05
Val	97^{a}	95^{a,b}	94^{b,c}	90^{d}	89^{d}	94^{b}	91^{c,d}	83^{e}	1·22	<0·05
Mean	98^{a}	96^{a}	97^{a}	92^{b}	93^{b}	96^{a}	93^{b}	85^{c}	0·90	<0·05
Dispensable amino acids (%)										
Ala	98^{a}	96^{a,b}	96^{a,b}	89^{d}	92^{c,d}	96^{a,b,c}	93^{b,c,d}	79^{e}	1·51	<0·05
Asp	99^{a}	96^{a,b}	97^{a,b}	88^{c}	93^{b}	95^{a,b}	88^{c}	80^{a,b}	1·63	<0·05
Cys	98^{a}	95^{a,b}	85^{c,d}	73^{e}	75^{e}	91^{b,c}	81^{d}	86^{c,d}	2·57	<0·05
Glu	98^{a}	96^{a,b,c}	94^{b,c,d}	90^{e}	96^{a,b}	97^{a}	92^{d,e}	93^{c,d}	1·19	<0·05
Gly	117^{a}	112^{a}	117^{a}	96^{b}	98^{b}	100^{b}	95^{b}	87^{c}	3·18	<0·05
Ser	95^{a,b}	92^{b,c}	88^{d}	80^{e}	91^{c,d}	96^{a}	92^{b,c,d}	89^{c,d}	1·90	<0·05
Tyr	99^{a}	96^{b,c}	98^{a,b}	95^{c,d}	93^{d}	96^{b,c}	93^{d}	90^{e}	0·97	<0·05
Mean	102^{a}	101^{a,b}	99^{a,b,c}	95^{d}	98^{b,c}	101^{a,b}	96^{c,d}	94^{d}	1·38	<0·05
Total amino acids	100^{a}	98^{a}	99^{a}	94^{b}	96^{b}	99^{a}	95^{b}	90^{c}	1·07	<0·05

WPI, whey protein isolate; WPC, whey protein concentrate; MPC, milk protein concentrate; SMP, skimmed milk powder; PPC, pea protein concentrate; SPI, soya protein isolate.
^{a,b,c,d,e,f} Mean values within a row with unlike superscript letters are different ($P<0·05$).
* Standardised ileal digestibility values were calculated by correcting values for apparent ileal digestibility for the basal ileal endogenous losses. Endogenous losses of amino acids were calculated from pigs fed the N-free diet as follows (g/kg DM intake): arginine, 0·59; histidine, 0·20; isoleucine, 0·29; leucine, 0·49; lysine, 0·40; methionine, 0·08; phenylalanine, 0·29; threonine, 0·49; tryptophan, 0·10; valine, 0·40; alanine, 0·62; aspartic acid, 0·72; cysteine, 0·17; glutamic acid, 0·94; glycine, 1·50; serine, 0·43; tyrosine, 0·23.

in WPI and WPC, and the SID of valine and glutamic acid was less ($P<0·05$) in MPC than in WPI, but for all other AA, no differences among MPC, WPI and WPC were observed. However, the SID of most AA was greater ($P<0·05$) in WPI, WPC and MPC than in SMP, PPC, soya flour and wheat, but for SPI, many AA had SID values that were not different from those in WPI, WPC and MPC. With the exception of arginine, tryptophan, alanine and glycine, the SID of all AA was greater ($P<0·05$) in SPI than in soya flour. The SID of methionine, tryptophan and cysteine was less ($P<0·05$) in PPC than in soya flour and the SID of aspartic acid and glutamic acid was greater ($P<0·05$) in PPC than in soya flour, but for all other AA, no difference between these two ingredients was observed. The SID of all indispensable AA and of alanine and tyrosine was less ($P<0·05$) in wheat than in all other ingredients.

The SID of CP was greater ($P<0·05$) than the STTD of CP for WPI, WPC and wheat (Table 5). In contrast, the STTD of CP was greater ($P<0·05$) than the SID of CP in MPC, SMP and SPI, whereas no difference between SID and STTD of CP was observed for PPC and soya flour.

The protein digestibility-corrected AA reference ratios calculated according to the recommendations from FAO/WHO[1] but using pigs instead of rats and based on the scoring pattern for preschool children (2–5 years old) are presented in the online Supplementary Table SA. However, the protein digestibility-corrected AA reference ratios calculated from STTD values of CP in pigs were also calculated according to FAO[8] and based on requirements of infants (birth to 6 months of age), children (6 months to 3 years of age) and older children (older than 3 years of age), adolescents and adults, and these values are presented in the online Supplementary Table SB. Likewise, the digestible indispensable AA reference ratios calculated according to FAO[8] and based on the same three age groups are presented in the online Supplementary Table SC.

If PDCAAS-like values calculated according to FAO/WHO[1] were truncated as recommended, values for WPC, MPC, SMP were less ($P<0·05$) than values for DIAAS, whereas PDCAAS-like values for PPC, SPI, soya flour and wheat were greater ($P<0·05$) than for DIAAS (Table 6). However, if PDCAAS-like values were not truncated, the PDCAAS-like value for WPC was not different from DIAAS, but PDCAAS-like values for MPC and SMP were greater ($P<0·05$) than DIAAS. If PDCAAS-like values were calculated according to the same scoring pattern as DIAAS[8], PDCAAS-like values for SMP, PPC, SPI, soya flour and wheat were greater ($P<0·05$) than values for DIAAS, whereas the PDCAAS-like value for WPI was less ($P<0·05$) than the DIAAS for WPI.

For values for DIAAS, the first-limiting AA in WPI and WPC was histidine, but for MPC, SMP, PPC, SPI and soya flour, the sulfur AA were first limiting, and lysine was first liming in wheat. If PDCAAS-like values were calculated using the same scoring patterns as used to calculate DIAAS, the first-limiting AA in the proteins was not different from those identified for DIAAS. However, if PDCAAS-like values were calculated using the original scoring patterns[1], either truncated or not truncated, the first-limiting AA for whey proteins was the aromatic AA and threonine was first limiting in MPC and the sulfur AA were first

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Table 5. Standardised ileal digestibility (SID) and standardised total tract digestibility (STTD) of crude protein (CP) in ingredients

	Ingredients							
Items	WPI	WPC	MPC	SMP	PPC	SPI	Soya flour	Wheat
SID of CP (%)	101	98	92	90	95	94	92	91
STTD of CP (%)	96	97	97	96	94	96	90	86
SEM	2·7	0·9	3·5	3·6	1·8	0·6	3·1	4·5
P	0·003	0·025	0·008	0·001	0·208	<0·001	0·168	0·022

WPI, whey protein isolate; WPC, whey protein concentrate; MPC, milk protein concentrate; SMP, skimmed milk powder; PPC, pea protein concentrate; SPI, soya protein isolate.

Table 6. Comparison of protein digestibility corrected amino acid scores (PDCAAS) and digestible indispensable amino acid scores (DIAAS) based on different requirement patterns*†

Ingredients	PDCAAS 1991‡	PDCAAS 1991, untruncated	PDCAAS 2013§	DIAAS	SEM	*P*
WPI	99[a] (AAA)	99[b] (AAA)	97[b] (His)	100[a] (His)	0·3	<0·0001
WPC	100[b] (AAA)	107[a] (AAA)	107[a] (His)	107[a] (His)	0·4	<0·0001
MPC	100[c] (Thr)	127[a] (Thr)	121[b] (SAA)	120[b] (SAA)	0·5	<0·0001
SMP	100[d] (SAA)	121[a] (SAA)	112[b] (SAA)	105[c] (SAA)	1·1	<0·0001
PPC	75[a] (Trp)	75[a] (Trp)	71[b] (SAA)	62[c] (SAA)	0·6	<0·0001
SPI	93[a] (SAA)	93[a] (SAA)	86[b] (SAA)	84[c] (SAA)	0·5	<0·0001
Soya flour	98[a] (Lys)	98[a] (Lys)	93[b] (SAA)	89[c] (SAA)	1·3	<0·0001
Wheat	50[a] (Lys)	50[a] (Lys)	51[a] (Lys)	45[b] (Lys)	1·3	0·013

WPI, whey protein isolate; AAA, aromatic amino acids (phenylalanine + tyrosine); WPC, whey protein concentrate; MPC, milk protein concentrate; SAA, sulfur amino acids (methionine + cysteine); SMP, skimmed milk powder; PPC, pea protein concentrate; SPI, soya protein isolate.
[a,b,c,d] Mean values within a row with unlike superscript letters are different ($P < 0·05$).
* Values for PDCAAS were calculated from the total tract digestibility of crude protein in pigs and values for DIAAS were calculated from the ileal digestibility of amino acids in pigs.
† First-limiting amino acid is in parenthesis.
‡ PDCAAS were calculated using the recommended amino acid scoring pattern for preschool children (2–5 years). The indispensable amino acids reference patterns are expressed as mg amino acid/g protein: histidine, 19; isoleucine, 28; leucine, 66; lysine, 58; sulfur amino acids, 25; aromatic amino acids, 63; threonine, 34; tryptophan, 11; valine, 35[(1)].
§ PDCAAS and DIAAS were calculated using the recommended amino acid scoring pattern for a child (6 months to 3 years). The indispensable amino acid reference patterns are expressed as mg amino acid/g protein: histidine, 20; isoleucine, 32; leucine, 66; lysine, 57; sulfur amino acids, 27; aromatic amino acids, 52; threonine, 31; tryptophan, 8·5; valine, 40[(8)].

Table 7. Comparison of protein digestibility-corrected amino acid scores (PDCAAS) and digestible indispensable amino acid scores (DIAAS)*†

	Ingredients							
Items	WPI	WPC	MPC	SMP	PPC	SPI	Soya flour	Wheat
Birth to 6 months‡								
DIAAS	67 (AAA)	71 (AAA)	85 (Trp)	81 (Thr)	45 (Trp)	68 (SAA)	73 (Leu)	37 (Lys)
PDCAAS	66 (AAA)	72 (AAA)	85 (Trp)	88 (Trp)	49 (Trp)	71 (SAA)	72 (Leu)	42 (Lys)
SEM	0·30	0·48	0·51	2·4	0·42	0·68	0·83	1·2
P	0·062	0·164	0·743	0·039	<0·0001	0·026	0·642	0·017
3 years and above§								
DIAAS	125 (His)	133 (His)	141 (SAA)	123 (SAA)	73 (SAA)	98 (SAA)	105 (SAA)	54 (Lys)
PDCAAS	122 (His)	134 (His)	142 (SAA)	132 (SAA)	84 (SAA)	102 (SAA)	109 (SAA)	51 (Lys)
SEM	0·44	0·68	0·73	1·6	0·62	0·98	1·4	1·7
P	<0·001	0·311	0·196	0·002	<0·0001	0·028	0·053	0·220

WPI, whey protein isolate; WPC, whey protein concentrate; MPC, milk protein concentrate; SMP, skimmed milk powder; PPC, pea protein concentrate; SPI, soya protein isolate; AAA, aromatic amino acids (phenylalanine + tyrosine); SAA, sulfur amino acids (methionine + cysteine).
* Values for PDCAAS were calculated from the total tract digestibility of crude protein in pigs and values for DIAAS were calculated from the ileal digestibility of amino acids in pigs.
† First-limiting amino acid is in parenthesis.
‡ PDCAAS and DIAAS were calculated using the recommended amino acid scoring pattern for an infant (birth–6 months). The indispensable amino acid reference patterns are expressed as mg amino acid/g protein: histidine, 21; isoleucine, 55; leucine, 96; lysine, 69; sulfur amino acids, 33; aromatic amino acids, 94; threonine, 44; tryptophan, 17; valine, 55[(8)].
§ PDCAAS and DIAAS were calculated using the recommended amino acid scoring pattern for children older than 3 years, adolescents and adults. The indispensable amino acid reference patterns are expressed as mg amino acid/g protein: histidine, 16; isoleucine, 30; leucine, 61; lysine, 48; sulfur amino acids, 23; aromatic amino acids, 41; threonine, 25; tryptophan, 6·6; valine, 40[(8)].

limiting in SMP and SPI. However, the first-limiting AA in PPC was tryptophan, whereas lysine was first limiting in soya flour and wheat.

Calculated PDCAAS-like values for infants were greater ($P < 0·05$) than values for DIAAS for SMP, PPC, SPI and wheat, whereas the value for DIAAS for WPI tended ($P = 0·062$) to be greater than the PDCAAS-like value (Table 7). For children older than 3 years, adolescents and adults, PDCAAS-like values for SMP, PPC and SPI were greater ($P < 0·05$) than DIAAS, and the PDCAAS-like value for soya flour tended ($P = 0·053$) to be

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greater than DIAAS. In contrast, the DIAAS for WPI was greater ($P < 0{\cdot}05$) than the PDCAAS-like value.

The first-limiting AA for DIAAS calculated for infants were the aromatic AA for the whey proteins, tryptophan for MPC and PPC, threonine for SMP, the sulfur AA for SPI, leucine for soya flour and lysine for wheat. The first-limiting AA for PDCAAS-like values calculated for infants in SMP was tryptophan, but for all other ingredients, the first-limiting AA in the calculation of DIAAS was also first limiting for PDCAAS-like values. For children >3 years old, adolescents and adults, the first-limiting AA for both DIAAS and PDCAAS-like values for all proteins were the same as those identified for children from 6 months to 3 years old.

Discussion

The amount and quality of protein consumed throughout the world varies depending on protein availability, AA composition of proteins and digestibility of AA[16]. In many parts of the world, plant proteins are the primary sources of AA in the diet[4,17,18], whereas animal proteins are the primary sources of AA in other parts of the world[18]. However, the composition and digestibility of both of these types of proteins differ[4,19], and both plant and animal proteins, therefore, need to be evaluated. In the present experiment we attempted to do that, but it is acknowledged that all proteins were fed as raw ingredients without the processing that these ingredients most often go through before consumption by humans. If processing changes the digestibility of the protein, results may be different. Other limitations of the experiment include the assumption that AA digestibility in growing castrated male pigs are representative of values obtained in both male and female humans of all ages.

