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Low-dose whey proteins supplementation can reduce oxidative stress in cystic fibrosis. A 36 months preliminary clinical trial

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Titolo
La supplementazione con proteine da siero di latte può ridurre lo stress ossidativo nella fibrosi cistica. Studio preliminare della durata di 36 mesi

Key words
Cystic fibrosis, oxidative stress, whey protein, GSH, d-ROMs test, BAP test

Parole chiave
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Summary
Cystic Fibrosis (CF) is the most common lethal autosomal-recessive disorder in the Caucasian population. The pulmonary disease is the most relevant clinical expression of CF. Cystic fibrosis airways are under the effect of oxidants that are generated from increased inflammation and infections present in the lungs. Low glutathione (GSH) levels in epithelial lining fluid and neutrophils/blood plasma can impair antioxidant defences thus magnifying the oxidative stress in patients suffering from CF. On these basis increasing or restoring normal GSH levels has been proposed as a potentially useful strategy in CF. Cow's milk whey proteins because its high levels of cysteine and cystine, that are both precursors of endogenous GSH synthesis, can increase tissue levels of GSH as required in CF. Therefore the primary aim of this study was to evaluate the efficacy of a diet supplementation with a cow's milk whey protein formula (WPF) on oxidative stress in CF patients. The secondary goal was to assess the effects of this supplementation on clinical status and its tolerability. This is a prospective, open, clinical study conducted among CF patients in regular follow up at CF Centre of Genova, Italy in the period 2007-2010. Participants received whey proteins with WPF, which was administered orally once a day. This study deals with 59 patients. Mean follow up period was 18 months (range 3-36). Oxidative balance was evaluated as BAP test and d-ROMs test while improvement of respiratory function was assessed as FEV_1% predicted for sex and age. In our data stress oxidative status showed to be favourably influenced by WPF administration as a matter of fact mean BAP value increased, even if in a not significantly way and, mainly, the d-ROMs value significantly decreased after WPF intake. Unfortunately oxidative stress condition in our data is not really associated with clinical status improvement. Conclusively we can say that, a nutraceutical formula, as WPF, has demonstrated a real effect on improving oxidative stress in CF patients and is well accepted and well tolerated.

Riassunto
La fibrosi cistica (FC) è la malattia genetica, autosomica recessiva, a prognosi infausta più frequente nella razza caucasica. L'espressione clinica più importante della FC è la malattia polmonare. Le vie aeree dei pa-

zienti con la FC sono soggette a sostanze ossidanti prodotte dai processi infiammatorio e infettivo cronici presenti a livello polmonare.
Inoltre nei pazienti affetti da FC il glutatione (GSH) presente nel fluido epiteliale bronchiale e nei neutrofili circolanti è notoriamente ridotto e tale condizione può compromettere le difese antiossidanti peggiorando lo stress ossidativo. Su queste basi ripristinare le normali concentrazioni di GSH rappresenta una strategia terapeutica utile in FC. La somministrazione di proteine di siero di latte vaccino dato l'alto contenuto di cisteina e cistina, che sono entrambe precursori del GSH, può determinare l'aumento dei livelli tissutali di GSH nella FC. Pertanto, l'obiettivo primario di questo studio è stato valutare l'efficacia di una supplementazione orale con una formula a base di proteine del siero di latte vaccino nel migliorare lo stress ossidativo. L'obiettivo secondario è stato verificare gli effetti di tale integrazione sulle condizioni cliniche e la sua tollerabilità. Questo studio clinico, prospettico, in aperto, è stato condotto nel periodo 2007-2010 su pazienti affetti da FC in regolare follow-up presso il Centro FC di Genova, Italia. Ai soggetti arruolati è stata somministrata per os, una volta al giorno, una formula a base di proteine di siero di latte vaccino. Sono stati arruolati 59 pazienti, la durata media dello studio è stata di 18 mesi (range 3-36). Lo stress ossidativo è stato valutato mediante i test d-ROMs e BAP mentre la funzionalità polmonare è stata valutata come FEV_1% del predetto per età e sesso. I nostri risultati dimostrano che il bilancio ossidativo è stato influenzato positivamente dalla integrazione con proteine di siero di latte. Il valore del BAP test, infatti, è aumentato anche se in modo non statisticamente significativo mentre il valore del d-ROMs test è diminuito in modo statisticamente significativo. Dai nostri dati purtroppo non emerge l'associazione tra il miglioramento dello stress ossidativo e il miglioramento clinico respiratorio. In conclusione possiamo affermare che la integrazione orale con una formula nutraceutica a base di proteine da siero di latte vaccino si è dimostrata efficace nel migliorare la condizione di stress ossidativo nei pazienti con FC ed è stata ben accettata e tollerata.

