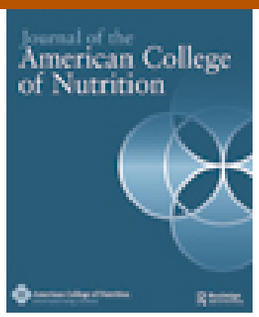

Journal of the American College of Nutrition

ISSN: 0731-5724 (Print) 1541-1087 (Online) Journal homepage: http://www.tandfonline.com/loi/uacn20

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To cite this article: Bruna Guida, Mauro Cataldi, Andrea Memoli, Rossella Trio, Martina di Maro, Lucia Grumetto, Ivana Capuano, Stefano Federico, Antonio Pisani & Massimo Sabbatini (2017): Effect of a Short-Course Treatment with Synbiotics on Plasma p-Cresol Concentration in Kidney Transplant Recipients, Journal of the American College of Nutrition, DOI: 10.1080/07315724.2017.1334602

To link to this article: http://dx.doi.org/10.1080/07315724.2017.1334602

Published online: 12 Sep 2017.

JOURNAL OF THE AMERICAN COLLEGE OF NUTRITION
https://doi.org/10.1080/07315724.2017.1334602

Effect of a Short-Course Treatment with Synbiotics on Plasma p-Cresol Concentration in Kidney Transplant Recipients

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ABSTRACT

Objective: We evaluated whether a short-term course with synbiotics may lower plasma p-Cresol concentrations in kidney transplant patients (KTRs) who accumulate this uremic toxin both because of increased production by their dysbiotic gut microbiome and because of reduced elimination by the transplanted kidneys.

Methods: Thirty-six KTRs (29 males, mean age 49.6 ± 9.1 years) with transplant vintage > 12 months, stable graft function, and no episode of acute rejection or infection in the last 3 months were enrolled in this single-center, parallel-group, double-blinded, randomized (2:1 synbiotic to placebo) study. Synbiotic (Probinul Neutro, CadiGroup, Rome, Italy) or placebo was taken at home for 30 days, as 5 g powder packets dissolved in water three times a day far from meals. The main outcome measure was the decrease in total plasma p-Cresol measured by high-performance liquid chromatography at baseline and after 15 and 30 days of placebo or synbiotic treatment.

Results: After 15 and 30 days of treatment, plasma p-Cresol decreased by 40% and 33% from baseline (both $p < 0.05$), respectively, in the synbiotic group, whereas it remained stable in the placebo group. After 30 days of treatment, no significant change was observed in either group in renal function, glycemia, plasma lipids, or albumin concentration. Treatment was well tolerated and did not induce any change in stool characteristics.

Conclusion: The results of this pilot study suggest that treatment with synbiotics may be effective to lower plasma p-Cresol concentrations in KTRs. Prospective larger scale, longer term studies are needed to establish whether cardiovascular prognosis could also be improved with this nutritional intervention.

ARTICLE HISTORY
Received 10 February 2017
Accepted 21 May 2017

KEYWORDS
Prebiotic; probiotic; synbiotic; kidney transplantation; p-Cresol; uremic toxins

Introduction

p-Cresol is a prototypical uremic toxin whose levels are higher in patients with chronic renal failure (CRF) than in normal subjects both because of enhanced synthesis and of impaired renal clearance [1–4]. Intestinal anaerobic bacteria through phenylalanine and tyrosine oxidation synthesize this toxin [5], which is then absorbed and metabolized into p-cresyl sulfate, the main form of circulating p-Cresol in humans [6]. Because of its prooxidant activity [7], p-Cresol exerts toxic effects on endothelial cells [8,9], causing endothelial dysfunction [10–13]. Moreover, it damages kidney tubular cells [14,15] and promotes tubule-interstitial fibrosis [16,17]. These effects can explain why high levels of p-Cresol predict a faster progression of renal dysfunction and a higher cardiovascular morbidity and mortality in patients with CRF [18]. It has therefore been suggested that p-Cresol could represent a new cardiovascular risk factor in CRF [11,18–21], and efforts are now directed to develop new strategies to lower its plasma concentrations. It has been recently shown that plasma p-Cresol concentrations remain high after kidney transplantation especially in the presence of residual renal function impairment [22,23]. Evidence also has been reported that it could worsen the cardiovascular risk of renal transplant recipients (KTRs) who experience a major cardiovascular event by 36 months after transplantation high in about 40% of cases [22–27].

