# Section II
# Clinical use

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# Whey proteins. Overview of clinical trials

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Le proteine da siero di latte. Rassegna di studi clinici

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**Summary**
Whey proteins, due to their documented biological properties that are exerted by their complex or due to the singular characteristics of their single fractions, have been and continue to be used with success in numerous pathological (e.g. metabolic, cardiovascular, degenerative and infective disorders etc.) and physiological conditions (e.g. sports activities). The purpose of this second section is in fact the presentation of a series of experimental and clinical studies carried out both on animals and in humans, in which the efficacy of whey protein formulations was evaluated in comparison to certain outcomes. Despite what has been done to date, further studies are needed in order to identify a more defined role - in terms of type, dose, administration modality, and pharmacokinetics etc. – of whey proteins in nutritional supplementation or, more precisely, as nutraceutics, especially in pathological situations, in association or in synergy with conventional medical or surgical treatments. Please note that in this section were not taken into account the clinical applications of whey proteins, respectively, in oncology, in asthenic syndrome of autoimmune diseases, in neurology and in cystic fibrosis  at which is dedicated the Section III of this issue.

**Riassunto**
Le proteine da siero di latte, grazie alle documentate proprietà biologiche esibite nel loro complesso o in virtù delle singolari caratteristiche di singole frazioni, sono state e continuano ad essere impiegate con successo in svariate condizioni sia patologiche (es. malattie metaboliche, cardiovascolari, degenerative, infettive ecc.) sia fisiologiche (es. attività sportiva). Scopo di questa seconda sezione è, appunto, la presentazione di una rassegna di studi sperimentali e clinici, condotti sia in modelli animali che sull'uomo, nei quali è stata valutata l'efficacia di formulazioni di proteine da siero di latte rispetto a determinati out come. Molto è stato fatto ma molto resta ancora da fare per l'individuazione di un ruolo sempre più definito – in termini di tipologia, dosaggio, modalità di somministrazione, farmacocinetica ecc. – delle proteine da siero di latte come integratori nutrizionali o, meglio, come nutraceutici, specialmente

in situazioni patologiche, in associazione o in sinergia con i trattamenti medici o chirurgici convenzionali. Precisiamo che in questa sezione non sono state prese in considerazione le applicazioni cliniche delle sieroproteine da latte rispettivamente in oncologia, nella sindrome da astenia da patologie autoimmuni, in neurologia e nella fibrosi cistica in quanto ad esse è dedicata la sezione III del fascicolo.

**Intestinal diseases of particular paediatric interest**

Maintaining an intestinal bacterial flora rich in Bifidobacteria and Lactobacilli in babies that cannot be breastfed is one of the traditional challenges of nutraceutics (1).

In fact, a randomised double-blinded clinical study performed on 102 apparently healthy newborns with an age of no more than 2 weeks demonstrated that the intake of a baby formula enriched with hydrolysed whey proteins is associated, compared to a traditional cow's milk formula, with a significantly greater increase of bifidobacteria in faeces, which is an indicator of an improvement of intestinal immune function (2) which, among other things, seems to be protective against atopic manifestations in subjects due to allergy familiarity (3).

An example of intestinal dismicrobism seems to be represented by episodes of so-called 'gas colic' in babies, notoriously associated with inconsolable crying due to pain. In this case, in a randomised double-blinded clinical trial *versus* placebo, carried out on 43 babies affected by this disorder, the administration of a hypoallergenic formula at 1 week led to a statistically significant reduction, compared to placebo, of the duration of crying episodes (4).

Moreover, significantly loose stools and also a statistically significant increase in the frequency of bowel movements, with positive effects on growth rate, were the effects recorded in a clinical randomised cross-over trial (3 weeks + 3 weeks) where 38 babies with a mean age of 1.7 months suffering from stipsis were given baby formula with hydrolysed whey proteins enriched with prebiotics; the control arm was given a standard paediatric formula. However, it must be noted that the study was completed only by 24 babies (5).

Furthermore, in a preliminary randomized double blinded cross-over study, carried out on 10 children with short bowel syndrome (caused by surgical resection), the addition of a whey protein hydrolysate was not associated with any particular advantage compared to a standard formula, despite these proteins theoretically being recommended more in this condition due to the fact that they prolong the time required for bowel transit (6).