In the current experiment, values for AA digestibility calculated from the total tract digestibility of CP were estimated from pigs although the rodent is the recommended model in the definition of PDCAAS[1]. However, it was the objective to determine if total tract digestibility values for CP can be used to accurately estimate ileal digestibility values of individual AA and if we had used a rodent to calculate PDCAAS values and the pig to calculate DIAAS values, any differences would have been confounded by using the two different animal models. It is, therefore, important that the comparison is done within the same animal and because the pig has been recommended as the preferred animal model to calculate DIAAS values[8], we chose to use the pig to also calculate PDCAAS-like values in this study.

As expected, dairy proteins had greater SID values than the plant proteins and they are, therefore, considered high-quality proteins for humans[20–22]. Protein quality in WPC, SMP and SPI or soya protein concentrate have been studied in rats, and results indicated that WPC had greater PDCAAS than SMP, SPI and soya protein concentrate[7,19]. Results of this experiment agree with previous results and also indicate that the PDCAAS-like value for WPC is greater than for SMP and that the whey proteins have a more balanced AA profile compared with whole milk protein. The major protein in SMP is casein, which has a low concentration of cysteine, and this may be the reason for the reduced PDCAAS-like value for SMP compared with WPC.

According to the FAO recommended AA patterns for older children, adolescents and adults and recommendations for nutrient claims, all dairy proteins tested in this experiment can be considered 'excellent/high' quality sources of protein, with DIAAS ≥100[8]. By the same guidelines, SPI and soya flour qualify as 'good' sources of protein, with a score ≥75 and <100. In contrast, proteins with DIAAS <75 are recommended to make no claims regarding protein quality[8], and PPC and wheat tested in this experiment fall into this category. However, it is recognised that the cut-off values for protein quality assessments that were proposed were arbitrarily chosen and not based on documented research[8].

The N-free diet was used to estimate endogenous AA losses. Values obtained using this procedure are estimates for the basal endogenous losses that are independent of the diet and secreted only in response to DM being present in the small intestine[3]. In addition to the basal endogenous losses, diet-specific endogenous losses may also occur, but these losses will not be included in the values obtained from the N-free diet, and therefore, diet-specific losses are debited against the ingredients in the calculations of SID values. Thus, if a specific diet or ingredient induces diet-specific endogenous losses because of high concentrations of dietary fibre or anti-nutritional factors, the SID values for that diet or ingredient will be reduced compared with values for a diet or ingredient that does not induce specific endogenous losses. However, because endogenous losses are really lost from the body, values for SID will give a better estimate of the AA that are available for metabolism than if values for diet-specific endogenous losses had not been debited against the ingredient or diet. The calculated values for the SID of glycine in several ingredients exceeded 100 % in the current experiment, which is not biologically possible, but these values are an artifact that is caused by an overestimation of endogenous glycine, which often happens when the N-free procedure is used to determine endogenous losses of AA[3].

For all proteins, SID values were different among both indispensable and dispensable AA indicating that one single value cannot be used to estimate the digestibility of individual AA as is assumed in the calculation of PDCAAS[1]. For all ingredients used in this experiment with the exception of wheat, threonine had a lower SID value than lysine, which is usually the case for proteins that are not heat damaged. This is a result of the greater concentrations of threonine than of lysine and other indispensable AA in mucin protein secreted into the small intestine[23]. Mucin protein is resistant to protease digestion, and therefore is included in the endogenous protein fraction that reaches the distal end of the ileum in pigs without being hydrolysed. We are not aware of data for the AA composition of mucin in humans, but it has been reported that the ileal digestibility of threonine in humans is less than that of other indispensable AA, which indicates that mucin in humans also may have a high concentration of threonine[9,10]. The observation that both lysine and tryptophan in wheat had a lower SID value than threonine may indicate that the wheat used in this experiment had been heat damaged during drying or grinding.

The differences between values for SID and STTD of CP that were observed are in agreement with reports indicating that the

apparent ileal digestibility of CP is different from the apparent total tract digestibility of CP[2,24]. In most cases, the total tract digestibility of CP is greater than the ileal digestibility because of absorption of ammonia from the hindgut[25,26], but as illustrated in this experiment, in some cases, N may be secreted into the hindgut resulting in a reduced value for STTD compared with SID. However, because N exchange in the hindgut does not contribute to the AA balance in humans and monogastric animals and because AA are absorbed only in the small intestine, the differences between STTD and SID values illustrate why values for STTD do not always represent absorption of AA. Thus, the use of STTD of CP to estimate the digestibility of all AA in the PDCAAS system will result in inaccuracies of estimates for AA digestibility, which has also been previously illustrated[7,21].

In addition to the lack of digestibility values for individual AA, a major limitation of the PDCAAS system is that all scores are truncated to 100 % with the rationale that any amount of AA beyond the requirement pattern confers no additional benefit to the individual consuming the protein[8,16,26,27]. This assumption, however, neglects the complementary effect that excess AA may have in combination with AA from other proteins[26,27], and as a consequence, PDCAAS values do not give credit for extra indispensable AA that a protein may add to a diet[26,28]. In contrast to the PDCAAS system, values for DIAAS are not truncated to 100 %, and therefore, give credit to a protein based on its value as a complementary source of AA with other sources of proteins in a mixed diet[7].

Despite the challenges with the PDCAAS procedures, which have been previously reviewed[5,26,27], it is important to recognise that criticism related to the scoring patterns that were originally suggested[1] can be easily overcome by adopting different scoring patterns. Indeed, in a later report from WHO/FAO, scoring patterns for several age groups of children, teenagers and adults were suggested[28]. Likewise, the problems associated with truncation can also be easily corrected by using untruncated values[26]. As a consequence, the principal methodological difference between values calculated for PDCAAS and values calculated for DIAAS is related to the assumption that the small intestinal absorption of individual AA can be predicted from the total tract digestibility of CP. As was clearly illustrated in this experiment, differences in the ileal digestibility among individual AA in all proteins exist with the digestibility of threonine being the least for most proteins. As a consequence, the ileal digestibility of AA cannot be accurately predicted from a single value obtained for the total tract digestibility of CP. It is also clearly illustrated that both STTD and SID of CP overestimate the ileal digestibility of AA for proteins with lower AA digestibility and as a consequence, values for PDCAAS that are predicted from the STTD of CP are expected to be less accurate for proteins with low AA digestibility than for proteins with greater AA digestibility. These principles are illustrated by the data in Table 6 where PDCAAS-like values are calculated according to the original recommendation[1] with scoring patterns for 2–5-year-old children and all values are truncated to 100. The observation that the PDCAAS-like values for WPC, MPC and SMP are much less than values for DIAAS is a consequence of truncation. However, if values are not truncated, none of these proteins have PDCAAS-like values that are less than values for DIAAS. Indeed, removing the truncation resulted in PDCAAS-like values that were greater than values for DIAAS for six of the eight protein sources, indicating an overestimation of protein quality by using PDCAAS-like values. Values for DIAAS were calculated based on the scoring pattern for children from 6 to 36 months[8], and because this scoring pattern is different from the original PDCAAS scoring pattern[1], this will influence the calculations. However, even if the PDCAAS-like values were calculated using the DIAAS scoring pattern, PDCAAS-like values for five of the eight proteins were greater than values for DIAAS. This observation is a consequence of the fact that total tract digestibility of CP is usually greater than the ileal digestibility of AA as discussed above, and as expected, the difference between PDCAAS-like values and DIAAS is greater for proteins with lower AA digestibility than for proteins with greater digestibility. Thus, it appears that the major inaccuracies in the calculation of PDCAAS are a consequence of the incorrect assumption that the ileal digestibility of all indispensable AA can be predicted from the total tract digestibility of CP. This inaccuracy will have greater impact on evaluation of proteins used in developing countries than in developed countries, because foods typically consumed in many developing countries have lower digestibility of CP than food typically consumed in developed countries[29].

If PDCAAS-like values and DIAAS values were calculated for children older than 6 months or for adults and if the same scoring pattern was used, no differences between the two methodologies in terms of predicting the first-limiting AA were observed with lysine being first limiting in wheat, histidine being first limiting in the whey proteins and the sulfur AA being first limiting in the whole milk proteins and the soya and pea proteins. However, if the original scoring pattern for PDCAAS was used, the predicted first-limiting AA were different for all proteins except SMP, PPC and wheat, which illustrates that the choice of scoring pattern will influence, which AA is predicted to be first limiting in a specific protein.

The observation that PDCAAS-like values and values for DIAAS were much less if the scoring pattern for infants (i. e. <6 months old) was used instead of scoring patterns for older children or adults illustrate the high-protein quality that is needed in proteins by infants. The fact that some of the proteins such as PPC and wheat, have very low DIAAS and PDCAAS-like values for infants is likely of minor consequence because these proteins are not expected to be used to a great extent in the feeding of infants.

In conclusion, data from this experiment indicate that PDCAAS-like values calculated from the total tract digestibility of CP in pigs and DIAAS values for dairy proteins are greater than for proteins obtained from soyabeans, peas or wheat. Data also indicate that for most proteins, significant differences between PDCAAS-like values and DIAAS were observed. Whereas some of the flaws in the calculation of PDCAAS can be corrected by using different scoring patterns, the fundamental problem with values for PDCAAS is that they are calculated using the incorrect assumption that the ileal digestibility of all AA can be predicted from the total tract digestibility of CP.

Because of this assumption, PDCAAS values do not accurately predict ileal AA digestibility and it appears that specifically for low-quality proteins, values for PDCAAS overestimate the protein quality. Thus, to better meet protein requirements of humans, specifically for individuals consuming diets that are low or marginal in digestible AA, values for DIAAS should be used to estimate protein quality of ingredients and diets.

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The contributions of the authors were as follows: J. K. M. conducted the animal work and laboratory work, analysed the data and wrote the majority of the manuscript. Y. L. prepared the experiment proposal, secured approval from appropriate animal welfare regulatory bodies and contributed to calculations, data analysis and interpretation of data. H. H. S. was the principal investigator. He designed the experiment, oversaw the development of the experiment and wrote the final version of the manuscript.

The authors declare no conflicts of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114517000125

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Protein quality assessment in follow-up formula for young children and ready to use therapeutic foods

Report of the Fao Expert Working Group
Rome, 6–9 November 2017

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Abbreviations and acronyms

AAA	Aromatic Amino Acids
AAS	Amino Acid Score
ANFs	Anti-nutritional Factors
CCNFSDU	Codex Committee on Nutrition and Foods for Special Dietary Uses
DIAAS	Digestible Indispensable Amino Acid Score
EAR	Estimated Average Requirement
EED	Environmental Enteric Dysfunction
FAO	Food and Agriculture Organization of the United Nations
FUF-YC	Follow up Formula for Young Children[1]
IAA	Indispensable Amino Acid
IAAO	Indicator Amino Acid Oxidation
LAL	Lysinoalanine
PDCAAS	Protein Digestibility-Corrected Amino Acid Score
RUTF	Ready to Use Therapeutic Food
SAA	Sulfur Amino Acids
SAM	Severe Acute Malnutrition
WHO	World Health Organization

[1] The name of the product used at the time of the adoption of the Report of the 38th session of the Codex Committee on Nutrition and Foods for Special Dietary Uses

Glossary – definition of terms used in the guideline

A

Amino acid score or Chemical score:

$$\text{Amino acid score} = \frac{\text{mg of amino acid in 1 g of test protein}}{\text{mg of amino acid in 1 g of requirement pattern}}$$

The amino acid score is calculated as above and expressed either as a ratio to unity (recommended), or on a percentage scale (WHO 1991).

B

Bioavailability: the term "bioavailability" encompasses three properties of foods that can alter the proportion of an amino acid that can be utilized; these are:

- Digestibility, which describes the net absorption of an amino acid.
- Chemical integrity, which describes the proportion of the amino acid that, if absorbed, is in an utilizable form.
- Freedom from interference in metabolism resulting from the presence in the food of substances that limit the utilization of the amino acid.

Of these, the greatest source of variation in bioavailability is, in most cases, digestibility (FAO 2013).

F

Fecal digestibility: defined in terms of balance of amino acids or nitrogen measured from the mouth to anus.

I

Ileal digestibility: defined in terms of balance of amino acids or nitrogen measured from the mouth to terminal ileum, which ends at the ileocaecal valve.

L

Limiting amino acid (LAA): the essential amino acid of a dietary protein source present in the lowest proportion as compared to the same quantity of another protein (real or hypothetical) selected as a standard. The apparent limiting amino acid in a protein is thus dependent on the standard chosen. The true limiting amino acid in a protein is, however, the amino acid limiting growth in a biological experiment (WHO 1991).

P

Protein requirement: the lowest level of dietary protein intake that will balance the losses of nitrogen from the body, and thus maintain the body protein mass, in persons at energy and other nutrient balance with modest levels of physical activity, plus, in children or in pregnant or lactating women, the needs associated with the deposition of tissues or the secretion of milk at rates consistent with good health (WHO 2007).

Protein digestibility: defined in terms of balance of amino acids or nitrogen across the small intestine. The difference between intake and losses provides a measure of the extent of digestion and absorption of food protein as amino acids by the gastrointestinal tract for use by the body (WHO 2007).

Executive summary

The Expert Consultation of the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU), 38th session, identified the need to determine protein quality of Follow-up Formula for Young Children (FUF-YC) and Ready-to-Use-Therapeutic Foods (RUTF), and subsequently sought scientific advice from the Food and Agriculture Organization of the United Nations (FAO) to address this need.

In the 2014 FAO report on "Research approaches and methods for evaluating the protein quality of human foods", the Working Group noted that the recommended Digestible Indispensable Amino Acid Score (DIAAS) values have not been established for all protein sources, and the transition to the method be made only with availability of data. Therefore, for the purpose of drafting guidelines, the currently available Protein Digestibility-Corrected Amino Acid Score (PDCAAS) values are to be adopted. In connection to this, FAO convened an Expert Working Group to discuss questions and related scientific issues, raised by CCNFSDU, and were tasked to provide practical guidelines and assistance to member countries and industry on how to determine protein quality of FUF-YC and RUTF.