Because the dysbiotic gut microbiota of patients with CRF and KTRs may produce p-Cresol in excess [22,23–28], therapeutic strategies normalizing the intestinal microflora should decrease plasma p-Cresol levels in these patients. Such normalization can be obtained by the administration of synbiotics; that is, preformed combinations of probiotics—"live microorganisms which, when administered in adequate amounts, confer a health benefit on the host"—and prebiotics—organic compounds, usually oligosaccharides made up of 4 to 10 monomeric hexose units—that act as nutrients for probiotics [29]. Consistent with this hypothesis, a significant decrease in total plasma p-Cresol levels was obtained in patients with CRF after a short-term treatment with a commercial preparation of

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Clinical trial registration number: NCT02179229.

synbiotics [30,31]. In the present study, we tested whether synbiotics may exert similar effects in KTRs.

Methods

Design

This was a single-center, parallel-group, 2:1 synbiotic to placebo randomized and double-blinded study. Uneven randomization was chosen to encourage patient participation [32]. The study protocol was approved by the Ethical Committee of the School of Medicine of the University Federico II of Naples and all patients gave written informed consent. The study was carried out in accordance with the WMA Helsinki Declaration as revised in 1996.

Thirty-six KTRs (29 males and 7 female, mean age 49.6 ± 9.1 years) in regular follow-up at the outpatient renal transplantation clinic of the University Federico II of Naples were enrolled in the study. Using a computer-generated random binary list, they were allocated to one of 2 arms: synbiotic ($n = 24$) and placebo ($n = 12$). Two patients in the synbiotic group dropped out for reasons unrelated to treatment.

Inclusion criteria were as follows: age > 18 years, transplant vintage > 12 months with stable graft function (serum creatinine < 2.5 mg/dl in the last 3 months), and no episode of acute rejection or infection in the last 3 months. Patients with diarrhea, diabetes, malignancy, pregnancy, food intolerance, autoimmune disorders, severe malnutrition, or clinical conditions requiring artificial feeding were excluded from the study.

Study protocol

At the time of enrollment, anthropometric parameters were assessed and body composition was determined with bioelectrical impedance analysis as detailed elsewhere [33]. Dietary intake, gastrointestinal symptoms, and stool characteristics were also quantified as detailed below. Blood chemicals and plasma p-Cresol concentration were measured as well. After baseline evaluation, patients began their treatment at home according to the schedule reported below. Blood samples for p-Cresol determination and dietary and gastrointestinal symptom assessment was performed after 15 and 30 days of treatment, whereas blood chemicals and anthropometric parameters were measured only at the end of the study, after 30 days of treatment.

Treatment schedule

Synbiotic (Probinul Neutro, CadiGroup, Rome, Italy) or placebo was taken at home for 30 days, as 5 g powder packets to be dissolved in water three times a day far from meals. Each Probinul Neutro packet contained lyophilized bacteria (5×10^9 *Lactobacillus plantarum*, 2×10^9 *Lactobacillus casei* subsp. *rhamnosus*, 2×10^9 *Lactobacillus gasseri*, 1×10^9 *Bifidobacterium infantis*, 1×10^9 *Bifidobacterium longum*, 1×10^9 *Lactobacillus acidophilus*, 1×10^9 *Lactobacillus salivarius*, 1×10^9 *Lactobacillus sporogenes*, and 5×10^9 *Streptococcus thermophilus*), prebiotic inulin (2.2 g; VB Beneo Synergy 1, Beneo Iberica, Barcelona, Spain) and 1.3 g of tapioca-resistant starch. Placebo powder was comparable to the synbiotic in color, texture, and taste and contained only cellulose. Both study groups remained on a standard diet and drug regimen during the study.