More recent studies have demonstrated how whey proteins, unlike those associated with the casein fraction or with soy, do not cause damage to DNA in colon cells isolated from the colon of rats (7).

Finally, the ability of whey proteins to protect the gastrointestinal apparatus and associated glands from the most common reactive processes seems mainly due to lactoferrin's properties that have been widely described in section I (8).

**Chronic-degenerative diseases associated with malnutrition**

Malnutrition is a common trait of the elderly especially if suffering from neurodegenerative disorders including Parkinson's disease. Due to their nutritional properties

whey proteins are good candidates to improve the metabolism and body mass composition in these individuals.

In a naturalistic prospective study (*Bartolotta et al, 2011, unpublished data*) 255 participants aged 60 to 80 years, suffering from neurodegenerative disorders (e. g. Amyotrophic Lateral Sclerosis, Parkinson's Disease, Alzheimer's disease and so on) or cancer were recruited from a total of 306 patients referred to the artificial nutrition unit Ospedale Nuovo Regina Margherita (Rome, Italy) between November 2009 and June 2010, to undergo whey protein treatment (Prother™) given orally or parenterally (PEG/NGP) once daily 20 to 40 grams/day depending on the severity of underlying disease.

Patients were eligible if they were medically stable, ready for hospital parenteral nutrition and had rehabilitation goals that required at least 12 sessions. They were ineligible if they lived outside of the health region or were unable to undertake the Bioelectrical Impedance Analysis (BIA) measurements. The project was approved by the Research Ethics Committee. All experimental procedures, possible risks and benefits were explained to the subjects before their written informed consent was obtained.

The efficacy was evaluated by measuring three-monthly some biochemical parameters of outcome like serum albumin and by highly innovative BIA device.

Bioelectrical impedance analysis is a simple, non-invasive technique primarily used for body composition analysis. Its ease of use and portability has made it a useful tool for evaluating the nutritional state of the elderly in both clinical and research environments. Bioelectrical impedance analysis uses body resistance (R) and reactance (Xc) to a flow of alternating electrical current to determine impedance and estimates body composition parameters from regression equations derived against a reference method. Phase angle (PhA) is calculated as the arctangent of the ratio of Xc to R (converted to degrees) with values for the majority of people lying between 3° to 15° (9).

In this trial the body composition was qualitatively evaluated according to the patterns of bioelectrical impedance vector analysis (10); resistance (Rz) and reactance (Xc) were directly measured in ohms (U) at 50 kHz using the bioelectrical impedance analyser (mod. EFG3, Akern srl, Firenze, Italy) according to the standard tetrapolar technique (11, 12). The BIA devices were checked at regular intervals against the calibration circuit standard with known resistance and reactance values supplied by the manufacturers to ascertain the accuracy of measurement of resistance and the intactness of the electrodes in ambulatory. Patients were measured in the morning after an overnight fast and in supine position with arms and legs abducted from the body. Source and sensor electrodes were placed on the dorsum of both hand and foot of the right side of the body.

Body weight was always measured at the end of the artificial nutrition therapy session. Body mass index (BMI) was calculated as the ratio body weight/height$^2$ (kg/m$^2$) (13). The 20 kg/m$^2$ cut-off was adopted for the diagnosis of malnutrition as it is the most commonly used in older people (14). Moreover, the 24 kg/m$^2$ level was utilized for the classification of individuals at risk of malnutrition, as suggested by Beck and Ovesen (15).

According to the RXc graph method, resistance and reactance measurements were normalised by the stature of subjects (i.e. R/H and Xc/H, expressed in ohms/m) and vectors were plotted as points on the gender-specific 50th, 75th and 95th tolerance ellipses calculated from the reference, healthy population (16). The vector's length (Z) was calculated according to the equation:

$$|Z| = \sqrt{(R/H)^2+(X_c/H)^2}.$$

The physiological role of PhA has not yet been well defined, but it is

considered a gauge of membrane integrity and intra- and extracellular water distribution. Originally correlated with various physiological variables and used to aid the diagnosis of some metabolic disorders, PhA has more recently shown promise as a prognostic tool and general indicator of health status.
Previous investigations have reported a positive relationship between PhA and recovery in critically ill patients (17) as well as survival times in those undergoing haemodialysis (18), or with HIV (19), lung cancer (20) and cirrhosis (21). Recent studies have also demonstrated that PhA is a strong prognostic indicator in advanced pancreatic (22) and colorectal (23) cancers. This work suggests that PhA may be more sensitive than biochemical markers as a technique to monitor disease progression and treatment efficacy.
In this study – where PhA values of recruited patients were compared with healthy reference values, a sensitive serum indicator of low-level inflammation – on the basis of the currently available data of patients who completed the study (analysis in progress) BIA data showed a trend of a shift of the mean Z vector in patients with severe impaired functionality (i.e. Alzheimer, Amyotrophic Lateral Sclerosis, Parkinson disease) with a further reduction of the phase angle and shortening of the modulus, thus indicating a bad nutritional status and a tendency to hyperhydration that was partially attenuated by whey protein therapy as given even by PEG/NGB. Further controlled studies must confirm these encouraging findings.

**Cardiovascular diseases**

Whey proteins can be useful in the control of arterial blood pressure and in the normalisation of lipemia. In this respect, in an 8 week preliminary study carried out on a group of healthy adult male volunteers (n=20) the effect of supplementation with fermented milk (containing *Lactobacillus casei* and *Streptococcus thermophilus*) to which a whey protein concentrate was added was evaluated comparatively, whereas in controls only fermented milk was used. In both cases milk was administered twice a day (24). At the end of the trial in the test group a lowering of the arterial blood pressure compared to basal pressure, an increase of HDL cholesterol concentration and a reduction of triglyceridemia were observed, effects that were all statistically significant. Differences in LDL cholesterol levels were not observed.

**Obesity**

Whey proteins represent an interesting source of nutrients in diet regimens proposed together with physical exercise to control obesity. In fact, due to their numerous metabolic effects, they can favour weight loss, thus improving the body mass index (25).
In a randomised double-blinded study, 106 female obese subjects aged between 25 and 50 on a calorie restricted diet (500 kcal/day), were invited to take a whey protein drink (with high levels of leucine, bioactive peptides and calcium) (n=53) twice a day for 12 weeks whereas the control arm (n=53) was asked to take a drink containing maltodextrines and less calories than compared to the previous one (26). Those subjects that had a weight loss of at least 2.25 kg were considered as being responders. At the end of the study a significant loss of weight was observed in both groups, but in the group that had taken whey proteins there was a greater reduction of fat mass (2.81 kg versus 1.62 kg in the overall group, equal to -6.1%, and 3.63 kg versus 2.11 kg in the subgroup of responders) with a more contained reduction of muscle mass than compared to controls (1.07 kg versus 2.41 kg, respectively). These effects were accompanied, again in the test group, by a better metabolic profile in terms of cholesterol and triglycerides levels.

**Diabetes**

In healthy subjects whey protein intake is associated with a reduction of post-prandial glycaemia levels that correlate with a stimulation of insulin release (27).
Similar effects are seen in patients with type 2 diabetes mellitus. In fact in a clinical trial glycaemia and insulin response were measured in a mixed group of in total 14 men and women with type 2 diabetes (aged between 27 and 69) after eating a high glycaemic index meal with or without the addition of whey proteins (28). Taking whey protein supplementation at breakfast was accompanied by significantly higher glycaemia levels (+21%) and an equally significantly higher insulinemic level (+31%) than compared to controls. Furthermore, whey protein intake, compared to the high glycaemic index meal alone, was accompanied by an increase in the response to insulin and to the glucose-dependant insulino-tropic polypeptide. Overall, these results indicate that whey protein intake, by positively interfering with the glucose metabolism, can be useful in controlling diabetes.
Later preclinical and clinical studies suggested that whey proteins are not only capable of reducing postprandial glucose levels and of stimulating insulin release both in normal and in type 2 diabetes patients but in diabetics they can also reduce dipeptydyl-peptidasic 4 activity (DPP-4) in the proximal intestine and therefore, increase levels of intact incretin. As recently demonstrated, this effect seems to be due to the ability of β-lactoglobulin to generate, through hydrolysis, peptides such as the tripeptide Ile-Pro-Ala that have an inhibitory effect on DPP-4 (29).