Introduction

Cystic fibrosis (CF) is the most common lethal autosomal-recessive disorder in the Caucasian population: its frequency is calculated about 1/3500 newborns (1). Cystic fibrosis is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Its main clinical manifestations include chloride and sodium increased concentration in the sweat, exocrine pancreatic insufficiency and, above all, chronic lung disease that, ultimately, may lead to severe pulmonary function failure and death (2).

Although the cause of CF is well established, the pathogenesis of lungs damage is still not fully understood and reactive biological processes such as inflammation and oxidative stress (OS) both triggered or worsened by recurrent bacterial infections as well as by malnutrition has been postulated to play a relevant role (3, 4).
According to the widest reported definition OS is the direct result of an unbalance between the production and the elimination – by antioxidant defences, like glutathione (GSH) – of oxidant chemical species, including reactive oxygen species (ROS) (5). This unbalance leads to cell and tissue damage and, ultimately, to more than one hundred diseases although it is not easy to establish whether oxidants are the cause or the effect of the observed biochemical abnormalities (6).
Cystic fibrosis airways are under the effect of oxidants that are generated from increased inflammation and infections that are present in the lungs (7). The CF mutations, moreover, cause a primary dysfunction in the system of one of the body's most important antioxidant and immune-signalling molecule: the reduced glutathione (GSH) (8, 9). Cystic Fibrosis mutations, in fact, significantly decrease GSH efflux from cells; this leads to deficiency of GSH in the epithelial lining fluid of the lung, as well in other compartments, including immune system cells and the gastrointestinal tract (10, 11). On the other hand low GSH levels in epithelial lining fluid and neutrophils/blood plasma can impair antioxidant defences thus magnifying the OS in patients suffering from CF.
On these basis increasing or restoring normal GSH levels has been proposed as a potentially useful strategy to counteract OS in CF and possibly to influence favourably its progression (12, 13). Since GSH is a peptide that if given orally is broken to its amino acids (e. g. glutamate, cysteine and glycine) by gastrointestinal secretions, different strategies have been used to generate endogenously this antioxidant, including N-acetylcysteine and whey proteins (14). In particular cow's milk whey proteins because its high levels of cysteine and cystine, that are both precursors of endogenous GSH synthesis, can increase tissue levels of GSH as required in CF (15).
In a previous preliminary report our group showed the ability of a low-dose whey protein formula to reduce OS in breast cancer patients as measured by means the determination of both total oxidant capacity (d-ROMs test) and biological antioxidant potential (BAP test) on blood plasma (16). Moreover a very recent study demonstrated that glycine and cysteine supplementation in type 2 diabetes is able to improve erythrocyte GHS synthesis and to reduce d-ROMs test values (17).
Therefore the primary aim of this study was to evaluate the efficacy of a diet supplementation with a cow's milk whey protein formula (WPF) on OS in CF patients. The secondary goal was to assess, in the same individuals, the effects of this supplementation on lung function and its tolerability.

Materials and methods

This was an open prospective clinical trial conducted among CF patients in regular follow up at CF Centre of Genova (Italy) in the period 2007-2010. To be eligible for the study, patients had to have a confirmed CF diagnosis and to be >10 years old. Patients with kidney failure or cirrhosis of the liver, cow milk allergy or ongoing O2 therapy were considered not eligible.
Patients suffering from CF who met inclusion and exclusion criteria were randomly assigned to receive a lactose/lipid free enriched-cystein WPF (Prother™), which was administered orally once a day at the dosage of 10 g/die in patients < 12 years old or 20 g/die in patients > 12 years old. The participants received adequate training for a correct intake of WPF,

which is formulated as powder contained in a small bags.