The fundamental questions around protein quality of FUF-YC and RUTF are recommendations related to protein and amino acid requirements, relevant amino acid scoring patterns to be used, and methods for protein and amino acid digestibility. The below questions and associated scientific issues were discussed by the Expert Working Group in the process of drafting the guideline and recommendations:

- what is the protein and amino acid requirement in infants and children of the target age group, which is 1–2.9 years for FUF-YC and 0.5-4.9 years for RUTF? How do the requirements change, especially in Severe Acute Malnutrition (SAM) for which RUTF is intended?
- Which reference amino acid pattern to use for determination of protein quality in FUF-YC and RUTF?
- What are the currently available methods to evaluate protein and amino acid digestibility for protein quality assessment? What are the limitations of these methods?
- How do anti-nutritional and environmental factors influence digestibility of food products?
- What is the PDCAAS target score for FUF-YC and RUTF?
- What are the cost implications of recommended methods to define protein digestibility?

Recommended amino acid scoring patterns to be used for calculation of PDCAAS

FUF-YC: The Expert Working Group recommends the use of protein, amino acid requirements and reference scoring pattern for children in the 1–2.9 year age group for determining protein quality. The reference amino acid pattern is computed utilizing a protein requirement of 0.86 g/kg/day (0.66 g/kg/day for maintenance and 0.20 g/kg/day for growth) and the maintenance and tissue pattern of amino acids (as reported in WHO/FAO/UNU 2007, summarized in Table 1).

RUTF: The Expert Working Group recommends the use of the reference amino acid pattern for a preferred weight gain value of 10 g/kg/day for catch-up growth and related protein requirement of 2.82 g/kg/day (0.82 g/kg/day for maintenance and 2.00 g/kg/day for growth). This is similarly computed using the maintenance and tissue pattern of amino acids (as reported in WHO/FAO/UNU 2007, summarized in Table 1). Formulations should preferably maintain a phenylalanine to tyrosine and methionine to cysteine ratio of 1:1, to ensure adequate Aromatic Amino Acid (AAA) and Sulfur Amino Acid (SAA) supply during catch-up growth.

Table 1 - Protein and amino acid requirement and amino acid reference pattern proposed for FUF-YC (1–2 year) and for RUTF (target weight gain value of 10 g/kg/d), in infants and children, 6 months to 5 years

Requirement	Protein (g/kg/d)	Amino acid (mg/kg/d)								
		His	Ile	Leu	Lys	SAA*	AAA*	Thr	Trp	Val
1–2 years	0.86	15	27	54	45	22	40	23	6.4	36
Catch-up growth	2.82	66	95	198	183	88	177	103	29	130
Amino acid reference pattern (mg/g Protein)[a]										
		His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
1–2 years		18	31	63	52	26	46	27	7.4	42
Catch-up growth		24	34	70	65	31	63	36	10	46

[a] calculated as amino acid requirement in mg/kg/d divided by total protein requirement in g/kg/d

*SAA = sulphur amino acids (methionine + cysteine), AAA = aromatic amino acids (phenylalanine + tyrosine)

Protein digestibility

The Expert Working Group proposes an algorithm that uses the best available methods to assess protein digestibility, depending on data availability. Member countries and/or industries are recommended to follow in order, starting with human true ileal digestibility values, growing pig true ileal digestibility values and rat true ileal digestibility values. If these are not available, human, pig, or rat fecal protein digestibility values should be used, in that order. One should also consider the possibility of generating prediction equations for ileal digestibility values, obtained from comparisons between pig and rat models and humans, that give scope for future research. It also recommends considering tested and agreed-upon *in vitro* methods of protein digestibility that are compared against *in-vivo* methods, once available.

The Expert Working Group recommends considering the influence of malnutrition, poor environments and infections on digestibility of formulations in infant and children while calculating and interpreting the PDCAAS.

The Expert Working Group recommends considering the effects of anti-nutritional factors (ANFs) on digestibility when calculating PDCAAS values. ANFs reduce protein digestibility mainly through a) inhibiting the action of digestive enzymes, b) binding with proteins causing precipitation and/or c) chelating nutrients, digestive enzymes and/or mineral cofactors. In such situations it may be necessary to include a correction for the bioavailability of the amino acids. It is prudent to note that the use of PDCAAS method is inappropriate for routine determination of protein quality in those protein sources that contain high levels of known ANFs, as the PDCAAS method would overestimate the protein quality of such products. Where possible, appropriate processing measures should be adopted to overcome these effects. Similar recommendation would apply to formulations that through processing and storage result in the generation of ANFs, such as those formed during the Maillard reaction, racemization and lysinoalanine.

PDCAAS: The Expert Working Group recommends using PDCAAS and appropriate digestibility values to determine protein quality of FUF-YC and RUTF. A high-quality protein source will have a PDCAAS score of 100. However, a PDCAAS score of ≥90 can still be considered adequate for these formulations. In formulations with PDCAAS score of <90 the quantity of protein should be adjusted to achieve the desired value. It should be noted that the ideal metric for protein quality assessment is the DIAAS. However, for practical and regulatory purposes at present, since true ileal digestibility values of individual amino acids are incomplete, the Expert Working Group recommends the use of PDCAAS.

Other recommendations

The Expert Working Group recommends member countries and industries to test the efficacy of a new formulation for its ability to support growth or related outcomes of interest in the target population, which, in this scenario, would be children of 1 to 2.9 years for FUF-YC and 0.5 to 4.9 years for RUTF and not just rely on fulfilling the protein quality recommendation.

The Expert Working Group recommends estimating true ileal nitrogen and amino acid digestibility values from animal models, where human data is not available. Rat models can be preferred as they are economical when compared to pigs, but where feasible, the recommendation is to conduct human studies that although limited by their cost, are desirable.

Future research recommendations

- It is necessary to generate a complete dataset on the true ileal digestibility for different protein sources.
- In order to allow for an algorithm to be operationalized, it is necessary to compare true ileal nitrogen and amino acid digestibility of foods within the full range of protein digestibility's between pig, rat and human, and to generate a robust statistical prediction equation.
- At present there are no data to show whether available models (adult human via naso-ileal intubation, pig ileal model or rat ileal model) are representative in children with malnutrition. There is a need for studies comparing ileal digestibility in children, both normal and malnourished, to adults and suitable animal models.
- It is important to develop an agreed-on *in vitro* method to predict true ileal nitrogen and amino acid digestibility values.
- There is clearly a need to further examine whether essential amino acid needs are increased (beyond current estimates) for adequate growth and development in malnourished children, where frequent episodes of gut insults occur due to poor environments.
- With introduction of formulations or food preparations that are enriched with single or multiple amino acids, one needs to consider setting scoring methods to accommodate added amino acids.
- It is important to determine the contribution of amino acids generated from the colonic microbiome towards the amino acid pool of the whole body, as there is considerable uncertainty around such a contribution towards host amino acid economy.

1. Introduction

In response to a request from the 38th Session of the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) the Food and Agriculture Organization of the United Nations (FAO) convened an Expert Working Group at the FAO Headquarters, Rome, Italy, from 6 to 9 November 2017.

Consistent with the need to provide safe food for young children, particularly during the complementary feeding period between 12 and 36 months and the period of rapid development to the age of 59 months, the meeting addressed questions related to protein quality evaluation for the development of the Codex Standards on

1) Follow-up Formula for Young Children (FUF-YC) (aged 12–36-months); and

2) Ready-to-Use-Therapeutic Foods (RUTF) (aged 6–59 months).

The topics discussed include the following:

- protein and amino acid requirement in the target age group;
- age group to be considered for amino acid reference profile in FUF-YC and RUTF;
- the measurement of protein and amino acid digestibility and bioavailability;
- the calculation of Protein Quality Score for FUF-YC and RUTF;
- the recommendations and guidelines for countries to use Protein Digestibility-Corrected Amino Acid Score (PDCAAS) in FUF-YC and RUTF.

Main objectives were:

- to determine the appropriate comparative protein or amino acid reference pattern to define protein quality for use in FUF-YC and RUTF;
- to provide guidance on the preferred protein quality assessment methodology that should be stipulated with the standards for FUF-YC and RUTF;
- to provide guidance on the measurement of protein and amino acid digestibility;
- to provide the appropriate reference amino acid profiles and the amino acid composition of common ingredients used for FUF-YC and RUTF;
- to provide cost implications for countries to use PDCAAS in FUF-YC and RUTF.

This report provides practical guidance on the measurement of protein quality in two distinct food products used to feed children in different conditions: RUTF and FUF-YC. RUTF is a therapeutic food to be provided under medical supervision to children with uncomplicated Severe Acute Malnutrition (SAM) between 6 and 59 months. It is recommended to feed RUTF during the recovery phase, to ensure adequate provision of required macro- and micronutrients. FUF-YC is intended to bridge or improve the nutrient gap in children's diets between 12 and 36 months, in those who are on complementary feeding with or without breastfeeding. However, FUF-YC is not intended to have the undesired consequence of replacing the natural home-based diet of the child.

While several different methods exist for the assessment of the quality of protein in a diet or food, the current accepted method is a chemical scoring approach. Expert Consultations conducted on Protein Quality (FAO/WHO 1991, WHO/FAO/UNU 2007) concluded that the preferred approaches to measuring protein quality are the PDCAAS and related methods such as Digestible Indispensable Amino Acid Score (DIAAS). These methods relate the indispensable amino acid content of an individual foodstuff or mixed diet to a reference amino acid profile after applying a correction term for protein digestibility.

In this regard, this report is only intended to outline how protein quality should be measured, by the definition of protein and amino acid requirement and scoring patterns according to the PDCAAS or DIAAS methods, for each of the foods (RUTF and FUF-YC).In presenting the recommendations regarding the treatment of SAM with RUTF, or whether FUF-YC should be a replacement for complementary foods, the Expert Working Group also considered the long-term health consequences of such feeding interventions, where they do not increase the risk of obesity and its consequences in later life.

The report also provides future research recommendations including the need to generate data on the true ileal digestibility for different protein sources so that DIAAS values can be used in the future.

2. Protein and amino acid requirements and amino acid reference patterns in the target populations for FUF-YC and RUTF

2.1. Overview of protein, amino acid and nitrogen metabolism in adult, infant and children

As with all living organisms the human body exists in a dynamic state in which it extracts from the environment the materials it needs to support its structure and function, with the end products being returned to the environment (Waterlow 1981; Jackson *et al.* 2015). In adults, energy and nutrient balance is achieved over extended periods of time, and during states of balance, energetically and chemically, there are losses from the body equivalent to that taken in, with size, structure and body composition remaining more or less constant (Reeds 1990; Waterlow 1995, 1999, 2006; WHO/FAO/UNU 2007).

During the infant and children period of growth there is net deposition of energy and all nutrients, as new tissues. During childhood, periods of insufficient nutrient consumption lead to deficits in growth (linear or weight gain) that may be aggravated by periods of ill health. In this circumstance, recovery is associated with a greater rate of net tissue deposition to correct any deficit incurred (Jackson and Wootton 1990; Jackson 1990; Graham *et al.* 1996).

The pattern of energy and nutrients required for maintenance and net tissue deposition defines the dietary intake required to make up for any deficit. The amount and pattern will vary with sex, age, composition of tissue deposition and recovery of functional competence and these will in turn determine the quantitative and qualitative pattern necessary to make good the deficit (Jackson 1993; Reeds 2000). The most obvious component of tissue lost or regained during these processes is protein and other amino acid derivatives (Reeds 1999, 2000).

Dietary proteins contribute to meeting nutritional needs through the provision of nitrogen and amino acids. The amount and pattern of proteins being turned over (synthesised and degraded) within the body and the needs for net deposition characterise the pattern that must be made available. In addition

to meeting the needs for protein turnover and net protein deposition, amino acids fulfil important functions as the precursors for structural and functional compounds that are metabolically active, such as neurotransmitters, glutathione, haem and creatine (Reeds 2000).

The categorization of amino acids into those that must be provided preformed in the diet (indispensable or dietary essential) and those that do not (dispensable or non-dietary essential) forms the basis for the concept of protein quality, the extent to which the dietary pattern of indispensable amino acids in the diet matches the pattern of the body's need for indispensable amino acids. In addition to the dietary intake of amino acids as an integral component of protein, the dispensable amino acids are synthesised endogenously as an integral feature of intermediary metabolism. Quantitatively, the rate of endogenous formation may exceed that taken in the diet substantially, up to an order of magnitude, and require complex inter-organ cooperativity. At all ages and in all states the demand for dispensable amino acids exceeds that for indispensable amino acids, although the relative proportions may vary widely during adulthood (Reeds 1990; Jackson 1995; Reeds 2000).

Normal adults can readily maintain nitrogen balance across the range of dietary protein intakes from 40 to 200 g/d when the need for energy and all other nutrients has been satisfied. As the protein intake decreases, balance is restored through a decrease in urinary excretion of urea. Higher levels of dietary protein intake, which provide amino acids at greater levels than what can be efficiently utilized, must be catabolised without placing undue metabolic stress on the body and excreted in a non-toxic form (Harper *et al.* 1970; Benevenga and Steele 1984).

An example of the fine balance between sufficient and excess can be illustrated from the need for sulphur containing amino acids, methionine and cysteine. During the catabolism of methionine excess methyl groups are buffered through methylation of glycine to form sarcosine; sulfhydryl groups are conjugated to serine in the formation of cysteine, which is held intracellularly conjugated with glutamine and glycine in the form of glutathione. Thus, the handling of generous amounts of methionine/cysteine generates a competitive demand for other possible pathways such as the formation of creatine, haem or collagen (Harper *et al.* 1970; Benevenga and Steele 1984; Meakins *et al.* 1998).