Diet composition and dietary assessment

At the time of enrollment, patients were already taking the standard diet that we recommend to all kidney transplant recipients who attend our nutrition unit for consultation. This Mediterranean pattern diet is based on foods rich in monounsaturated fatty acids, such as olive oil—the primary source of fat in a Mediterranean diet—or in complex carbohydrates and fibers such as whole grains, fruit, vegetables, and legumes [34]. In accordance with current nutritional guidelines for kidney-transplanted patients [35–37], our dietary plan fits an energy intake higher than 25/kcal/kg/ideal body weight/day, with 55% of carbohydrates and total fat not exceeding 30% of calories (fatty acids < 10% of calories and dietary cholesterol limited to 300 mg/day). In addition; protein intake is restricted to 0.8 g/kg of ideal body weight/day. Our diet includes both insoluble and soluble fibers in a ratio of about 3 to 1 because of the high content of insoluble fibers in whole grains and vegetables. Patients are left free to drink water as they need and the average volume of water intake ranges between 1.5 and 2.0 L/day.

Diet composition was determined by expert dietitians who interviewed each patient using a detailed food frequency questionnaire that includes 130 foods and beverages [38]. This questionnaire inquires about amount and frequency of foods and beverages consumed everyday by the patient. Data on nutrient composition of the different foods were obtained using the tables of the Italian National Institute of Nutrition, Souci's Food Composition and Nutrition Tables, and the European Institute of Oncology as reported elsewhere [33,39]. Data were analyzed with an Excel-based computer program.

Gastrointestinal symptom assessment

Gastrointestinal symptoms and stool characteristics were quantified using a questionnaire similar to that described by Nakabayashi et al. [30] and Guida et al. [31]. More specifically, upper and inferior abdominal pain, borborygm, and flatus were scored as frequent (= 1), occasional (= 2), or almost absent (= 3), whereas ease of defecation was scored as difficult (= 1), easy (= 2), or very easy (= 3). Defecation frequency was scored as follows: 1 = once every 3 days; 2 = once every 2 days; 3 = once/day; 4 = >twice/day. Stool shape was recorded according to the Bristol stool scale [30].

Analytical determinations

Blood chemicals were assayed by standard analytical methods in venous blood samples. Specifically, serum albumin was determined by nephelometry, whereas plasma lipids were measured with Architect i2000SR (Abbott Park, IL). Estimated glomerular filtration rate (eGFR) was evaluated by the modification of diet in renal disease formula. Total p-Cresol was measured by high-performance liquid chromatography after heat–acid denaturation and ethyl acetate extraction [40]. Because acidification released p-Cresol conjugates from plasma proteins and converted p-cresyl

sulfate and p-cresyl glucuronide into p-Cresol, this method measured total plasma p-Cresol concentration that is mainly contributed by p-cresyl sulfate [41].

Statistical analysis

Data were assayed for normality distribution with the Shapiro-Wilk test and reported as means ± SD if normally distributed and as medians (25th–75th percentiles) if not. p-Cresol concentrations were log-transformed before analysis. Student's *t* test and Mann-Whitney *U* test were used for 2-group statistical comparisons of normally and nonnormally distributed data, respectively. Time-dependent changes in normally distributed data were analyzed by 2-way repeated measures analysis of variance using the time of evaluation and placebo or synbiotic treatment as within-subject and between-subject factors, respectively. Mann-Whitney *U* test was used to compare gastrointestinal symptom scores in synbiotic and placebo groups at each time point. Conversely, time-dependent changes in these variables in each of these 2 groups were evaluated with Kruskall-Wallis nonparametric one-way analysis of variance. The threshold for statistical significance was $p < 0.05$. Data were analyzed using SPSS 20.0 (SPSS Inc., Chicago, IL).

