

## Salivary Phosphate-Binding Chewing Gum Reduces Hyperphosphatemia in Dialysis Patients

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### ABSTRACT

In uremic patients, hyperphosphatemia is associated with cardiovascular calcification and increased cardiovascular mortality. Despite the use of phosphate binders, only half of hemodialysis (HD) patients achieve recommended serum phosphate levels. A hyperphosphoric salivary content, which correlates linearly with serum phosphate, has been reported in HD patients. We hypothesized that binding salivary phosphate during periods of fasting in addition to using phosphate binders with meals could improve the treatment of hyperphosphatemia. We assessed the phosphate-binding capacity of the natural polymer chitosan by <sup>31</sup>P nuclear magnetic resonance and established that 10 and 20% (wt/vol) middle viscosity chitosan solutions bind 30 and 50% of the phosphate contained in PBS, respectively. Thirteen HD patients with serum phosphate levels >6.0 mg/dl despite treatment with sevelamer hydrochloride chewed 20 mg of chitosan-loaded chewing gum twice daily for 2 wk at fast in addition to their prescribed phosphate-binding regimen. Salivary phosphate and serum phosphate significantly decreased during the first week of chewing; by the end of 2 wk, salivary phosphate decreased 55% from baseline (73.21 ± 19.19 to 33.19 ± 6.53; *P* < 0.00001), and serum phosphate decreased 31% from baseline (7.60 ± 0.91 to 5.25 ± 0.89 mg/dl; *P* < 0.00001). Salivary phosphate returned to baseline by day 15 after discontinuing the chewing gum, whereas serum phosphate levels took 30 d to return to baseline. Parathyroid hormone and serum calcium concentrations were not affected by the gum. In conclusion, adding salivary phosphate binding to traditional phosphate binders could be a useful approach for improving treatment of hyperphosphatemia in HD patients.

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Hyperphosphatemia is recognized as a contributor to vascular calcification in patients with chronic kidney disease (CKD) and hemodialysis (HD) patients and is independently associated with cardiac mortality.<sup>1</sup> Serum PO<sub>4</sub> levels >6.5 mg/dl, serum calcium-phosphate products >70 mg/dl, and parathyroid hormone (PTH) >495 pg/ml all are associated with increased extracellular calcification.<sup>2</sup> Altered mineral metabolism and hyperphosphatemia stimulate the phenotypic conversion of smooth muscle cells to an osteogenic cell type through an increased intracellular phosphorus intake *via* Pit-1, a sodium-dependent phosphate co-transporter,<sup>3</sup>

thereby leading to vascular calcification. In terms of cardiac mortality, the higher the serum PO<sub>4</sub> level,

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the higher the relative mortality risk. It has been reported that, among HD patients, a percentage as high as 40% presenting serum PO<sub>4</sub> level >6.5 mg/dl, showed a relative risk 1.27 times higher than patients with serum PO<sub>4</sub> level in the range of 2.4 to 6.5 mg/dl.<sup>4</sup> These data strengthen the need for strict management of hyperphosphatemia, which is currently mainly based on dietary restriction, dialysis, and the use of phosphate binders.<sup>5</sup> Calcium- and metal-free polymers, such as sevelamer hydrochloride or carbonate, and lanthanum carbonate should be the preferred options: the still widely used calcium-based phosphate binders are currently under scrutiny because of their possible contribution to vascular calcification through calcium load.<sup>6</sup> Nevertheless, only approximately 50% of HD patients achieve the serum PO<sub>4</sub> levels currently recommended by Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines.<sup>7</sup>