**Osteoporosis**

Due to the elevated calcium bioavailability that milk can guarantee, milk is considered as one of the pivotal food stuffs in the prevention of osteoporosis (30). It is reasonable to hypothesise, however, that also other milk components may be involved in this effect, among which foremost proteins, which are heavily needed by the bone matrix.
In this respect, one of the first baby formulas to be tested, was the so-called milk basic protein (MBP) a whey protein concentrate purified through ion exchange chromatography, composed mainly of lactoferrin and lactoperoxidase, that is capable *in vitro* of stimulating osteoblast proliferation and differentiation and of inhibiting bone reabsorption (31-34).
Later *in vivo* studies demonstrated that MBP but also a normal whey protein preparations can strengthen the structure of the femur bone in previously ovariectomised young rats (35-37). Instead, in an experimental periodontitis model an increase in alveolar bone was observed (38).
As far as humans are concerned, the intake of a drink containing 0.3 g of MBP for 16 days in 30 adult subjects led to an increase in serum osteocalcin levels and in the levels of procollagen I carboxy-terminal pro peptide (PICP), considered as biochemical markers of osteon formation (39). Similar results were recorded in a similar study performed on 33 adult women versus placebo after taking 40 mg BMP (considered by authors the equivalent of 400-800 mL of whole milk); in this case a significant reduction in urinary excretion of cross-linked N-telopeptides was also observed and these are considered markers of osteoclastic activity (40). Again, in a double-blinded study, 40 mg of BMP a day for 6 months were able to increase radial bone density in 30 adult women (41). In premenopausal women, BMP intake was associated with an increase of bone density which can be attributed to both an increase in formation processes and a reduction of bone reabsorption (42). However, subsequently, also other whey protein fractions were identified that are biologically active on the bone and these are the so-called acidic proteins. In a study performed on 6 month old ovariectomised rats, the

ingestion of these components of cow's milk taken in a dosage of 3 g/kg/day for 4 months was associated, compared to controls where animals were not ovariectomised, with an improvement in mechanical performance, bone density and with a reduction in plasma levels of collagen I telopeptide C (43). Finally more recently, a whey protein formulation was accompanied by a premature increase of IGF-1 in elderly women with a recent hip fracture (44).

**Viral hepatitis**

Nutritional whey protein supplementation can represent an important tool in the prevention and treatment of viral forms of hepatitis. Initial *in vitro* studies had documented that bovine lactoferrin was capable of inhibiting HCV infection in a human cell line of hepatocytes (45), whilst whey proteins resulted as being efficient in preventing hepatic damage due to D-galactosamine in a rat model (46).
Therefore, a pilot trial carried out on 11 patients with chronic hepatitis C recorded a significant reduction in ALT and viral RNA levels (HCV) only in those subjects with a low viral load after a daily 8 week treatment with bovine lactoferrin at a dosage of 1.8 or 3.6 grams/day (47).
Instead, results that were more difficult to interpret emerged from a dose-response study with 1.8, 3.6 and 7.2 grams of lactoferrin a day (48) whilst in another open trial the intake of 12 g of whey protein twice a day for 12 weeks led to a reduction in serum lipid peroxide levels only in patients with HBV (and not in those with HCV) associated with an increase of IL-2 and NK activity (49).
It is clear that further studies are needed to better understand the role of whey proteins or of their single fractions in association with the conventional treatment of viral forms of hepatitis.

**HIV infection**

Glutathione deficiency is common in subjects with HIV. A study demonstrated significantly higher GSH levels in 30 subjects with HIV undergoing nutritional supplementation with 45 g of whey proteins a day for 2 weeks (50). The authors of the same study in another 6-week trial yet again found significantly higher GSH levels than compared to basal levels (51).