Results

As shown in Table 1, there was no significant difference between synbiotic and placebo groups in demographic, anthropometric, and biochemical variables or body composition. The majority of our patients were in CRF stage II/III (20/22 in the synbiotic group and 8/12 in the placebo group), whereas only a few were in stage IV (2 in the synbiotic group and 4 in the placebo group). Renal function, lipid profile, and plasma concentration of albumin and glucose remained stable during the study in both groups and were not affected by treatment (Table 2). In patients taking the synbiotic, p-Cresol concentration was lower than baseline after 15 and 30 days of treatment (−40% and −33% vs basal, respectively, both $p < 0.05$), whereas it remained stable in the placebo group (−25% and −10% vs basal, respectively, ns; Fig. 1). No change was observed in energy or nutrient intake with the only exception of a marginal decrease in lipid intake after 15 days of follow-up in the placebo group (Table 3). Notably, there was no change in the ingested amount of phenylalanine and tyrosine, the main source for p-Cresol synthesis by intestinal bacteria [5] (Table 3). Because of the high fiber content of Probinul Neutro, fiber intake increased, whereas the protein–fiber ratio decreased during treatment in patients in the synbiotic group. Treatment with synbiotics was well tolerated with no relevant gastrointestinal adverse effect; borborygmi were reported as occasional by most patients (60%), whereas abdominal pain was virtually absent in both groups.

Table 1. Baseline characteristics of the study population.[a]

	Synbiotic	Placebo
n	22	12
Sex (male/female)	16/6	12/0
Age (years)	54.0 ± 8.9	47.3 ± 8.5
eGFR (ml/min)	50.6 ± 17.6	58.5 ± 24.0
Body weight (kg)	71.7 ± 10.8	79.6 ± 4.3*
Heigth (cm)	168.0 ± 0.1	176 ± 0.04*
BMI (kg/m^2)	25.6 ± 1.5	25.2 ± 3.4
Waist circumference (cm)	87.7 ± 12.8	101.8 ± 4.9
TBW, % BW	58.0 ± 3.2	59.6 ± 5.8
ECW, % TBW	45.2 ± 2.6	47.1 ± 4.9
FM, % BW	23.1 ± 4.8	19.3 ± 7.6
FFM, % BW	76.9 ± 4.8	80.7 ± 7.9
Immunosuppressive drug treatment		
Corticosteroids (%)	83	91
Cyclosporin (%)	67	73
Tacrolimus (%)	33	9
Mycophenolic acid (%)	83	36
Everolimus (%)	—	18
CKD stage		
II/III	20	10
IV	2	2

eGFR = estimated glomerular filtration rate, BMI = body mass index, TBW = total body water, BW = body weight, ECW = extracellular water, FM = fat mass, FFM = fat-free mass, CKD = chronic kidney disease.
[a]Data are expressed as means ± SD.
*$p < 0.05$.

Discussion

This pilot study provides preliminary evidence that a short-course treatment with an oral synbiotic significantly lowers plasma p-Cresol levels in KTRs.

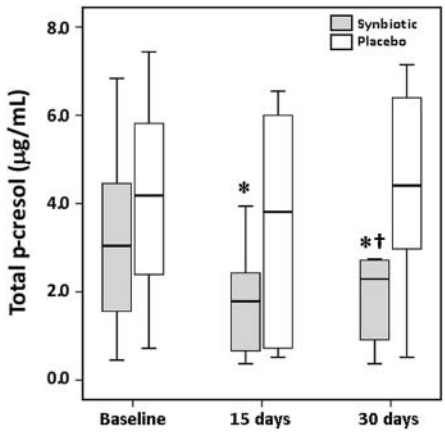
The most likely explanation for our findings is that synbiotics modified the composition of the dysbiotic gut microbiota that produces p-Cresol in excess in KTRs [42–44]. Although we did not perform a metagenomic study, evidence has been reported that the probiotic microorganisms contained in synbiotic colonize the gut of patients affected with CRF and replace the preexisting pathologic flora [45]. Upon gut colonization by these beneficial microrganisms, a marked decrease in p-Cresol generation in the gut is expected to occur. Indeed, these microorganisms belong to species such as *Lactobacillus* that do not express the enzymes required to convert aromatic amino acids in this uremic toxin. In addition, the probiotic microorganisms *Lactobacillus* and *Bifidobacterium* sp. produce lactic acid and short-chain fatty acids that lower the pH of gut lumen, setting up unfavorable conditions for the growth of p-Cresol-producing harmful bacteria such as *Clostridium difficilis* [46].