An increased salivary PO<sub>4</sub> excretion has been reported in patients with CKD<sup>8</sup> and HD patients,<sup>9</sup> independent from food intake. Salivary PO<sub>4</sub> levels correlated with serum creatinine and GFR in CKD<sup>8</sup> and with serum PO<sub>4</sub> levels in HD patients, the salivary PO<sub>4</sub> content being at least five times the serum PO<sub>4</sub> level.<sup>9</sup> In this article, we suggest that in HD patients with serum PO<sub>4</sub> ≥6 mg/dl and salivary PO<sub>4</sub> ≥30 mg/dl, a sustained intestinal absorption of immediately bioavailable PO<sub>4</sub> could account for an additional load to the dietary PO<sub>4</sub> intake, *via* the ingestion of the 500 to 700 ml of saliva produced daily. Among other underestimated dietary contributions to PO<sub>4</sub> burden,<sup>10</sup> a looping of gastrointestinally secreted and intestinally absorbed PO<sub>4</sub> could partially explain the unsatisfactory efficiency of sevelamer hydrochloride administered at meals to reduce the intestinal absorption of PO<sub>4</sub> generated by food intake in long-term HD patients. To this end, this preliminary open study tested the hypothesis that adding salivary PO<sub>4</sub> binding during fasting periods to sevelamer PO<sub>4</sub> binding at meals could optimize the efficacy of the latter, thereby facilitating the reduction of serum PO<sub>4</sub> toward the recommended levels. The study was performed with a small uremic HD cohort of patients who had serum PO<sub>4</sub> levels >6.0 mg/dl for at least 6 mo despite daily treatment with sevelamer hydrochloride in the range of 3200 to 4800 mg at meals and were undergoing hemodialysis three times a week for not less than 12 mo. The HD patients were directed to chew a newly formulated gum acting as a slow delivery system for a middle viscosity, highly deacetylated chi-

tosan polymer, tested *in vitro* for its PO<sub>4</sub>-binding capability. The 20-mg chitosan-loaded chewing gum was administered twice daily during fasting periods (*i.e.*, between meals) for 2 wk as an add-on to sevelamer hydrochloride treatment, and the effect on salivary and serum PO<sub>4</sub> level was evaluated.

## RESULTS

### In Vitro Characterization of Chitosan PO<sub>4</sub> Binding

Viscosity is an index of chitosan molecular weight.<sup>11</sup> PO<sub>4</sub>-binding capability was determined in low and medium molecular weight chitosan, using the A and B protocols developed by Zhang and Zhang<sup>12</sup> to quantify the chitosan PO<sub>4</sub>-binding capability in an aqueous solution at neutral pH, where the polymer is totally insoluble, and in an acidic solution, where chitosan is easily soluble and shows gelling properties. Data are summarized in Table 1. In protocol A, low- and middle-viscosity chitosan with a degree of deacetylation ≥75% was tested at pH 6.5, thus mimicking the physiologic pH of the human mouth. In these experimental conditions, chitosan is practically insoluble, and its PO<sub>4</sub>-binding capability relies on the degree of chitosan deacetylation and related polyelectrolytic structure, mucoadhesivity, and retention time onto the gastrointestinal mucosa, as described by Grabovac *et al.*<sup>13</sup> <sup>31</sup>P nuclear magnetic resonance (<sup>31</sup>P-NMR) analysis showed that 2, 10, and 15% (wt/vol) low-viscosity chitosan was able to remove 10, 15, and 20%, respectively, of the 10 mM PO<sub>4</sub> contained in 1× reference PBS (Sigma; corresponding to 10 mM PO<sub>4</sub> in 10 ml [pH 6.5]). Two, 10, and 15% (wt/vol) middle-viscosity chitosan was able to remove 15, 25, and 33%, respectively, of the 10 mM PO<sub>4</sub> contained in 1× PBS solution. These results indicate that increasing chitosan concentrations are able to bind increasing amounts of PO<sub>4</sub> from PBS, at pH 6.5. Moreover, the higher the chitosan viscosity, the stronger its PO<sub>4</sub>-binding capability. For this reason, protocol B was performed with the middle viscosity chitosan, dissolved in 0.2 M acetic acid (pH 4.2). When PBS was then added to the chitosan solution to test its PO<sub>4</sub>-binding capability in an acidic environment, thus mimicking chitosan behavior in the stomach, 10 and 20% (wt/vol) middle-viscosity chitosan solutions were able to bind 30 and 50%, respectively, of the 10 mM PO<sub>4</sub> contained in PBS. Altogether, these results confirm the good binding capability

**Table 1.** Results of the “in vitro” determination of PO<sub>4</sub> binding capability by low and medium viscosity chitosan

Chitosan (g/ml)	PBS	Low Viscosity		Medium Viscosity	
		PBS	Chitosan	PBS	Chitosan
Protocol A (%)					
2	1	0.90	0.10	0.85	0.15
10	1	0.85	0.15	0.75	0.25
15	1	0.80	0.20	0.67	0.33
Protocol B (%)					
				Liquid Phase	Chitosan
10	1			0.70	0.30
20	1			0.50	0.50

of chitosan, depending on its degree of deacetylation, molecular weight, and pH conditions.