The ability of a diet to support whole body nitrogen equilibrium in adults or positive nitrogen balance during childhood is necessary but not a sufficient characterisation of its adequacy. The use of nitrogen balance to assess the protein adequacy of a diet requires that the needs for energy and all other nutrients has been satisfied, and hence "protein" or "nitrogen" is the first limiting consideration. It is well characterised that around the marginal requirement for energy or marginal requirements for protein there is a complex interaction between the two with increased energy intake sparing protein or increased protein intake sparing energy. If any other nutrient is limiting, there is inefficiency in achieving nitrogen balance or the net retention of amino acids as tissue. This is important in practice, for example when increased gastrointestinal losses of potassium or magnesium have not been taken into account.

The efficiency with which different forms of dietary amino acids can be utilized may be predicted by the nature of the balance between the metabolic demand and that supplied directly or indirectly from the diet. Beyond nitrogen balance, the nature of growth, both its quantity and quality mark the extent to which dietary protein and its constituent amino acids match the body's needs (Reeds 1990, 2000). Further functional indices related to the capacity for maintaining the integrity of the organism, resilience to infection, inflammation and immunity should inform these judgements. Measures of long-term health are increasingly considered important, such as the risk of obesity in childhood or chronic disorders including cancer in later life.

2.2. Protein requirement in infant and children 1–2.9 years

The Estimated Average Requirement (EAR) for protein, in the age range of 1 to 2.9 years is calculated as the sum of maintenance requirement plus the protein deposited during growth (Table 2).

EAR = maintenance + tissue protein deposition (deposition / efficiency of utilization)

It is assumed that the maintenance requirement in this age range is equal to the adult value of 0.66 g/kg/d, derived from observations of nitrogen balance versus nitrogen intake in 235 individuals (WHO/FAO/UNU 2007). Observed values in children were close to this figure. The value for the average protein deposited during growth is taken from estimations of protein accretion by whole body potassium counting (Butte *et al.* 2000) in this age range. The efficiency of utilization of protein for deposition during growth was calculated as the average from several studies, to be 58 percent in healthy infant and children (WHO/FAO/UNU 2007).

The recommended level (exceeding the requirement of 97.5 percent of the population) is then estimated assuming that the requirement follows a log normal distribution i.e., safe level is the average level plus 1.96 standard deviation, with total variability of maintenance and deposition calculated from the root mean square of CV of 12 percent for the maintenance needs (as used in case of adults) and 24 percent for the protein deposition rates between 1–2.9 y.

Table 2 - EAR and safe level of protein intake for children aged 1–2.9 years (sexes combined)

	g protein/kg body weight/day (g/kg/d)			
Age (y)	Maintenance[a]	Growth[b]	Total (EAR)	Safe level[c] 1.96SD
1	0.66	0.29	0.95	1.14
1.5	0.66	0.19	0.85	1.03
2	0.66	0.13	0.79	0.97

[a] from N balance studies

[b] adjusted for efficiency of utilization of 58% from N balance studies (WHO/FAO/UNU 2007)

[c] SD calculated as in text

2.3. Amino acid requirement in infant and children 1–2.9 years

Nitrogen balance studies have provided the only empirical data available for determination of indispensable amino acid requirements in children. However, due to problems in interpreting the data, they were not utilized; instead the factorial approach was used to calculate the indispensable amino acid requirement from 6 months through to 18 years (WHO/FAO/UNU 2007). The factorial approach based on the maintenance and growth components of the protein requirement was used to estimate the indispensable amino acid requirements (WHO/FAO/UNU 2007) (Table 3).

Maintenance component

The amino acid requirements for maintenance was assumed to be similar to adults based on the observation that the average maintenance nitrogen requirement of children (110 mg/kg/d) across a wide age range from 6 months to 18 years was similar to the value of 105 mg/kg/d found for adults (WHO/FAO/UNU 2007). Thus the adult maintenance protein requirement of 0.66 g/kg/d times the adult

maintenance amino acid pattern (amino acid requirement x maintenance protein requirement) was used to calculate the maintenance portion of the amino acid requirements (WHO/FAO/UNU 2007).

Growth component

The growth component was estimated using the best available data on the rates of protein deposition at different ages (Butte *et al.* 2000). The amino acid composition of the body proteins and the efficiency of protein utilization of 0.58 were obtained from nitrogen balance studies conducted in children from 6 months to 12 years old (WHO/FAO/UNU 2007). The amino acid requirement for each indispensable amino acid in Table 3 was thus calculated as the sum of the adult maintenance protein requirement (g/kg/d) times the maintenance amino acid pattern (mg/g protein), plus growth (tissue deposition rate in g/kg/day) adjusted for efficiency of deposition (0.58) times the human tissue amino acid pattern (mg/g protein) (WHO/FAO/UNU 2007).

Table 3 - Amino acid requirement in children 1–2.9 years determined by factorial calculation (WHO/FAO/UNU 2007)

AA pattern (mg/g Protein)	His	Ile	Leu	Lys	SAA*	AAA*	Thr	Trp	Val
Tissue amino acid pattern[a]	27	35	75	73	35	73	42	12	49
Maintenance amino acid pattern[b]	15	30	59	45	22	38	23	6	39

Protein requirement (g/kg/d)		Amino acid requirement (mg/kg/d)d								
Maintenance	Growth c	His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
0.66	0.20	15	27	54	45	22	40	23	6.4	36

[a] amino acid composition of whole-body protein (WHO/FAO/UNU (2007)

[b] adult maintenance pattern calculated as the amino acid requirement for adults (mg/kg) i.e. the mean protein requirement for adult (0.66 g/kg) (WHO/FAO/UNU (2007)

[c] calculated as average growth rate for age range adjusted for efficiency of protein utilization of 58%(WHO/FAO/UNU (2007)

[d] sum of amino acids contained he the dietary requirement for maintenance (maintenance protein x the adult scoring pattern) and growth (tissue deposition adjusted for a 58% efficiency of utilization x the tissue pattern) (WHO/FAO/UNU (2007)

*SAA = sulphur amino acids (methionine + cysteine), AAA = aromatic amino acids (phenylalanine + tyrosine)

Support for adopting the factorial approach

Estimates from factorial approach are supported by findings from stable isotope studies. For instance, the total branched chain amino acid (leucine, isoleucine and valine) requirement estimated from the indicator amino acid oxidation (IAAO) method for adults (Riazi *et al.* 2003) and children (Mager *et al.* 2003) were 144 and 147 mg/kg/day respectively. An estimate of the growth component of 6–10 year-old children of 10 mg/kg/day (Mager *et al.* 2003) gives a total estimate by the factorial approach of 154 mg/kg/day (144+10) (WHO/FAO/UNU (2007). Similarly, for lysine, the estimated daily requirement derived from the IAAO method was 35 mg/kg/d in both children and adults (Elango *et al.* 2007; Kriengsinyos *et al.* 2004). An estimate of the growth component of 6.1 mg/kg/d for growth in the 9–13 year-old children gives a total need of 41 mg/kg/d (35+6.1) by the factorial approach.

2.4. Protein and amino acid requirement in catch-up growth and poor environments in infant and children 0.5–4.9 years

Growth deficits that occur due to undernourishment in children are classified into two categories of thinness or wasting (weight-for-height), and shortness or stunting (height-for-age), which are less that 2SD below the respective appropriate reference growth standards (FAO/WHO/UNU 2007). Severe wasting is defined as weight for length/height less than 3SD below the WHO standard for age and sex (WHO 2006, 2009). Both types of growth deficits are predominantly due to a combined effect of environmental factors and poor nutrition (FAO/WHO/UNU 2007).

Once the adverse effects are removed, catch-up growth is enabled where the growth deficits are corrected, although improvement in weight occurs more rapidly than height (FAO/WHO/UNU 2007). Factors that determine slower catch-up in height are still unknown, height changes occur over a longer period, and peak velocity for height may not be gained until weight-for-height is restored (FAO/WHO/UNU 2007). The focus in the following sections will be primarily on the catch-up growth requirements for protein and amino acids in terms of weight deficit.

Following the initial management of the severely wasted child as per the World Health Organization (WHO) guidelines (Ashworth *et al.* 2003), rates of catch-up growth can be rapid depending on the amount of nutrient that can be consumed. In the presence of adequate energy and micronutrients, the protein needs during catch-up growth have been factorially calculated (FAO/WHO/UNU 2007). The Expert Working Group agreed on a preferred weight gain value of 10 g/kg/d considering the usual weight gain of 10–15 g/kg/day, during the recovery phase of SAM (WHO 1999) (Table 4). The calculations do require assumptions to be made on composition of weight gain, whether it is lean or fat, the amount of the maintenance protein and energy values, and finally the efficiency of the utilization and deposition of protein and energy. The calculations for amino acid requirement use maintenance amino acid pattern and tissue amino acid composition for maintenance and growth requirement, respectively. Full details for the calculations are presented in the 2007 FAO/WHO/UNU report.

Table 4 - Protein and amino acid requirement for catch-up weight gain of 10 g/kg/d in infant and children 6 months to 5 years

AA pattern (mg/g Protein)	His	Ile	Leu	Lys	SAA*	AAA*	Thr	Trp	Val
Maintenance	15	30	59	45	22	38	23	6	39
Tissue	27	35	75	73	35	73	42	12	49

Requirement	Protein (g/kg/d)[a]	Amino acid (mg/kg/d) [(b)]								
		His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
Maintenance	0.82	12	25	48	37	18	31	19	5	32
Growth	2.00	54	70	150	146	70	146	84	24	98
Total requirement	2.82	66	95	198	183	88	177	103	29	130

[a] Target protein requirement to achieve a catch-up weight gain of 10 g/kg/d is calculated by considering a compositional weight gain of 73:27, lean/fat equivalent to 14% protein and 27% fat, 14% deposited tissue adjusted for a 70% efficiency of utilization, and a safe level of maintenance at 1.24X0.66 g/kg/d = 0.82, with 0.66 g/kg/d being the adult maintenance protein needs.

[b] The amino acid requirement for catch-up growth was factorially derived (Table 3) from the maintenance (0.82 g/kg/d) and growth requirement (2.0 g/kg/d), related to adult maintenance amino acid pattern, and tissue amino acid pattern, respectively.

*SAA = sulphur amino acids (methionine + cysteine), AAA = aromatic amino acids (phenylalanine + tyrosine)

Severe Acute Malnutrition (SAM)

In SAM, there have been reports of differences in protein metabolism between edematous versus non-edematous forms. While the non-edematous malnourished child can increase body protein breakdown to supply amino acids for survival, edematous malnourished children have a slower rate of body protein breakdown (Manary *et al.*1998; Jahoor *et al.* 2008), resulting in lower plasma indispensable amino acids (Jahoor *et al.* 2008).

During the growth recovery phase *de novo* synthesis of several dispensable amino acids could be limiting *in vivo* thus becoming conditionally indispensable (i.e. tyrosine, cysteine). This in turn could limit the synthesis of acute phase proteins and anti-oxidant molecules, which are required during periods of recovery from infection.

The impact of supplementing Aromatic Amino Acids (AAA) phenylalanine, tyrosine and tryptophan have been examined in the catch-up growth phase of recovery from SAM (Hsu *et al.* 2014), as acute phase proteins are rich in these amino acids. Supplementation with AAA at 330 mg/kg/d (phenylalanine at 140 mg/kg/d, tyrosine at 130 mg/kg/d, and tryptophan at 60mg/kg/d) showed significant increases in acute phase protein synthesis, compared to a similar dose of alanine. This suggests that there is an increased demand for the AAA during the catch-up growth phase.

The requirements for tyrosine during catch-up growth were examined in a dose response study, in the presence of 140 mg phenylalanine/kg/d (Badaloo *et al.* 2010). The requirement for tyrosine was 99 mg/kg/d, suggesting that the phenylalanine: tyrosine needs during catch-up growth are at 59:41, similar to body protein (55:45) and in requirements determined in neonates (56:44) (Roberts *et al.* 2001). It was discussed at the meeting that the diet formulations should attempt to balance the phenylalanine: tyrosine ratio to be closer to 1:1, to ensure adequate AAA during growth and recovery.

Glutathione, the primary cellular anti-oxidant molecule, synthesis is rate limited by the availability of cysteine (Jahoor *et al.* 2012). In edematous malnutrition supplementation of cysteine increased glutathione synthesis, but methionine supplementation, the pre-cursor of cysteine, did not (Badaloo *et al.* 2002; Green *et al.* 2014). In addition, methionine remethylation, transmethylation and transulfuration pathways were not affected by edematous malnutrition (Jahoor *et al.* 2006a, 2006b). The overall conclusion from this set of studies is that while methionine is the indispensable amino acid and is also necessary for polyamine synthesis or s-adenosylmethionine (universal methyl donor), the balance of methionine/cysteine in the diet would be important to be closer to 1:1 to ensure adequate Sulfur Amino Acid (SAA) supply during catch-up growth.

Amino acid needs in poor environments

Children living in poor environments could have altered amino acid needs due to small intestinal malabsorption and chronic intestinal inflammation (Crane *et al.* 2015). While it is understood that childhood stunting is multifactorial in its causes (Millward 2017), childhood Environmental Enteric Dysfunction (EED) leading to increased gut permeability has been implicated in lower serum concentrations of some amino acids in stunted children (Semba *et al.* 2016a). Furthermore, rural stunted Malawi children had lower serum amino acid concentrations of all essential amino acids when compared to non-stunted children (Semba *et al.* 2016b).

It is unclear whether supplementation with protein and amino acids would benefit these children (Arsenault and Brown 2017). But, there is evidence that lysine requirements are increased by ~20 percent in chronic-malnourished Indian children aged ~7.5y due to gut parasite infestation (Pillai *et al.* 2015). It is of note that children in the study were asymptomatic, but with a weight-for-age and height-for-age <2SD (Pillai *et al.* 2015), suggesting that children living in poor environments with increased rates of small intestinal insults may increase needs for the most limiting amino acid (lysine) in plant-based diets.