Because of the high fiber content of the synbiotic preparation, another factor that could have played a role in lowering p-

Table 2. Biochemical parameters in the synbiotic (n.22) and placebo (n.12) groups at baseline and at the end of the study.

		Baseline	T30
Total Cholesterol (mg/dL)	Synbiotic	184.4 ± 34.3	191.1±37.8
	Placebo	191.0 ± 22.9	195.8 ± 19.1
HDL-Cholesterol (mg/dL)	Synbiotic	62.7 ± 21.1	59.4 ± 20.1
	Placebo	65.3 ± 13.9	70.3 ± 17.6
Triglycerides (mg/dL)	Synbiotic	91 (83.0 – 136.0)	132.0 (77.0 – 209.0)
	Placebo	151.5 (130.0 – 163.0)	148.5 (141.0 – 157.0)
Glucose (mg/dL)	Synbiotic	77.0 (69.0 – 83.0)	81.0 (73.0 – 89.0)
	Placebo	76.0 (64.0 – 99.0)	75.0 (64.0 – 102.0)
Albumin (mg/dL)	Synbiotic	4.53 ± 0.3	4.35 ± 0.3
	Placebo	4.60 ± 0.4	4.60 ± 0.2
eGFR (ml/min)	Synbiotic	50.6 ± 17.6	53.5 ± 16.0
	Placebo	58.5 ± 24.0	57.3 ± 22.1

Data are expressed as means ± SD or median and interquartile range, as appropriate.



Median and interquartile levels of plasma p-Cresol in the synbiotic and placebo groups

	T0	T15	T30
SYNBIOTIC	3.0 (1.6-4.5)	1.79 (0.7-2.43)	2.3 (0.9-2.72)*†
PLACEBO	4.2 (2.4-5.8)	3.82 (0.72-6.0)	4.4 (3.0-6.4)

*p<0.01 vs baseline, †p<0.01 vs placebo.

Figure 1. Box plot of p-Cresol plasma concentrations in the symbiotic ($n = 22$) and placebo ($n = 12$) groups at different times of the study. $^{*}p < 0.01$ vs baseline. $^{\dagger}p < 0.01$ vs placebo.

Cresol is the increase in fiber intake. Indeed, in our patients, we observed a significant decrease in the ratio between protein and fiber intake, an index that has a strong positive correlation with p-Cresol plasma levels [47] and cardiovascular risk in CRF [48].

If confirmed in larger studies, our evidence that synbiotics lower p-Cresol plasma levels in KTRs could have relevant implications for the treatment of these patients. It is well known, indeed, that cardiovascular risk remains high even if kidney transplantation is fully successful [24,25,27], and evidence suggests that this can be due in part to the accumulation of p-Cresol [23]. An important argument to suggest the use of synbiotics in KTRs as a tool to lower plasma p-Cresol is that they were very well tolerated. This is an important advantage in comparison with other nutritional approaches that have been proposed to lower p-Cresol in CRF, such as diet supplements with pea hull and inulin that cause a significant increase in stool frequency [49]. It is also worth noting that by normalizing gut dysbiotic microbiota, synbiotics could also decrease the occurrence of serious gastrointestinal complications such as the severe diarrhea caused by *Clostridium difficilis* [50–52]. Treatment with the synbiotic did not induce major changes in any of the metabolic parameters that were evaluated, although a nonsignificant trend for an increase in tryglicerides was observed. Remarkably, changes in plasma lipids upon synbiotic treatment have been reported already in previous studies with conflicting results [53].

Table 3. Time-dependent changes in BMI and daily energy and nutrient intake in patients belonging to the synbiotic ($n = 22$) and placebo ($n = 12$) groups.