### Clinical Evidence

As expected by the low chitosan dosage administered (40 mg/d), no patients reported or showed any adverse event related to the chewing gum, confirming reports of chitosan as safe when used in diets to control hyperlipidemia, at dosages at least 10-fold higher than that used in this study.<sup>14,15</sup> In the 6 mo before chewing gum exposure, patients were administered a protein diet content of 1.2 g/kg per d. Patients' age; gender; dialytic age; sevelamer hydrochloride daily dosage and number of pills per day; and average serum PO<sub>4</sub>, Ca<sup>2+</sup>, and PTH levels in the 6 mo before chewing gum exposure are reported in Table 2. Patients were consecutively recruited to the study when they requested a repeat prescription of sevelamer. All of the patients recruited to this study had their sevelamer consumption recorded, this being the drug regularly provided by the hospital pharmacy on a schedule defined by the individual daily dosage. The dialysis protocol, sevelamer daily dosing, and protein diet content remained unchanged for the duration of the study and the follow-up period. For both sevelamer and the chitosan-loaded chewing gum, the medical staff dispensed to patients at study entry the number of tablets needed for treatment during the study period. With the exception of patient 5, who required only 180 sevelamer tablets to cover the study period fully, all of the others were supplied with two 180-tablet sevelamer packages, the second being provided at the end of week 5 for patients 2 and 9 or at the end of week 4 for all of the others. Thirty chewing gum tablets were provided in a bottle to each patient. Patients were instructed to supplement their regular sevelamer intake at meals with the chewing gum, to be chewed for 60 min, one in the morning and the other in the afternoon, between major meals. The numbers of chewing gum and sevelamer tablets was counted to assess consumption at the end of weeks 2 and 6, respectively. Each patient returned two

chewing gum tablets (28 tablets consumed during 14 d). The returned sevelamer tablets were as follows: 12 from patient 5 (168 tablets consumed during 42 d of study period [*i.e.*, 4 tablets/d (3200 mg/d)]), 150 from patients 2 and 9 (210 tablets consumed during 42 d of study period [*i.e.*, 5 tablets/d (4000 mg/d)]), and 108 from all of the others (252 tablets consumed during 42 d of study period [*i.e.*, 6 tablets/d (4800 mg/d)]). Salivary and serum PO<sub>4</sub> levels during chewing gum use (weeks 1 and 2) and after discontinuation (weeks 4 and week 6) are summarized in Table 3 and Figure 1. When compared with baseline serum values, any reported difference can be attributed to additional salivary PO<sub>4</sub> binding (chitosan-loaded chewing gum, weeks 1 and 2) over and above that accounted for by the established diet and sevelamer treatment (unchanged from week 0 to week 6). Compared with baseline values, salivary PO<sub>4</sub> content declined significantly from 73.21 ± 19.19 to 52.02 ± 12.89 mg/dl in week 1 (−28.8%; *P* < 0.01) and to 33.19 ± 6.53 mg/dl in week 2 (−54.7%; *P* < 0.00001). A significant difference in salivary PO<sub>4</sub> levels was also recorded between week 1 and week 2 (−36.2%; *P* < 0.0001). In week 4 (*i.e.*, 15 d after chewing gum discontinuation), salivary PO<sub>4</sub> levels (68.91 ± 16.05 mg/dl) returned to baseline values, remaining practically constant also at week 6 (*i.e.*, 30 d after chewing gum discontinuation; 75.33 ± 18.11 mg/dl). Compared with baseline values, serum PO<sub>4</sub> levels significantly decreased at week 1 from 7.60 ± 0.91 to 5.38 ± 0.81 mg/dl (−29.2%; *P* < 0.00001) and to 5.25 ± 0.89 mg/dl (−30.9%; *P* < 0.00001) at week 2. Serum PO<sub>4</sub> levels were lower than baseline (5.64 ± 1.02 mg/dl; −25.8%; *P* < 0.0001) 15 d after chewing gum discontinuation (week 4) but returned to baseline values 30 d after chewing gum discontinuation (week 6; 7.55 ± 0.75 mg/dl).