It is instrumental to examine earlier data collected from the neonatal piglet model where it was shown that the portal drained viscera (primarily the small intestine) extracted significant amounts of essential and non-essential amino acids (Stoll *et al.* 1998). Using a similar neonatal piglet model, the apparent needs for threonine (Bertolo *et al.* 1998), branched-chain amino acids (BCAA; leucine, isoleucine and valine) (Elango *et al.* 2002), and SAA (methionine+cysteine) (Shoveller *et al.* 2003) were shown to be increased by 60 percent, 44 percent and 31 percent, respectively, associated with gastric feeding, compared to intravenous feeding.

The increased need for threonine has been attributed to the need to form a major secretory component of small intestinal mucin proteins. In a follow up study, piglets receiving threonine deficient diets had significantly increased episodes of diarrhoea, reduced mucosal mass, reduced mucin protein, and reduced mucin-producing goblet cells in duodenum and ileum (Law *et al.* 2007). There is clearly a need to further examine whether essential amino acid needs are increased (beyond current estimates) for adequate growth and development in malnourished children, where frequent episodes of gut insults occur due to poor environments.

2.5. Summary: proposed amino acid reference pattern for FUF-YC for infant and children 1–2.9 years and RUTF for infant and children 0.5–4.9 years

The Expert Working Group proposes the amino acid reference patterns reported in Table 5 calculated from protein and amino acid requirement and based on the age group of 1–2 years for FUF-YC and on a preferred weight gain of 10 g/kg/d for SAM children receiving RUTF in recovery.

Table 5 - Protein and amino acid requirement and amino acid reference pattern proposed for FUF-YC (1–2 year) and for RUTF (target weight gain value of 10 g/kg/d, 6 months to 5 years)

Requirement	Protein (g/kg/d)	Amino acid (mg/kg/d)								
		His	Ile	Leu	Lys	SAA*	AAA*	Thr	Trp	Val
1–2 years	0.86	15	27	54	45	22	40	23	6.4	36
Catch-up growth	2.82	66	95	198	183	88	177	103	29	130
Amino acid reference pattern (mg/g Protein) [a]										
		His	Ile	Leu	Lys	SAA	AAA	Thr	Trp	Val
1–2 years		18	31	63	52	26	46	27	7.4	42
Catch-up growth		24	34	70	65	31	63	36	10	46

[a] calculated as amino acid requirement in mg/kg/d divided by total protein requirement in g/kg/d

*SAA = sulphur amino acids (methionine + cysteine), AAA = aromatic amino acids (phenylalanine + tyrosine

3. Protein digestibility methods for FUF-YC and RUTF

3.1. Overview of protein and amino acid digestibility – apparent and true digestibility, fecal and ileal digestibility – influence of malnutrition and poor environment

The scoring approach considers the content of bio-available amino acid in food and diet that represents the dietary intake which is made available to the organism for metabolism after digestion and absorption and is oriented to sequential anabolic and catabolic pathways.

Bioavailability is traditionally associated with digestibility that measures digestive losses expressed as the proportion of ingested nitrogen or amino acids that is absorbed in the intestine following protein consumption:

Digestibility (%) = (ingested – digestive losses) / ingested %

Apparent and true digestibility, fecal and ileal digestibility

Digestion is a complex process due to the continuous movements and exchange of protein, amino acids and urea between the gut lumen and the systemic pools of the body (Figure 1). From the perspective of nitrogen metabolism and flow, the small and large intestine are considered as two functionally separate pools operating in series. In the healthy adult, the nitrogen flux through the small intestine may be around 25–30 g/d with as much as 50 percent being derived from the diet and the balance from endogenous secretions in various forms. The flow through the ileo-caecal valve is estimated to be about 10 percent of the total flux.

Digestibility can be determined by measuring the digestive losses in the faeces or at the level of the terminal ileum. The digestibility of protein has largely been determined from fecal digestibility (difference between nitrogen ingested and excreted in the feces). In addition, apparent versus true protein digestibility differentiates between dietary and endogenous origin of nitrogen and amino acids in the intestinal lumen and in digestive losses. True fecal digestibility measure true dietary fecal losses by the difference between total and endo-genous fecal losses. Endogenous fecal losses were traditionally determined by using a protein free diet.

Amino acids and short peptides (di- and tri-peptides) are end products of food protein digestion that are absorbed in the small intestine. Unabsorbed amino acids and peptides are mostly metabolized by colonic bacteria with the production of ammonia, bacterial metabolites and amino acids. Ammonia and many of the bacterial metabolites can be absorbed by the colon whereas amino acid absorption in the colon remains questionable. The protein digestibility values obtained by the

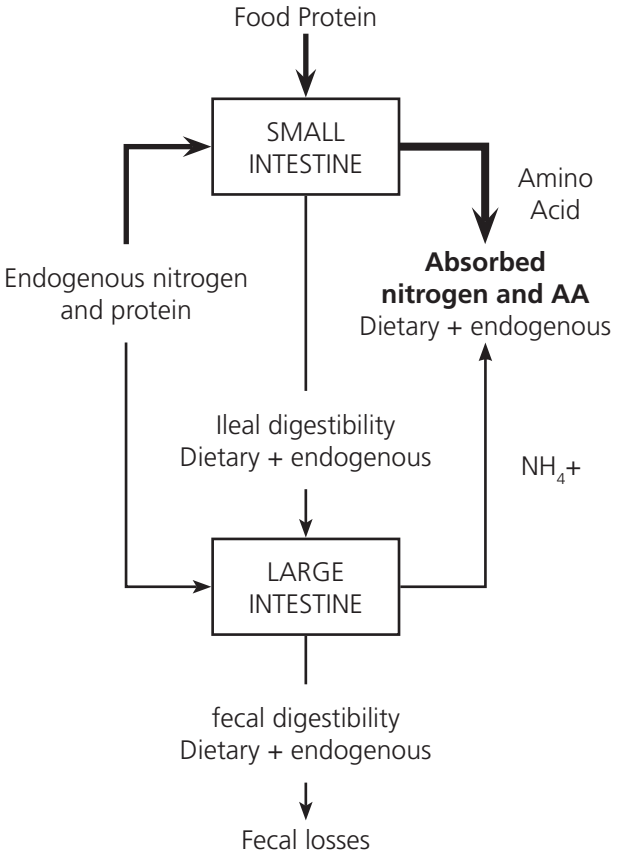

Figure 1 - True ileal protein digestibility

fecal analysis method are thus overestimated when compared to the ileal analysis method. The ileal digestibility is considered more accurate for dietary amino acid digestibility and availability (FAO 2014).

Differences between fecal and ileal digestibility are particularly important for protein sources which are poorly digested in the upper intestine, increasing the quantity to be fermented in the colon. In addition, in the PDCAAS approach the same digestibility, usually fecal, of the protein is applied to each amino acid. More recent developments consider that all amino acids from a same

dietary protein source are not similarly absorbed and that each amino acid should be treated as an individual nutrient. This has led to consider the true individual ileal digestibility of each amino acid as more accurate (FAO 2014).

Influence of malnutrition, poor environments and infections on digestive capacities in infant and children

Both the quality and the quantity of complementary foods can positively influence body weight and linear growth. However, dietary quality, specifically protein quality and micronutrient content is a critical component, in that poor-quality food cannot be easily compensated for by quantity. Many complementary feeding studies and programs fail to demonstrate adequate effects of protein supplementation on growth; for example, the effect of complementary feeding performed in seven efficacy trials around the world with and without fortified foods showed modest population effect sizes (standardized mean difference, Cohen's d) of about 0.26 and 0.28 for weight and height respectively (Dewey and Adu-Afarwuah 2008). Many factors are potentially responsible for this, including social, family and individual level determinants, as well as biological variables, such as coexisting morbidity.

One possibility is that the quality of food provided is effectively reduced because of the child's inability to digest what is consumed. This might be due to EED that results from unsanitary environments, with persistent intestinal immune activation and increased intestinal permeability (Crane *et al.* 2015), and is thought to reduce the ability to digest and absorb protein, thereby impacting linear growth. It is well known that undernourished children have low disaccharidase activity in their intestines, along with poor jejunal absorption of sugars (James 1972).

The secretion of many pancreatic enzymes, such as trypsin, chymotrypsin, amylase and lipase was found to be lower in undernourished children, aged 1–3 years, from Senegal and Ivory Coast, in comparison with well-nourished age and sex matched French children (Sauniere and Sarles 1988). The defect in secretion of enzymes, as well as ions, prompted the authors to term this condition as a silent exocrine pancreatic insufficiency, which showed a variable response to feeding, and never quite recovered to match the French children's level of enzyme secretion. Finally, it is possible that in addition to digestive capacity, there will be a poor absorption of amino acids and dipeptides due to decreased villous surface area (Crane *et al.* 2015)

Poor digestibility could also occur because of intestinal parasites. The mucosal changes that occur because of intestinal parasites are similar to that described in EED. For example, the presence of moderate burdens of Ascaris suum, an intestinal parasitic nematode, in experimentally infected pigs has been shown to cause flattening of villi as well as villous atrophy and fusion (Martin *et al.* 1984), all of which could lead to a loss of brush border enzymes and a reduced surface area for digestion and absorption. Deworming Indian school children led to a reduction in their lysine requirement, after two weeks (Pillai *et al.* 2015). However, the mechanism of this relatively acute effect is unknown. Another possible cause of malabsorption could be bacterial overgrowth of the small intestine due to the presence of worms, though this is more commonly associated with infections such as Giardia duodenalis, a parasite that colonizes the small intestine and transmitted through contaminated water or food (Gendrel *et al.* 1992; de Boisseu *et al.* 1996).

3.2. Antinutrient effects on protein digestibility of human foods

Antinutrients or Anti-nutritional Factors (ANFS) are dietary factors that reduce the bioavailability of nutrients. These may be naturally occurring in plants and seeds or formed during processing and storage of ingredients or foods (including formulas). In general, naturally occurring ANFs diminish dietary protein quality via one or more of three mechanisms. They may reduce protein digestibility by inhibiting the action of digestive enzymes in the gut. Alternatively, they may chelate nutrients preventing their digestion and absorption. Some ANFs damage the digestive tract, reducing the efficiency of digestion and absorption.

Protease inhibitors, such as trypsin inhibitor inhibit the action of proteases. This ANF is found in significant quantities in soyabeans and in lower quantities in other plant-based protein sources such as peas and beans. The concentration and activity of trypsin inhibitor varies greatly between batches and cultivars of soyabeans (Anderson and Wolf, 1995). Trypsin inhibitor is thermolabile, and the heat processing applied to products such as soyabeans (extrusion, steam processing or flaking, boiling, autoclaving etc.) typically inactivate up to 80 percent of this ANF (Gatel 1994). It is noted that currently there are no regulatory upper safe limits established for dietary trypsin inhibitors.

Tannins are polyphenolic compounds. Condensed tannins (flavolans or procyanidins) are present in cereal grains and legume seeds (such as sorghum, millet and many beans and peas). Condensed tannins bind with proteins causing precipitation, thus reducing protein and amino acid digestibility. They are generally heat resistant and potential methods to reduce their content in foods (dehulling, soaking in water or alkaline solutions, germination, addition of chemicals to bind with the tannins) are ineffective or too costly for routine application (Jansman and Longstaff 1993). An alternative is the development of cultivars with low levels of condensed tannins, as has occurred with faba beans (Crépon *et al.* 2010).

Phytic acid, or phytate, is found in oilseeds and grain legumes. It chelates with several nutrients, including protein and synthetic amino acids that are often added to infant formulas. It can chelate with digestive enzymes and/or mineral cofactors and in this manner, decreases the activity of digestive enzymes. It also interferes with zinc homeostasis (Manary *et al.* 2002). Phytic acid is relatively heat stable, with extrusion reducing phytate content by around 20–30 percent (Batista *et al.* 2010). Fermentation has also been demonstrated to reduce phytate content by

23–26 percent (Antony and Chandra 1999). The most effective manner to minimize the effect of phytic acid is via the addition of the exogenous enzyme phytase to the diet/formula. It should be noted that phytase is thermolabile, thus must be added following any processing that involves heat.

Processing and storage of protein sources of formulas can result in the generation of ANFs, such as those formed during the Maillard reaction, racemization and lysinoalanine. The Maillard reaction occurs between reducing sugars and lysine. The "early" Maillard reaction renders lysine nutritionally unavailable. With severe processing, carbonyls may be formed that react with other amino acids, decreasing their nutritional bioavailability (for review see Moughan 2005). Infant formula requires heat processing in their manufacturing processes (eg. spray-drying, sterilization, treatment at ultra-high temperatures, extrusion). Moreover, the processing of some ingredients, such as soyabean, to minimize the quantities of other ANFs (such as trypsin inhibitor) may provoke the Maillard reaction. During conventional amino acid analysis, a proportion of the nutritionally unavailable lysine residues will convert back to lysine, causing an overestimation of the amount of lysine in these products. This degree of overestimation of the lysine that is nutritionally available for processed milk products has been shown to be up to 14 percent (Rutherfurd and Moughan 2005). It is necessary, therefore, to determine the quantity of available lysine in infant formula, using methods such as that described by Moughan and Rutherford 1996.

Heat and/or alkaline treatments can cause racemization, which involves the conversion of L-amino acids to their D-amino acid isomer, and the formation of lysinoalanine (LAL). Protein bound D-amino acids are reported to be hydrolysed at a slower rate than their L-amino acid counterparts and have a slower

absorption from the digestive tract (see Gilani *et al.* 2012). The formation of LAL in foods results in a decrease in the bioavailability of lysine, cysteine and threonine, along with reduced protein digestibility. Increased LAL in foods also poses a risk of kidney damage (see Gilani *et al.* 2012).

In protein sources and formulas that contain minimal quantities of ANFs, nitrogen and protein digestibility is a good measure of the bioavailability of most amino acids. However, in protein sources that contain ANFs (either naturally occurring or because of processing), it is necessary to include a correction for the bioavailability of the amino acids when calculating PDCAAS values. As discussed by FAO (2013), it is likely to be inappropriate to use the PDCAAS method for routine determination of protein quality in protein sources that contain high levels of known ANFs, as the PDCAAS method will overestimate the protein quality of such products.