		Baseline	15th Day	30th Day
BMI (kg/m^2)	Synbiotic	25.2 ± 3.4	25.2 ± 3.5	25.2 ± 3.4
	Placebo	25.6 ± 1.5	25.3 ± 1.4	25.4 ± 1.3
Body weight (kg)	Synbiotic	71.7 ± 10.8†	71.8 ± 11.2†	71.8 ± 10.8†
	Placebo	79.6 ± 4.3	78.7 ± 4.6	78.8 ± 4.5
Waist circumference (cm)	Synbiotic	87.7 ± 12.3†	92.4 ± 13.7	92.7 ± 6.9
	Placebo	101.5 ± 4.5	100.8 ± 3.6	99.2 ± 2.7
Energy (kcal/day)	Synbiotic	2589.0 ± 496.0	2360.0 ± 373.0	2274.0 ± 482.0
	Placebo	2308.0 ± 108.0	2777.0 ± 477.0*	2488.0 ± 402.0
Energy/ideal body weight (kcal/kg)	Synbiotic	38.9 ± 4.7	35.9 ± 4.0	34.4 ± 5.5
	Placebo	31.9 ± 1.6	38.5 ± 7.4	34.5 ± 6.1
Lipid (% energy)	Synbiotic	28.0 ± 3.9	27.2 ± 2.9	27.1 ± 3.2
	Placebo	31.3 ± 2.9	26.7 ± 4.6*	27.8 ± 3.3
Carbohydrates (% energy)	Synbiotic	54.2 ± 4.3	55.6 ± 4.1	55.0 ± 4.7
	Placebo	52.6 ± 2.8	56.1 ± 5.5	55.8 ± 3.3
Protein (% energy)	Synbiotic	16.4 ± 2.6	16.4 ± 3.1	16.7 ± 2.6
	Placebo	14.9 ± 1.3	15.1 ± 2.7	14.4 ± 1.1
Protein (g/day)	Synbiotic	106.2 ± 28.3	96.7 ± 24.3	94.4 ± 23.8
	Placebo	85.8 ± 8.5	102.5 ± 8.6	90.6 ± 16.4
Phenylalanine (g/day)	Synbiotic	4.0 ± 0.1	3.4 ± 0.1	3.4 ± 1.0
	Placebo	3.2 ± 0.5	4.0 ± 0.4	3.4 ± 0.8
Tyrosine (g/day)	Synbiotic	2.9 ± 1.0	2.4 ± 0.8	2.4 ± 0.7
	Placebo	2.3 ± 0.4	2.7 ± 0.4	2.4 ± 0.5
Fiber (g/day)[a]	Synbiotic	22.8 ± 7.8†	32.6 ± 4.6*†	30.6 ± 4.2*†
	Placebo	16.6 ± 3.3	21.9 ± 6.3	18.1 ± 7.0
Protein/fiber ratio[a]	Synbiotic	4.8 ± 1.2	3.0 ± 0.8*†	3.1 ± 0.7*†
	Placebo	5.4 ± 1.3	5.0 ± 1.4	5.5 ± 1.7

BMI = body mass index.
[a]Also including the fiber content of the synbiotic preparation.
$^{*}p < 0.01$ vs baseline.
$^{\dagger}p < 0.01$ vs placebo.

This was a small-scale pilot study, and its findings will have to be confirmed in a larger number of patients. Because p-Cresol accumulation in KTRs parallels the severity of renal failure [22], an important issue that will have to be addressed by future investigations is whether synbiotics are similarly effective in patients with different degrees of renal function impairment. Indeed, the majority of our patients were in stage II/III CRF, with only few in stage IV and, because of the small size of our sample, we did not perform any patient stratification according to CRF stage. Another important point that remains to be clarified is whether synbiotic treatment does really impact the cardiovascular prognosis of KTRs. Indeed, because of the very short duration of our pilot study, we did not assess any surrogate or clinically relevant endpoint.

In conclusion, we showed that a short-course treatment with a symbiotic preparation given daily by mouth significantly reduced circulating p-Cresol plasma concentration in kidney transplant patients. Because of the evidence suggesting a close link between cardiovascular risk and p-Cresol in these patients, our results prompt further prospective studies to explore whether lowering the circulating concentrations of this uremic toxin synbiotics could also improve the cardiovascular prognosis after kidney transplantation.

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