Primary endpoint of the study was the improvement of oxidative status, evaluated as d-ROMs test (18) and BAP test (19), while improvement of respiratory function, evaluated as percentage of FEV_1 predicted value for gender, age and height was the secondary endpoint.

d-ROMs test and BAP test (Diacron International, Grosseto, Italy) were performed on plasma from capillary blood samples by using a dedicated photometer (20).

The d-ROMs test measures the oxidant ability of a plasma sample towards N,N-diethylparaphenylendiamine as used as an indicator (chromogen) (18). The phenomenon is associated with the progressive and gradual color change to pink reaction mixture (plasma + chromogen), initially colourless (18). The colour change is measured photometrically as absorbance variation/min at 505 nm (18). Especially alcoxyl e hidroperoxyl radicals, derived from hydroperoxides (ROOH), contribute to determine the oxidizing capacity measured by d-ROMs test (18). The results are expressed as CARR U, where 1 CARR U is equivalent to 0.08 mg/dL of hydrogen peroxide (21, 22). Normal range was established between 250 and 300 CARR U (21).

The BAP test for the determination of biological antioxidant potential is a photometric test that allows to determine the plasma concentration of antioxidants in their sense of agents capable of reducing the iron from ferric to its ferrous form (19). So conceived BAP test provides an overall measure of many antioxidants such as bilirubin, uric acid, vitamins C and E and proteins. Optimal value is considered > 2200 µmol/L of reduced iron by using vitamin C as a standard (19).

d-ROMs test, BAP test, and FEV_1 were evaluated at the moment of study entry (T=0) and then every three months. FEV_1 values were also collected quarterly in the year before T=0.

For each eligible patient, data on demographic and clinical features at study entry were collected. In more details, exocrine pancreatic insufficiency was defined as any need of pancreatic enzymes replacement; abnormal glucose metabolism was defined by the presence of two abnormal oral glucose tolerance tests in the last six months and *Pseudomonas aeruginosa* airway chronic colonization was defined by the presence of more than 50% positive isolates in the previous year (at least four).

All the study patients continued their CF standard treatment, vitamin supplementation included. Diet was free. No other calorie supplement was prescribed during the study. Any other therapy prescribed during the follow-up period by the physician responsible for the CF Centre was allowed, except for antioxidant agents.

Patients also had to fill in a complete four days dietary diary at T=0 and after 12 months of assumption, in order to calculate the calorie intake.

Finally adherence to the WPF prescribed supplementation, during the follow up period, was evaluated on the basis of anamnestic data.

Any adverse event or clinical symptom had to be promptly reported and, anyway, it was collected during the quarterly control.

The study duration was scheduled for a period of 36 months (T=+36). Enrolment was possible until 33 months from the beginning. Patients had to be followed until the end of the study or until the evidence of WPF intolerance or any other event no compatible with the study protocol.

Statistical analysis

Raw data were expressed as mean ± SD and/or SE. Firstly, a descriptive analysis of single variables was performed, using unpaired Student t test for comparisons between subgroups means and CHI square test for proportions.

The mean annual values of FEV_1% either before and after and

of BAP, d-ROMs tests variation after the beginning of WPC intake were assessed as an estimated average of linear regression beta coefficients of single patients. The null hypothesis of mean = 0 was tested by an unpaired data t test. The difference between FEV_1% trends before and after WPC intake, was assessed with comparison between means of respective (before and after supplementation) linear regression beta coefficients within patients by a paired data t test.

For the subgroup analysis, all the comparisons between the mean annual values of FEV_1%, BAP and d-ROMs tests variation (linear regression beta coefficients) were performed by paired data t tests. Statistical significance was at 95% level.

Further analysis was exploited by comparison of the mean variations of FEV_1%, BAP and d-ROMs tests after 12 and 18 months of WPF supplementation (t12 – t0 and t18 – t0), in all patients as a whole and in subgroups of FEV_1% < OR >40%, by t unpaired tests.