3.3. Measurements of protein digestibility in human adults and children – current approaches and future developments

The direct determination of true ileal nitrogen and amino acid digestibility requires the collection of ileal digesta. In the human, this is performed by using naso-ileal intubation methods or collection of digesta from humans that have previously undergone an ileostomy operation. These methods are however invasive and ethically non-relevant for their use as routine methods (FAO 2013, 2014). Alternatively, minimally invasive or non-invasive alternative methods are discussed for amino acid bioavailability. Stable isotope-signature based method for bioavailability were proposed including the IAAO – non-invasive, based on free amino acid mixture, and the dual- tracer approach that can lead to a non-invasive method (FAO 2014).

In the naso-ileal intubation methods, human subjects are equipped with a double-lumen intestinal tube introduced through the nose up to the terminal ileum, with one lumen used to perfuse a non-absorbable marker of the flux of intestinal effluents, and the other used to aspirate ileal effluent samples. In addition, the method uses intrinsic and uniformly nitrogen or carbon stable isotope-labelled dietary protein source to differentiate dietary protein-bound dietary amino acids and nitrogen from endogenous protein, amino acids and derived metabolites (particularly ammonia and urea) already present in the intestinal lumen (Fuller and Tomé 2005; Bos *et al.* 2005, 2007; Fromentin *et al.* 2012). Nitrogen and amino acid true digestibility's are calculated from the cumulated amounts recovered at the ileal level and thus not absorbed in the small intestine. True ileal digestibility measured for different protein sources were: milk and meat protein 95 percent, egg, soy and pea protein, 91 percent, wheat protein, 85–90 percent, rapeseed protein, 84 percent (Oberli *et al.* 2015; Fromentin *et al.* 2013; Gaudichon *et al.* 2002; Juillet *et al.* 2008; Bos *et al.* 2005; Bos *et al.* 2007). In addition, true ileal digestibility values of dietary amino acids were also measured after the ingestion of milk or soy protein, with digestibility of amino acids ranging from 91 percent (glycine) to 99 percent (tyrosine) for milk protein, and from 89 percent (threonine) to 97 percent (tyrosine) for soy protein (Gaudichon *et al.* 2002).

An alternative option that allows the collection of digesta from humans to determine true ileal nitrogen and amino acid digestibility coefficients involves humans with a permanent ileostomy, as described in Moughan *et al.* (2005). This method can be used for fibrous/coarse foods, which is not the case for naso-ileal intubation methods. However, it is possible that digestibility results obtained in ileostomates could differ slightly than those in the "intact" human (i.e. via naso-ileal intubation), due to the presence of the ileostomy. Tracer based approaches have also been used to study the digestibility of 13C-and 15N-labelled egg protein (Evenepoel *et al.* 1998), where the ileal effluent was collected in ileostomates and analysed for their residual labelled protein content, in a classical oro-ileal balance.

The IAAO method is based on the concept that when one IAA is deficient for protein synthesis, then the relative surplus of other amino acids including the indicator amino acid (usually L-[1-13C-phenylalanine) is oxidized (Elango *et al.* 2012). A reference slope is constructed from the IAAO response measured with graded intakes of free (crystalline) limiting amino acid (e.g. methionine or lysine) from a reference crystalline AA mixture patterned after egg protein. The metabolic availability is calculated from the ratio of the IAAO response to the addition of amino acid intake from test proteins (substituted for a portion to the free amino acid mixture) to that of free (crystalline) amino acids. The metabolic availability measured by the IAAO method is an estimate of the proportion of the amino acid available for protein synthesis. Hence the IAAO method measures not only digestibility, which has the potential to overestimate protein quality (Rutherfurd 2012, Moughan 2003) but also accounts for all losses due to cellular metabolism (Elango *et al.* 2012). Thus, the method accounts for some amino acids, e.g. lysine that form Maillard products, which are not available for protein synthesis though absorbed. The method was validated in pigs (Moehn *et al.* 2005) and applied in humans for the measurement of methionine metabolic bioavailability in casein and soy protein (Humayun *et al.* 2007) and of lysine in cooked white rice and oven-browned cooked rice (Prolla *et al.* 2013).

The dual-tracer method of measuring small intestinal amino acid digestibility (FAO 2014) follows the principles of other dual-tracer applications such as those used to study starch digestion (Priebe *et al.* 2008). This tracer approach had been used in earlier studies of protein digestion, albeit for a single amino acid, where phenylalanine digestibility was measured by the dual-tracer method in humans with cystic fibrosis, using uniformly labelled 15N-spirulina (Engelen *et al.* 2014). For measuring the digestibility of different amino acids, by the dual-tracer technique, an intrinsically isotope-labelled test protein is simultaneously fed with a different isotope-labelled 'standard' protein, whose digestibility is known (Devi *et al.* 2018). Then, the postprandial ratio of the appearance of differently labelled amino acids in the blood allows for the evaluation of the true digestion and absorption of the test protein, since the splanchnic uptake and metabolism of the different amino acids can be corrected for, when using this ratio approach. As test and standard proteins are delivered simultaneously, it is assumed that their splanchnic extraction terms will be the same. In addition, since this method only measures the appearance of labelled amino acids from the intrinsically labelled test and standard protein, it is not confounded by endogenous protein secretion, and is hence a measure of true ileal digestibility.

3.4. Animal models for protein and amino acid digestibility, with special reference to infants and young children – current approaches and future developments

Due to the lack of available data on true ileal nitrogen and amino acid digestibility coefficients of foods determined in the human, and difficulties in the use of humans for routine determinations, data are generated in animal models. The two animal models that have been most commonly used for protein quality evaluation are the pig and rat.

The digestive tract of the piglet is very similar to that of the milk-fed infant (Moughan *et al.* 1992). Moreover, for infant formulas, the bottle-fed piglet has been shown to be a pertinent model for the human infant (Darragh and Moughan 1995). It should be noted, however, that it is very complex to work with very young piglets, thus for routine evaluations, older growing pigs are typically used. The growing pig has been shown to give values of nitrogen and amino acid digestibility close to human values (Deglaire *et al.* 2009; Rowan *et al.* 1994), at least for highly digestible proteins (FAO 2014). However, statistical prediction equations need to be generated that relate nitrogen and amino acid digestibility values determined in the pig with those determined in the human, encompassing the full

range of digestibility seen in human protein sources, especially for protein sources with lower nitrogen and amino acid digestibility (60–85 percent). Overall, the use of the pig model offers the advantages that their digestive physiology is very similar to that of the human (Deglaire and Moughan 2012; Guilloteau *et al.* 2010). They are meal feeders, readily eat human foods and provide large samples of ileal digesta.

The rat is another potential animal model for the determination of true ileal nitrogen and amino acid digestibility. In order to determine true ileal nitrogen and amino acid digestibility in the rat, the protein sources need to be ground, to prevent selection of particles by the rats. This grinding may affect the digestibility of the nitrogen and amino acids. Moreover, the rat is not a meal feeder, and has the risk of coprophagy occurring, although the latter can be minimized in experimental studies. Currently, there is no data that compares the digestibility values determined in the rat and those in infants or young children, and no published direct comparisons of true ileal nitrogen and amino acid digestibility of protein sources between the rat and the adult human.

3.5. Nitrogen to protein conversion factor

For nutritional objectives related to protein quality, the protein content in a foodstuff is the source of amino acids, and estimating protein content aims at the estimation of total amino acid content. The protein content in a foodstuff is usually estimated by multiplying the nitrogen content by a nitrogen-to-protein conversion factor, considering that the majority of nitrogen is associated with amino acids in protein. This nitrogen-to-protein conversion factor is traditionally set at 6.25. This historical factor (dating back to the 19th century) assumes the nitrogen content of proteins to be 16 percent. There are however different limitations to this approach.

Firstly, nitrogenous compounds in foodstuffs do not only comprise protein or amino acids, but also include numerous substances such as nucleic acids, amines, urea, ammonia, nitrates, nitrites, phospholipids, nitrogenous glycosides, etc. Analysis of protein and non-protein nitrogen content of breast milk from mothers of term infants shows that total nitrogen in human milk represents both protein, about 75 percent, and non-protein nitrogen, which is made up of urea (up to 50 percent of the non-protein nitrogen), amino acids and other nitrogenous compounds (SCF 2003; WHO/FAO/UNU 2007). Non-protein nitrogen fraction in different biomass products (mushroom, vegetables, algal samples, plant leaves, food products, and cereal products) represents from 5 to 50 percent of total nitrogen (Chen *et al.* 2017).

Secondly, if some proteins (considered as biochemical entity) are constituted only by amino acids which are the compounds that contain nitrogen, for other protein the amino acid chain is associated to a "prosthetic" group that usually does not contain nitrogen (mineral, sugar, fatty acid, etc.). For protein with a prosthetic group the mass of the protein is different if we consider only

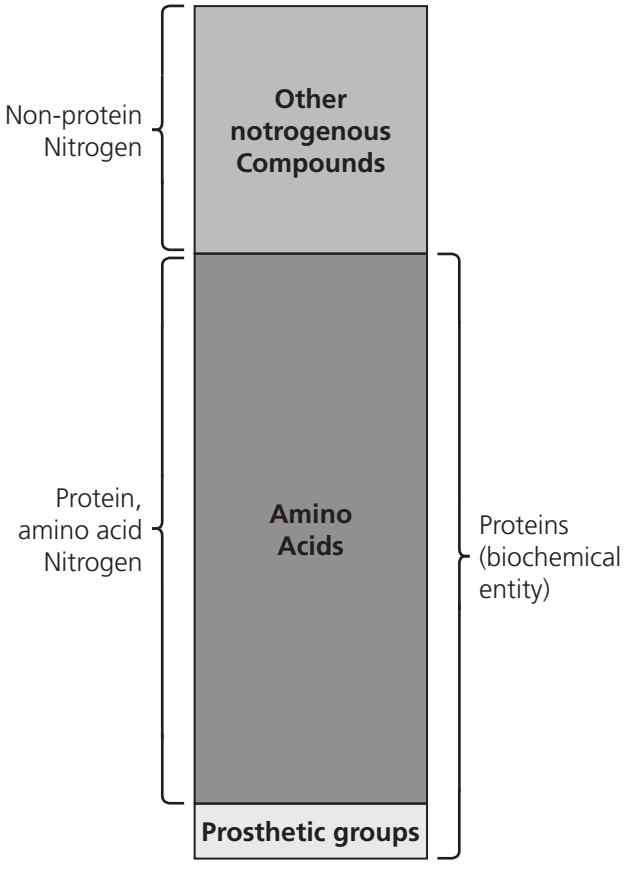

Figure 2 - Nitrogenous compounds in foodstuff

the amino acid part or the biochemical entity including the amino acid part and the prosthetic group, but the nitrogen content remains the same. The conversion factor related to the biochemical entity is higher than the conversion factor related to amino acids. Calculation in different products of the ratio of protein (as the sum of anhydrous amino acids) to amino acid nitrogen provide conversion factors in the range 5.0-6.15 (Fujihara *et al.* 2008; Chen *et al.* 2017). In contrast, values provided from Jones (1941) obtained from the ratio of protein (as molecular entities) to Kjeldahl nitrogen are in the higher range of 5.7–6.38.

Lastly the different amino acid chains of pure proteins differ in terms of their nitrogen contents that results from differences in their amino acid composition, because the nitrogen content of amino acids can vary considerably, being high in arginyl, histidyl, glycyl, and asparagyl residues and low in phenylalanyl and tyrosyl residues. This explains that, even if only the amino acid content is considered, the ratio of protein amino acids to amino acid nitrogen provide a relatively large range from 5.0 to 6.15 for different products (Fujihara *et al.* 2008; Chen *et al.* 2017).

3.6. Recommended methods for protein and amino acid digestibility for FUF-YC and RUTF and costs involved in digestibility measurements

The Expert Working Group agreed that true nitrogen and amino acid digestibility determined at the ileal level (the end of the small intestine) should ideally be used to correct for protein availability in the formulation of FUFs and RTUFs, as per the recommendations of FAO (2013; 2014). However, at present these data do not exist for most of the protein ingredients that are used in these formulas.

True ileal nitrogen and amino acid digestibility data determined in the adult human, whether determined using the naso-ileal intubation method or with ileostomised humans (once validated), could provide informative estimates of nitrogen and amino acid availability when evaluating the protein quality of FUFs and RUTFs. However, as discussed in a recent FAO report (FAO 2013), at present there is a limited amount of data available on ileal digestibility of nitrogen and amino acids for foods determined in humans (Deglaire *et al.* 2009; Gaudichon *et al.* 2002; Rowan *et al.* 1994). These methods are invasive and ethically non-relevant for their use as routine methods (FAO 2013, 2014). In future, stable isotope-signature based method for bioavailability such as the Indicator IAAO, and the dual-tracer approach could be used in humans provided they have been previously validated in comparison to direct methods.