**Sports and exercise**

An adequate equilibrium between protein synthesis and degradation is essential not only for the correct maintenance of an adequate lean mass but also for good muscle performance, particularly, but not only in subjects that do a lot of sport. It is in this field that whey proteins have long been used with success.
In acute conditions for example, whey protein intake was accompanied, compared to casein and placebo, by a stimulation of the net muscle protein synthesis and by a significant increase in the capture of phenylalanine immediately after physical strength training leg exercise in a group of 25 young peoples (52).
These effects were in some way confirmed very recently in a double blinded cross-over study carried out on 10 trained cyclists (aged 29 ± 6 years) that randomly completed two 90 minute tests, drinking a drink containing 25 g of carbohydrates alone or with the addition of 10 grams of whey protein immediately before and 30 minutes after exercise. In the subjects who had taken whey proteins an increase in myofibrillar muscle proteins was observed and this increase was greater than that observed in the control group; this effect was also accompanied by a significant increase in insulinemia (53).
Similarly, in 26 apparently healthy body builders that had taken whey proteins at a dosage of 1.5 g/kg a day *vs.* carbohydrates, muscle strength significantly increased at 11 weeks and this was associated

with signs of hypertrophy (54).
Instead in another double-blinded study 42 male weightlifters aged between 18 and 21 underwent a 12 week resistence programme and were asked to take whey proteins (at a dosage of 1.2 g/kg of body weight) or a specifically formulated supplementation for sport activity containing whey proteins (at a dose of 1.3 g/kg of body weight) to which other ingredients had been added or a maltodextrine placebo formulation. At the end of the trial whey protein intake, compared to placebo, was associated with an improvement of lean mass and of the score related to at least one of the 4 parameters related to muscle strength (55).
In 2 preliminary studies performed on HIV positive women, the intake of a non-denatured whey protein formulation, at a dosage of 1.0 g/kg of body weight as part of a physical exercise programme was associated with an improvement in quality of life and a tendency to do more sport with however, non-univocal effects on lean mass (56, 57).
Peak muscle strength and work performance at 30 seconds improved significantly compared to basal conditions in 18 apparently healthy subjects that had taken a whey protein formulation (10 grams, twice a day) for 3 months. These effects were not observed following the ingestion of casein, used as the control arm. It can be noted that body weight remained unvaried in all recruited subjects whereas in the test group a percentage reduction of fat mass was recorded and worthy of mention also an increased intracellular glutathione synthesis which is probably, at least in part, responsible for the improvement in muscle performance was observed (58).
This effect opens the way to important considerations on the health implications of physical exercise. In fact, whereas moderate physical exercise stimulates the immune system (59) and allows exuberant reactive processes such as inflammation and production of oxygen reactive species to be kept under control, intense and prolonged training programmes, especially in untrained subjects, can lead to extremely negative effects mainly due to oxidative stress (sports paradox) (60-65) which is contrasted within cells by GSH whose levels of its reduced form which is functionally active are reduced after intense physical exercise (64). Furthermore, overtraining is accompanied by an increased frequency of infections that is associated with lower levels of secretory IgA and glutamine (66-68), a mediator involved in gastrointestinal functions and of which whey proteins have a reasonable amount (69).
In conclusion, whey proteins, notwithstanding certain advertising campaigns proposing an incorrect image of fitness in gyms, represent a useful ally in diets of nutritional regimens associated with correct physical activity as they are capable of i) positively modulating muscle protein synthesis and therefore, of maintaining or potentiating lean mass to the detriment of fat mass and of ii) keeping oxidative stress under control.

## Other whey protein clinical applications

The use of whey proteins or single fractions of whey proteins is slowly but progressively growing in a series of clinical conditions. For example, lactoferrin can lead to an improvement of immune response that is impaired after major surgery (70) whilst complete whey protein formulations are potentially useful in favouring the healing of post-surgical lesions, due to precious essential aminoacids (71). Studies carried out on Humans demonstrate that whey proteins improve cognitive function and the ability of subjects to cope with high levels of emotional stress (72, 73) and since it is believed that coping with emotional stress is improved by serotonin it has been hypothesised that the effect is mediated by the relatively high levels of tryptophan in these proteins, especially α-lactalbumin (74) (see also Section I).
In the various clinical trials con-

sidered, whey proteins were used in humans at doses between 30 and 90 grams a day depending on the clinical condition being treated. Treatment was always well-tolerated apart from a few gastrointestinal disturbances (74) and only one case of hepatotoxicity has been reported in literature (which was due to a continued treatment associated with creatine) (75).
Clearly, despite casein having allergizing activity, whey proteins are not recommended in subjects who are intolerant to cow's milk (76). Lactose intolerance however, does not limit their use, if the formula is delactosed (es. Prother™).

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