Results

In the study period WPF intake was proposed to 73 patients. Twelve of them decided not to participate to the trial because the palatability of WPF. After a few weeks from the beginning of the study one patient died for acute respiratory distress and one patient was transferred to another CF Centre. Therefore this study dealt with 59 patients, i.e. 36 males and 23 females.

Demographic, clinical, instrumental and laboratory data at the study entry are shown in Table 1.

No difference is shown between males and females. FEV_1, d-ROMs test, and BAP test values, at the moment of study entry, were not significantly correlated.

Mean follow up period was 18 months (range 3-36). All the patients concluded the study, except 10 patients. One patient dropped out at T=+9 because he was submitted to lung transplantation. The other 9 patients stopped WPF intake voluntary for low compliance (1 patient at T=+6, 2 patients at T=+9; 1 patient at T=+12; 2 patients at T=+15; 2 patients at T=+21; 1 patients at T=+33).

Thirty-five patients completed 12 months and 20 patients completed 18 months of follow up.

Results about the mean estimated annual values of the considered parameters (d-ROMs test, BAP test, and FEV_1%) as evaluated in all the patients in the study with available data are shown in Table 2.

Mean FEV_1% annual estimated variation value before T=0 was significant (-1.61; 95% CI -3.13/-0.09). Mean FEV_1% annual estimated variation value after T=0 was not significant (-1.69; 95% CI -4.05/0.66). The difference between the year before and the year

Table 1 - Demographic data of patients at the study entry.

	Male	Female	Male + female	P
Patients (N)	36 (61%)	23 (39%)	59	
Age (yrs, mean ± SD)	26.0 ± 10.0	29.0 ± 12.0	27.0 ± 13.3	0.3
Pseudomonas aeruginosa colonization (N, %)	16/36 (44.5%)	15/23 (65.2%)	31/59 (52%)	0.2
Abnormal glucose metabolism (N, %)	11/36 (30.5%)	12/23 (52.2%)	23/59 (38%)	0.2
FEV_1% (mean ± SD)	55.9 ± 21.8	53.2 ± 23.4	55.0 ± 22.3	0.62
d-ROMs test values (CARR U, mean ± SD)	380.3 ± 79.4	416.4 ± 111.8	394.0 ± 94.0	0.15
BAP test values (μmol/L, mean ± SD)	2527.7 ± 602.8	2488.5 ± 558.3	2520 ± 579	0.8

after T=0 was again not significant (-0.23; 95% CI -3.14/2.68).
Mean d-ROMs test estimated annual values after T=0 significantly decreased (-35.0; 95% CI. -52.76 /-17.14 p = 0.0003) while mean BAP test estimated annual values after the start of WPF administration (T=0) increased, but not significantly (17.3; 95% CI -218.6/253).
Mean estimated annual variations of the considered parameters on the basis of gender, abnormal glucose metabolism, and *Pseudomonas aeruginosa* airway chronic infection at study entry are compared in Table 3.
Because no significant differences were detected, searching for a more satisfying interpretation of

Table 2 - Mean estimated annual variations of the considered parameters in all the patients study with available data.

	Pts (N)	Mean value	SD	SE	Lowest value	Highest value	p value
FEV_1% variation before T=0	58/59	-1.61	5.78	0.76	-3.13	-0.09	0.04
FEV_1% variation after T=0	57/59	-1.69	8.89	1.18	-4.05	0.66	0.16
Difference FEV_1% variation before/after T=0	56/59	-0.23	10.87	1.45	-3.14	2.68	0.88
BAP test results variation after T=0	56/59	+17.29	880.49	117.66	-218.60	+253.19	0.88
d-ROMs test results variation after T=0	56/59	-34.95	66.47	8.88	-52.76	-17.14	0.0003

Table 3 - Mean estimated annual variations of the considered parameters compared on the basis of gender, abnormal glucose metabolism, and *Pseudomonas aeruginosa* airway chronic infection at study entry.