When these data do not exist, it is necessary to use true ileal nitrogen and amino acid digestibility data determined in an animal model. As true ileal nitrogen and individual amino acid digestibility values measured in rat and pig are generated and published, these values should be preferred. When individual amino acid digestibility values

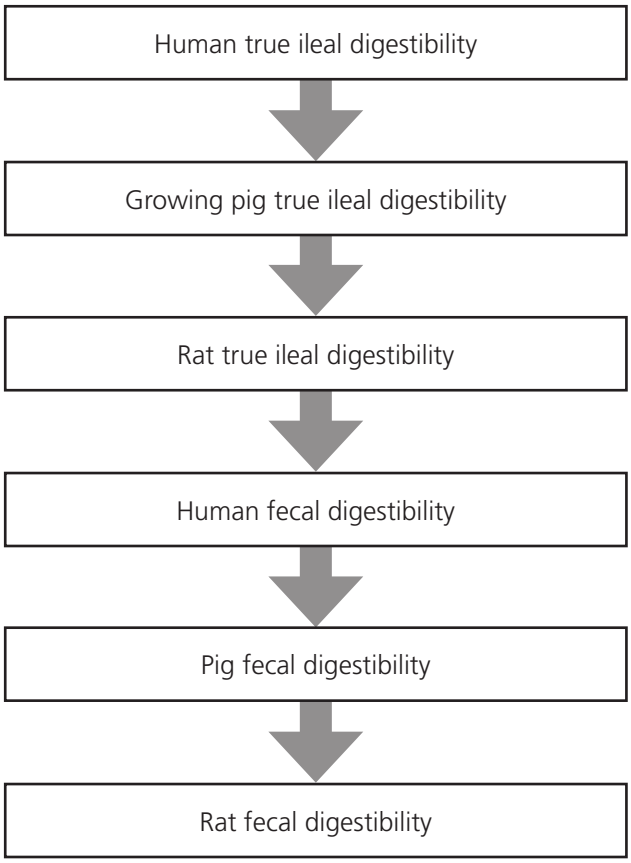

Figure 3 - Algorithm for protein quality assessment with the available digestibility values

are available they should be preferred with DIAAS being used to determine protein quality. Example calculations for the calculation of DIAAS are presented in the FAO report (FAO 2013).

A further alternative for the correction of nitrogen and amino acid availability is data generated using *in vitro* methods. In future, due to ethical considerations with studies involving humans or animal models, *in vitro* methods are likely to become the preferred methods. However, at present there are many different *in vitro* models, and these differ in reaction conditions and, most likely, in the digestibility results generated. There is no agreed-on model for the determination of true ileal nitrogen and amino acid digestibility values using *in vitro* methods, and little data is available on digestibility values to allow the use of these methods at present.

The Expert Working Group proposes an algorithm (Figure 3) that uses the best available methods to assess protein digestibility, depending on data availability for defining protein quality of FUF-YC and RUTF. Member countries and/or industries are recommended to follow in order, starting with human true ileal digestibility values, growing pig true ileal digestibility values, rat true ileal digestibility values. If these are not available, human, pig, or rat fecal protein digestibility values should be used, in that order. One should also consider the possibility of generating prediction equations for ileal digestibility values, obtained from comparisons between pig and rat models and humans that give scope for future research. A cautionary note ought to be considered in formulations utilizing plant-based protein sources, owing to the effect of anti-nutritional factors as explained in section 2.2. Also, one must be aware of the adverse effects of poor environment and infections on intestinal function in children, as digestibility values may differ in such instances.

Cost of digestibility measurements

Where human data on true ileal nitrogen and amino acid digestibility of protein sources are not available, ileal digestibility data could be determined in pig or rat. Pig models are however more expensive to work with than the rat model, and, in the future, there may be limitations on their use for ethical reasons. Rat is an economical model, and where there is no data on true ileal nitrogen and amino acid digestibility values for foods or ingredients determined in the human or pig, ileal data determined in the rat should be used to correct nitrogen and amino acid availability for the FUFs and RUTFs.

On average the total costs involved in conducting digestibility studies using a dual stable-isotope approach is ~8 000 USD per subject. This includes costs of procuring labelled reference protein (for example spirulina), producing ^{2}H labelled test protein using deuterium; in addition there are experimental and analytical costs.

4. Procedures and recommendations

4.1. Use of PDCAAS in assessing protein quality of formulated products

While several methods exist for the assessment of the quality of proteins in a diet or food, the current accepted method is a chemical scoring method.

The chemical amino acid score is the ratio for each amino acid (mg/g protein) in the food ingredient or formulation and a reference pattern of amino acids (mg/g protein). The PDCAAS is computed by correcting the lowest chemical amino acid score of one of four essential amino acids (lysine, tryptophan, SAA and threonine) by the protein fecal or ileal digestibility.

Chemical amino acid score % = 100 x [(mg of amino acid in 1 g test protein) / (mg of amino acid in reference pattern)].

PDCAAS% = weighted protein digestibility for the food formulation * limiting Amino Acid Score (AAS).

In the more recently modified score DIAAS the content of each IAA, and in particular the most commonly limiting four indispensable amino acids (lysine, tryptophan, SAA and threonine) is corrected by its specific ileal digestibility. Then each value is related to same amino acid in the reference amino acid pattern.

DIAAS% = 100 x [(mg of digestible dietary indispensable amino acid in 1 g of the dietary protein) / (mg of the same dietary indispensable amino acid in 1g of the reference protein)].

Scores are truncated to 100 percent. A PDCAAS or DIAAS score below 100 indicates that at least one amino acid is limiting in the food or diet and a score of 100 that there is no limiting amino acid in the food or diet. A key difference between the DIAAS and the PDCAAS is that DIAAS requires the use of true ileal digestibility of each amino acid determined in humans then in growing pigs or in growing rats in descending order of preference (as per the suggested algorithm) (FAO 2013).

4.2. Computing PDCAAS in food formulations (e.g. RUTF)

Computing the PDCAAS to assess protein quality is recommended as part of the assessment of the nutritional composition of a new food formulation used either as a FUF-YC or RUTF. In this section we provide steps for computing the PDCAAS for food formulations. The method to compute PDCAAS has been outlined in detail with associated caveats in the 2007 protein requirements (WHO/FAO/UNU 2007). To compute the PDCAAS, first the AAS for the indispensable amino acids must be estimated. The AAS determines the effectiveness with which absorbed dietary nitrogen meets the indispensable amino acid requirement at safe levels of protein intake.

The following steps and Table 6 outlines an example for the PDCAAS computation procedure of 25 percent RUTF. Once a formulation and its ingredients (amounts per 100 g) are identified, the protein and amino acid content for each of the ingredients in the formulation should be extracted from appropriate food composition data (preferably from the US Department of Agriculture, Agricultural Research Service, Nutrient Data Laboratory, USDA National Nutrient Database for Standard Reference, Release 28).

1. For the computation, the amino acids lysine, tryptophan, threonine, SAA (methionine and cysteine combined) are required, although, to assess the amino acid pattern (mg/g protein), computations could include all amino acids (see Table 6).
2. Data on fecal digestibility should be integrated with the food composition data. Appendix 1 provides estimates of human and rat fecal digestibility, which are not the most accurate data, however, at this moment, this is the best available and thus recommended option.
3. The total protein content and the amino acid content for the food formulation are then calculated.
4. The amounts of each amino acid are then converted to mg/g of protein (for each ingredient). In other words, amino acid pattern for the protein source is calculated.
5. The protein value of each food ingredient is then multiplied by the digestibility value for that ingredient to calculate the amount of digestible protein present in that food item.
6. By multiplying digestible protein values with calculated amino acid pattern mg/g values, the total digestible amino acid content (total mg per food ingredient) is computed for each amino acid.
7. Weighted digestibility is then calculated, weighted by the protein contribution of each ingredient. The standard assumption here is that digestibility of foods does not change when foods are consumed in mixed diets. However as noted earlier (section 2.5), digestibility can be affected by processing and under the presence of anti-nutrient factors.
8. The digestible amount of each amino acid per gram of digestible protein is then calculated (units: mg/g protein). This value should be divided by the recommended reference pattern (see Table 4 or section 1.5) to calculate the unit less AAS. The AAS is calculated for lysine, SAA, threonine, and tryptophan.
9. The amino acid with the lowest score is the limiting amino acid. The AAS of this amino acid is then used in computing PDCAAS, which is the product of the lowest AAS and the weighted digestibility of the food formulation.
10. If the PDCAAS is over 100 percent, it should be rounded down to 100.

Table 6 - Computation of PDCAAS for 25 percent Milk RUTF (25% Milk in 100 g product)[2]

Ingredient	Amount	Protein	Fecal digestibility	Digestible protein	Lys	SAA*	Thr	Trp
Food composition data								
	g	g		g		Amino acids g		
Dried skim milk	100	36.2	0.95	34.4	2.868	1.575	1.63	0.51
Peanut butter	100	22.2	0.95	21.1	0.681	0.723	0.53	0.23
Formulation: 25% Milk RUTF -100 g product								
	g	g		g		Amino acids mg/g protein		
Dried skim milk	25	9.0		8.6	79.3	43.6	45.1	14.1
Peanut butter	26	5.8		5.5	30.7	32.6	23.6	10.4
						Total digestible amino acids mg		
Dried skim milk					681	374	388	121
Peanut butter					168	179	130	57
Sum		14.8		14.1	849	553	517	178
Digestible amino acid mg/g digestible protein					60	39	37	13
Recommended reference pattern (10 g/kg/day weight gain) mg/g protein					65	31	36	10
Amino acid score					0.93	1.27	1.02	1.27
Weighted digestibility	0.950							
PDCAAS	88							

*SAA = sulphur amino acids (methionine + cysteine)

[2] The composition for 25% RUTF was obtained from Oakley *et al,* 2010 where it is referred to as a standard RUTF formulation Contains 25 g of milk per 100 g portion of RUTF

4.3. Protein quality assessment in diets of developing countries – FUF-YC and RUTF used for treating SAM in children aged 0.5–4.9 years

In 2016, at a global level, 52 million children 6–59 months suffered from SAM (with or without complications) with 14 million in Sub-Saharan African and 36 million children in South Asia (UNICEF, 2017). Food products used within the treatment regime need to be carefully assessed for nutrient density (quality protein, fat and micronutrients). SAM and underweight is most prevalent in those populations in the developing world that subsist primarily on cereals and cereal products with very low levels of animal source food consumption. Such diets are likely to be very low in micronutrients as well as high quality protein (Ghosh and Uauy 2016).

At a public health level, there is a need for a better understanding on how adjusting for protein quality could change pattern of protein availability and intakes. Furthermore, a clear set of guidelines is needed to ensure that products targeting specific conditions (e.g. treatment of SAM) to achieve nutrition outcomes in meeting specific standards derived in an evidence-based manner. Adjusting amino acid requirements for physiologic status to evaluate food aid products has been shown to assess protein quality more accurately (Callaghan *et al.* 2017) and protein quality scores have been reported to be highly correlated with the rate of weight gain in recovery from SAM (Manary *et al.* 2016).

Global availability and individual intakes corrected for protein quality

Ecological analysis examining trends in global availability (from 1961 to 2005) of protein correcting for protein quality using the PDCAAS methods show differences in availability of energy and utilizable (good quality) protein across regions and countries. Correcting for quality of protein led to a reduction in total protein by 11 percent globally with as much as a 17 percent reduction in total protein availability in Africa (Ghosh and Uauy 2016). Adjusting for energy deficit and infections leads to a further reduction in protein availability in Sub-Saharan Africa and South Asia (Ghosh *et al.* 2012). Estimates of protein inadequacy (computed from availability) are found to be significantly lower using total protein versus protein adjusted for PDCAAS (utilizable protein). Furthermore, while both total protein and utilizable protein were negatively associated with prevalence of stunting at the national level, the association of utilizable protein unlike total protein is independent of total energy availability.

Assessment of protein intakes adjusted for protein quality using individual level data shows mixed results. An analysis of protein quality of diets of children under five from Kenya, Uganda and Bangladesh found about 30% risk of inadequacy (Suri *et al.* 2012). More recent analysis has shown highest prevalence of inadequate protein intake being found only in breast feeding children aged 6–8 months (24% in Bangladesh, 16% in Peru), with very low or no risk of inadequacy in older children (Arsenault and Brown 2017). The authors concluded that a risk of inadequate protein intake was likely an effect of low intake from complementary foods, and that the quality of complementary foods and protein density (protein intake/100 kcal/day) in infants in Peru and Guatemala (but not Bangladesh) was significantly higher in those infants who met the EAR for protein than those who did not (Arsenault and Brown 2017). Key caveats in both sets of findings is that none of the country data are representative at the national or sub-national level, there is a mixture of rural, urban and peri-urban data with likely purposive sampling. Protein intakes in infants and young children across many different national surveys and cross-sectional surveys showed much higher levels of protein intake than required but it is unclear if any of these estimates have been adjusted for protein quality (Suthutvoravut *et al.* 2015). Excess protein intakes have also been documented in infancy and early childhood in most developed countries and in some developing countries and were discussed in relation with potential enhance weight gain and later risk of obesity (Koletzko *et al.* 2009a; Koletzko *et al.* 2009b). Lower protein in FUF-YC was associated with lower weight gain up to 2 years of age (Koletzko *et al.* 2009a). These findings are particularly relevant in the discussion of protein quality assessment of FUF-YC.

Effectiveness and protein quality of RUTF: plant versus animal based formulations

RUTF used for outpatient treatment of SAM without complications are required to meet specifications as laid out in the guidelines on community based management of SAM (WHO *et al.* 2007). Specifically, within the context of energy and protein requirements, per 100 g, RUTF products must provide 520–550 kcals with 10–12 percent total energy originating from protein. Furthermore, 50 percent of the total protein must come from milk or a milk-based product. The latter recommendation is based on findings from studies that have found a milk based RUTF (50 percent of total protein) to be as efficacious (or even more) as standard therapy for children recovering from malnutrition after being stabilized (Ciliberto *et al.* 2005; Diop *et al.* 2003; Lenters *et al.* 2013). A study conducted in Senegalese children found significantly higher weight gain in children fed RUTF compared to F100 along with an average lower duration of rehabilitation (Diop *et al.* 2003). In Malawi, children who received home based therapy with RUTF had significantly higher gains in WHZ score, were less likely to relapse or die and had a lower prevalence of respiratory infections and diarrhoea compared to children who received standard therapy (F-100) (Ciliberto *et al.* 2005). A systematic review found that children given RUTF were 51 percent more likely to achieve nutritional recovery than the standard care group (Lenters *et al.* 2013).