	FEV_1%			BAP test values			d-ROMs test values		
	n	Mean value (IC 95%)	p	n	Mean value (IC 95%)	p	n	Mean value (IC 95%)	p
Gender									
F	21	-0.84		20	-34.87		20	-28.42	
M	36	-2.19		36	46.27		36	-38.58	
Difference		1.35 (-3.18 to 5.89)	0.55		-81.15 (-501.8 to 339.51)	0.70		10.16 (-29.81 to 50.12)	0.61
AGM*									
Yes	23	-3.24		22	259.75		22	-45.50	
No	32	-0.72		32	-128.63		32	-27.88	
Difference		-2.52 (-7.37 to 2.33)	0.30		388.38 (-120.96 to 897.72)	0.13		-17.62 (-56.24 to 21)	0.36
Pa ACI**									
Yes	30	-1.91		29	83.29		29	-33.75	
No	26	-1.34		26	-46.89		26	-36.64	
Difference		-0.57 (-5.56 to 4.42)	0.82		130.19 (-370.29 to 630.67)	0.60		2.88 (-34.1 to 39.86)	0.88

*Abnormal glucose metabolism; ** *Pseudomonas aeruginosa* airway chronic infection.

these findings, patients were divided in 2 groups with regard to FEV_1% value at the starting of WPF intake, i. e. group 1 (patients with FEV_1% value ≤ 40%; N=17) and group 2 (patients with FEV_1% value >40%; N=40); this is in agreement with international literature where FEV_1%=40 has been considered the threshold value of severe respiratory insufficiency.
Mean estimated annual variations of the 3 considered parameters (FEV_1%, d-ROMs test and BAP test) in Group 1 and Group 2 are shown in Table 4.
Significant differences where therefore found between Group 1 and Group 2 about FEV_1% and d-ROMs test values.
When the considered parameters were analysed only in those patients who completed 12 months of supplementation a significant reduction of d-ROMs test mean value was confirmed as reported in Table 5.
Similar results were observed by expanding the analysis to 18 months of follow-up, as reported in Table 6 where a significant increase of BAP test value was also detected.
Daily caloric intake was evaluated in 28 patients. It did not significantly decreased in 10/28 patients and did not significantly increased in the remaining 18/28 patient when the comparison was made between T=0 and T=+12 (-58 Kcal/Kg/die, range -4/-147, and +117 Kcal/Kg/die, range 19/312 respectively).
The adherence to WPF intake in the patients who accepted to start the treatment was very good. Also

Table 4 - Mean estimated annual variations in Group 1 and Group 2.

	FEV_1%			BAP test values			d-ROMs test values		
	n	Mean value (IC 95%)	p	n	Mean value (IC 95%)	p	n	Mean value (IC 95%)	p
FEV_1% T = 0									
FEV_1% ≤ 40	17	2.42		16	344.10		16	-72.92	
FEV_1% > 40	40	-3.44		40	-113.43		40	-19.76	
Difference		5.86 (1.13 to 10.59)	0.016		457.54 (-136.38 to 1051.45)	0.13		-53.16 (-92.76 to -13.57)	0.009

Table 5 - Outcomes before and after 12 months of WPF supplementation.

Parameter	N	Before WPF		12 months after WPS		After vs. before		p value
		Mean	SD	Mean	SD	Mean	SD	
• FEV_1%	35	57.2	21.5	58.9	23.9	1.7	15.2	0.51
• d-ROMs	35	419.1	122.8	358.9	105.9	-60.1	104.6	0.00
• BAP test	35	2525.9	737.4	2691.0	616.8	165.0	736.7	0.20

Table 6 - Outcomes before and after 18 months of WPF supplementation.

Parameter	N	Before WPF		18 months after WPS		After vs. before		p value
		Mean	SD	Mean	SD	Mean	SD	
• FEV_1%	20	58.3	20.1	59.2	24.7	0.9	16.2	0.81
• d-ROMs	20	426.9	116.3	334.4	117.1	-92.5	100.0	0.00
• BAP test	20	2329.5	769.1	2839.3	859.3	509.8	920.7	0.03

those patients who voluntary dropped out the study, regularly assumed it until their defection.
No significant adverse events were reported.