While currently almost all RUTF available for therapeutic purposes are made of a combination of peanut paste and dried skim milk, efforts are being placed on formulating RUTF using lower cost milk products or locally available legumes such as soya bean, chick peas, cereal flours such as rice, millet, oats, wheat and sorghum (WHO and UNICEF 2007). Studies comparing different levels of milk in RUTF, using different types of milk products (e.g. whey protein concentrate WPC 34) as well as formulating RUTF solely using plant based proteins or a combination of plant based proteins that are enriched with single or multiple amino acids (Bahwere *et al.* 2017; Bahwere *et al.* 2016; Bahwere *et al.* 2014; Irena *et al.* 2015; Oakley *et al.* 2010). A comparison of 10 percent milk (~ 20 percent milk protein) to 25 percent Milk RUTF or standard RUTF (>50% milk protein) found that 10 percent RUTF was less effective in the treatment of SAM in Malawian children

6–59 months (Oakley *et al.* 2010). While rates of recovery were similar (81 to 84 percent) these were significantly different with duration of recovery being shorter in the group with 25 percent Milk RUTF. On the other hand, substituting dried skim milk with whey protein concentrate led to recovery rates and weight gain that were non-inferior than standard RUTF (Bahwere *et al.* 2014). Effectiveness studies comparing non-milk-based RUTF to standard RUTF in non-inferiority cluster randomized trials did not find equivalence in recovery or weight gain in Zambian children (Irena *et al.* 2015), a finding that was further confounded by the age of children, but did in children in the Democratic Republic of Congo (Bahwere *et al.* 2016). The equivalency was also observed in another study conducted in Malawi that examined the efficacy of plant-based RUTF (soya-maize and sorghum) which was enriched with essential amino acids (Bahwere *et al.* 2017). These findings indicate promising avenues for further research.

An assessment of protein quality of different milk-based RUTF and plant-based RUTF was conducted using the new proposed reference pattern (Table 4) which utilizes the preferred weight gain value of 10 g/kg/day and protein needs of 2.82 g/kg/day (0.82 g/kg/day for maintenance +2.0 g/kg/day for growth). Amino acid pattern, scores and PDCAAS of the milk-based RUTF including standard F-100, RUTF, 10 percent Milk RUTF and whey protein RUTF (Bahwere *et al.* 2014; Oakley *et al.* 2010; WHO 1999) and the plant based RUTF (Bahwere *et al.* 2017; Bahwere *et al.* 2016; Irena *et al.* 2015) were computed using the method outlined in the WHO 2007 protein requirements (WHO/FAO/UNU 2007). Weighted digestibility was computed using true fecal digestibility values (Axtell *et al.* 1981; WHO/FAO/UNU 2007). All products almost met the amino acid reference pattern (Table 5) and most AAS were above 1, except in the case of 10 percent Milk RUTF where lysine was 0.83 as well as in the case of Soy-Maize-Sorghum RUTF where it was 0.9. PDCAAS estimates were computed (in all products, lysine was the lowest score) and estimates are presented in Table 7.

4.4. Summary on guidelines and recommendation for protein quality assessment in FUF-YC and RUTF

The Expert Working Group recommends the following in relation to protein quality assessment in FUF-YC and RUTF:

a. to use PDCAAS and appropriate fecal digestibility values to define protein quality of FUF-YC and RUTF.

b. To use reference amino acid requirements and scoring patterns of children in the 1–2.9 year age group for determining protein quality of FUF-YC (Table 5).

c. To use reference amino acid requirement and scoring patterns related to catch up growth of 10 g/kg/day for determining protein quality of RUTF (Table 5).

d. To consider effects of anti-nutritional factors and impaired gut function in the presence of poor environment and infections on digestibility.

e. A high-quality protein source will have a PDCAAS score of 100. However, a PDCAAS score of ≥90 can still be considered adequate for these formulations. In formulations with PDCAAS score of <90 the quantity of protein should be adjusted to achieve the desired value.

f. The efficacy of new formulations should not rely on protein quality considerations alone, and should be tested for their ability to support catch up growth in the target population, which in this scenario would be children of 1 to 2.9 years for FUF-YC and 0.5 to 4.9 years for RUTF.

Table 7 - Protein quality assessment of RUTF formulations found in the literature [3]

Amino acid reference pattern mg/g protein	His	Ileu	Leu	Lys*	SAA$*	AAA$	Thr*	Trp*	Val	PDCAAS
Proposed reference (10 g/kg/day weight gain)	24	34	70	65	31	63	36	10	46	
Amino acid pattern mg/g protein										
F100 (dried skim milk)	27	61	98	79	44	97	45	14	67	
Milk-based RUTF										
25% milk RUTF (>50% protein from milk)	26	48	87	60	39	95	37	13	55	
10% milk RUTF (~20% protein from milk)	25	42	78	54	39	89	35	12	47	
WPC RUTF (whey protein concentrate)	23	44	89	62	49	67	50	15	44	
Plant based and enriched RUTF										
Soya-maize-sorghum RUTF	24	44	77	59	43	82	39	13	46	
Soy-maize-sorghum-amino acid RUTF	26	42	74	71	36	77	35	11	45	
Soy-maize-sorghum-milk-amino acid RUTF	27	44	80	73	37	78	37	11	48	
Amino acid score										
F100 (dried skim milk)	1.13	1.78	1.40	1.22	1.41	1.53	1.25	1.41	1.45	100[a]
Milk-based RUTF										
25% milk RUTF (>50% protein from milk)	1.10	1.40	1.24	0.93	1.27	1.50	1.02	1.27	1.19	88[2]
10% milk RUTF (~20% protein from milk)	1.06	1.24	1.11	0.83	1.25	1.41	0.98	1.24	1.01	76
WPC RUTF (whey protein concentrate)	0.94	1.29	1.27	0.96	1.58	1.06	1.39	1.50	0.97	91
Plant based and enriched RUTF										
Soy-maize-sorghum RUTF	1.02	1.29	1.10	0.90	1.38	1.30	1.09	1.31	0.99	77
		1.2	1.0						0.9	
Soy-maize-sorghum-amino acid RUTF[b]	1.06	3	6	1.09	1.17	1.22	0.98	1.14	7	84
		1.2	1.1						1.0	
Soy-maize-sorghum-milk-amino acid RUTF[b]	1.10	9	4	1.13	1.19	1.24	1.04	1.14	5	89

$SAA = sulphur amino acids (methionine + cysteine), AAA = aromatic amino acids (phenylalanine + tyrosine)

*Amino acids used for the computation of PDCAAS

[a] The value was truncated from 116 to 100 at the final step of PDCAAS computation

[b] These estimates are derived from analytical protein and digestible amino acid data that allowed for computation of digestibilities. No ingredient breakdowns were available for these products, thus these may not be entirely comparable to the other products. All other estimates are based on computations starting from formulation recipes and are thus comparable to each other

[3] PDCAAS values may be lower than other published values due to difference in reference pattern used

5. Future research recommendations

Following the provision of practical guidance on the measurement of protein quality in FUF-YC and RUTF used to feed children in different conditions, the Expert Working Group summarized research recommendations for future work:

- It is necessary to generate a complete dataset on the true ileal digestibility for different protein sources so that DIAAS values can be used in the future, as this data becomes available.
- In order to allow for an algorithm to be operationalized, it is necessary to compare true ileal nitrogen and amino acid digestibility of foods within the full range of protein digestibility's between pig and human, and to generate a robust statistical prediction equation.
- At present there are no data to show whether available models (adult human via naso-ileal intubation, pig ileal model or rat ileal model) are representative in children with malnutrition. There is a need for studies comparing ileal digestibility in children, both normal and malnourished, to adults and suitable animal models.
- It is important to develop an agreed-on *in vitro* method to predict true ileal nitrogen and amino acid digestibility values.
- There is clearly a need to further examine whether essential amino acid needs are increased (beyond current estimates) for adequate growth and development in malnourished children, where frequent episodes of gut insults occur due to poor environments.
- With introduction of formulations or food preparations that are enriched with single or multiple amino acids, one needs to consider setting scoring methods to accommodate added amino acids.
- It is important to determine the contribution of amino acids generated from the colonic microbiome towards the amino acid pool of the whole body, as there is considerable uncertainty around such a contribution towards host amino acid economy.

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Appendix 1

Table 8 - True digestibility values for various protein sources in humans (WHO 2007)

Protein source	True digestibility (%)	Protein source	True digestibility (%)
American mixed diet	96	Oatmeal	86
Beans	78	Oats, cereal	72
Brazilian mixed diet	78	Peanut butter	95
Chinese mixed diet	96	Peanuts	94
Corn, cereal	70	Peas, mature	88
Corn, whole	87	Rice, cereal	75
Cottonseed	90	Rice, polished	88
Egg	97	Soy flour	86
Farina	99	Soy protein isolate	95
Filipino mixed diet	88	Sunflower seed flour	90
Indian rice + beans diet	78	Triticale	90
Indian rice diet	77	Wheat flour, white	96
Indian rice diet + milk	87	Wheat gluten	99
Maize	85	Wheat, cereal	77
Maize + beans	78	Wheat, refined	96
Maize + beans + milk	84	Wheat, whole	86
Meat, fish	94		
Milk, cheese	95		
Millet	79		

Table 9 - Digestibility values of various protein sources as determined by the rat balance method (WHO 1991)

Protein source	Digestibility (%)	Protein source	Digestibility (%)
Beef (roast)	100	Rapeseed protein concentrate	95
Beef salami	99	Rolled Oats	94
Casein	99	Rice-wheat-gluten	93
Corn-pea	83	Rice-soyabean	90
Corn-soybean	93	Skim milk	95
Chicken franks	96	Sausage	94
Egg white solids	98	Soybean	90
Fababean (autoclaved)	86	Soybean protein isolate	98
Lentil (autoclaved)	85	Sunflower meal	90
Macaroni – cheese	95	Tuna fish	97
Pea flour	88	Wheat	93
Pea, Century (autoclaved)	83	Wheat-flour-casein	95
Peanut	96		
Peanut meal	91		
Peanut butter	98		
Pinto bean (canned)	79		
Potatoes – beef	86		

Appendix 2

List of participants

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Appendix 3

Call for experts

FAO Expert Working Group on Protein Quality Assessment in Follow up Formula for Young Children and Ready to Use Therapeutic Foods

Call for experts

As follow-up to a request submitted by the Codex Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) *, FAO is seeking experts to participate in a four day working group session to be held at the FAO Headquarters, Rome, Italy, from 6 to 9 November 2017.

The objective of this working group will be to provide scientific advice on setting up guidelines for Codex members to determine protein quality using the Protein Digestibility–Corrected Amino Acid Score (PDCAAS) in Follow- up formula (FUF) for young children (12–36 months) and Ready to Use Therapeutic Foods (RUTF).

Interested experts should apply by submitting their curriculum vitae (CV) to the FAO Nutrition and Food Systems Division. Before applying, please check the selection criteria, process, and application guidelines detailed here below.

Selection criteria

Applicants should meet the following general criteria:

- advanced University/College degree in nutrition science, food science, or related fields;
- good knowledge of the English language, both written and oral;
- experience with *in vitro/in vivo* models/assays on the digestion and efficiency of utilization of protein and amino acids;
- experience in research and application of the PDCAAS method in assessing protein quality in foods;
- scientific publications in peer-reviewed journals, in particular published in the past ten years;
- ability to prepare scientific documents and to work in an international environment with scientists from various disciplines;
- leadership, or invited participation, in national or international scientific bodies, committees and other expert advisory bodies pertinent to the scope of this work is desirable.

Selection process

FAO places great value on the technical quality and independence of the participating experts as well as on the transparency of its selection process.

Applicants' CV will be reviewed on the basis of the criteria listed above by a selection panel consisting of three or more individuals including at least two independent, internationally recognized external experts appointed by FAO. In selecting experts, FAO will consider, in addition to scientific and technical excellence in the topic of the review, balanced geographic representation, including developing and developed countries, as well as gender. Experts may be requested to assist in the preparation of background papers.

Appointment of experts

The experts will be invited to contribute only in their individual scientific capacity. Experts will not represent their government, nor their institution. Attendance expenses (travel and per diem) will be covered by FAO. No other remuneration is foreseen.

Application guidelines

Interested experts should submit their CV to Dr Warren T K Lee (warren.lee@fao.org), cc.Ms Cristiana Fusconi (cristiana.fusconi@fao.org), by 15 September 2017.

CVs should include a description of education and work experience and a list of peer-reviewed publications relevant to the factors indicated above (please do not include copies of your publications in your submission, unless specifically requested at a later date).

Experts will be asked to indicate in writing any possible conflict of interest (financial and intellectual) that may affect their scientific independence as an expert. For transparency purposes, experts will be required to also indicate their employment (past or present) in any commercial enterprise or private or civil sector association; benefit of research/study grants; shareholdings in commercial enterprises active in fields related to food and nutrition. These declarations will be evaluated and retained by the FAO Secretariat. In addition, experts will be requested to sign a confidentiality undertaking to ensure proper handling of dossiers and information.

Meetings and correspondences will be conducted in English, no translation service will be provided.

All applications should be sent electronically to:

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*Link to the Report of the 38th session of the Codex Committee on Nutrition and Foods for Special Dietary Uses

Consistent with the need to provide safe food for young children, particularly during the complementary feeding period between 12 and 36 months and the period of rapid development to age 59 months, the Food and Agriculture Organization of the United Nations (FAO) convened an Expert Working Group the FAO Headquarters, Rome, Italy, from 6 to 9 November 2017. The meeting addressed questions related to protein quality evaluation in two distinct products used to feed children in different conditions: Ready to Use Therapeutic Food (RUTF) and Follow up Formula for Young Children (FUF-YC). Specific meeting objectives were:

- To determine the appropriate comparative protein or amino acid reference pattern to define protein quality for use in FUF-YC and RUTF.
- To provide guidance on the preferred protein quality assessment methodology that should be stipulated with the standards for FUF-YC and RUTF.
- To provide guidance on the measurement of protein and amino acid digestibility.
- To provide the appropriate reference amino acid profiles and the amino acid composition of common ingredients used for FUF-YC and RUTF.
- To provide cost implications for countries to use PDCAAS in FUF-YC and RUTF.

This report provides future research recommendations including the need to generate data on the true ileal digestibility for different protein sources so that Digestible Indispensable Amino Acid Score (DIAAS) values can be used in the future.

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