Discussion

Many experiences of antioxidant therapy in CF are reported in the recent international literature without any conclusive results (23). One study showed that oral N-acetylcysteine, a GSH prodrug, because it acts as a cysteine donor, decreases neutrophil elastase in CF sputum (24). There are also some positive experiences in literature about the use of inhaled GSH in CF (25-27). Hartl et al. demonstrated that GSH for inhalation in 17 CF patients caused PGE_2 reduction as evaluated in broncho-alveolar liquid but not affected oxidative status (28).
Recently it was reported that 10 CF patients were treated for 56 days with an antioxidant formulation containing beta carotene, gamma tocopherol, coenzyme Q_{10}, vitamin D_3 and vitamin K (29). An increase of circulating antioxidants and a decrease of myeloperoxidase in sputum, without any change in FEV_1 and in sputum bacterial count were observed.
In the antioxidant's field the use of an oral cow's whey proteins has been more and more pointed out. Pressurized whey proteins can increase lymphocyte GSH level in healthy individuals at the dosage of 45 grams/die for 15 days as described in an open-label dose response study (15).
In a prospective double blind clinical trial an oral supplementation with whey proteins increased plasma GSH levels in HIV infected patients (30).
Mancuso et al. in a double-blind cross-over study in 27 patients affected by mitochondrial myopathies and 42 controls demonstrated a significant reduction in oxidative stress levels after a 30-days supplementation with a whey based cysteine donor (31).
According to the available literature antioxidant therapy with whey proteins seems not provide conclusive results, as a whole, about its efficacy in CF disease.
A whey protein isolate administered for three month in 21 CF patients in a blind controlled clinical trial caused increasing in GSH level in lymphocytes but no change in FEV_1 and nutritional status (32).
Finally, in a recent pilot open-label study of 1-month dietary supplementation with pressurized whey, conducted on 24 CF patients, whey proteins seemed to improve nutritional status and to increase predicted FEV_1% in children (33).
The goal our study was to investigate the impact of a oral nutraceutical formula based on cow's milk whey proteins with a high content of cysteine on a series of 59 CF patients followed up 36 months.
At our knowledge no previous study about this kind of antioxidant supplementation, dealt with so a large number of enrolled patients followed for so a long time period.
According to our data oxidative balance – that was the first time measured with d-ROMs and BAP test in CF patients – showed to be favourably influenced by WPC administration. As a matter of fact, after WPC intake d-ROMs values significantly decreased while BAP test values significantly increased as expected even if only after prolonged therapy. In this object we should consider that d-ROMs test values showed higher than the normal range in nearly all the enrolled patients while BAP values at study entry were substantially normal in our patients.
Noticeably – although the heaviness of statistics appeared not so high – we observed that patients of group 1, the same patients with a significantly greater d-ROMs decrease, showed a significant better FEV_1% outcome compared with patients of group 2. We are not able to explain, on the basis of the current knowledge, this apparent greater impact of WPC treatment in our patients with more

severe clinical condition. Pharmacokinetic study are in progress to explore this interesting findings (*data not shown*).

Our data showed a satisfying acceptance of WPC by our CF patients. In fact, the nearly wholeness of patients accepted to assume the supplementation and showed a good adherence during all the study period.

Finally this study demonstrated a really good tolerability of WPC because no adverse event was registered. In particular a reduction of daily caloric intake, as reported in other studies was not observed in our patient.

Unfortunately we did not measured GSH levels and this can be a limit of this trial. However other studies already demonstrated this effect (17, 30, 32, 33) and we were aimed to use a more suitable approach to measure OS in clinical practice. In fact dosing of GSH requires more complicate equipment than a common photometer that we used directly in our ambulatory with a real-time analytical answer.

In conclusion, the intake of a lactose/lipid free cystein-enriched WPF was safely associated to an improvement of OS in CF patients as evaluated at a glance. Taken together these findings suggest that this formula can be prescribed without problems, also in patients with such a heavy therapeutic daily protocol. Of course the lack of a clear significance in improving clinical status in our patients must be studied in a randomized controlled clinical trial, in order to correct the possible bias linked with the progressive worsening of the clinical condition in CF disease.

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