ANOVA was computed from logarithmically transformed data. Mean PTH and serum Ca concentrations did not change significantly at weekly intervals (up to 42 d) compared with baseline, whereas calcium-phosphate product values significantly decreased by 28.4% at week 1 and 30.6% at week 2 (*P* <

**Table 2.** Patients characteristics<sup>a</sup>

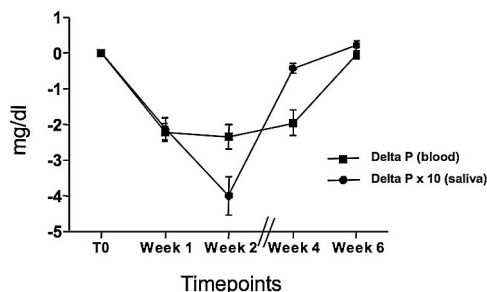
Patient	Gender	Age (yr)	Dialytic Age	Sevelamer HCl Daily Dosage (mg [no. of pills/d])	Serum PO <sub>4</sub>	Ca <sup>2+</sup>	PTH
1	F	65	12	4800 (6)	7.08 ± 0.12	8.75 ± 0.26	658.33 ± 11.25
2	M	63	18	4000 (5)	6.32 ± 0.12	8.58 ± 0.21	223.33 ± 25.03
3	M	58	18	4800 (6)	7.85 ± 0.76	8.17 ± 0.36	287.67 ± 30.87
4	M	62	96	4800 (6)	8.95 ± 0.54	8.03 ± 0.47	703.33 ± 42.27
5	M	55	17	3200 (4)	6.07 ± 0.08	8.27 ± 0.23	251.00 ± 26.50
6	M	62	22	4800 (6)	7.52 ± 0.12	8.88 ± 0.08	358.33 ± 7.53
7	M	30	14	4800 (6)	7.27 ± 0.41	8.87 ± 0.36	375.00 ± 18.71
8	M	78	264	4800 (6)	6.93 ± 0.31	8.60 ± 0.21	828.00 ± 56.36
9	F	56	204	4000 (5)	6.58 ± 0.60	9.13 ± 0.56	143.00 ± 3.46
10	F	58	180	4800 (6)	8.30 ± 0.30	8.90 ± 0.09	735.00 ± 28.81
11	M	62	72	4800 (6)	7.40 ± 0.09	8.82 ± 0.08	665.00 ± 12.25
12	M	71	84	4800 (6)	7.22 ± 0.33	8.75 ± 0.14	678.33 ± 14.72
13	F	69	27	4800 (6)	6.88 ± 0.10	8.90 ± 0.24	133.83 ± 4.92

<sup>a</sup>Data are means ± SD.

**Table 3.** Parameters at baseline (T0), during chewing gum use (weeks 1 and 2), and after chewing gum discontinuation (weeks 4 and 6;  $n = 13$ )<sup>a</sup>

Parameter	Baseline T0	Chewing-Gum Add-on		Follow-up (after Chewing Gum Discontinuation)	
		Week 1	Week 2	Week 4	Week 6
Serum Ca (mg/dl)	8.41 ± 0.98	8.35 ± 0.54	8.45 ± 0.63	8.45 ± 0.70	8.58 ± 0.70
PTH (pg/ml)	508.42 ± 283.88	444.75 ± 238.80	397.67 ± 254.20	382.92 ± 221.80	496.35 ± 285.75
Salivary P (mg/dl)	73.21 ± 19.19	52.02 ± 12.89	33.19 ± 6.53	68.91 ± 16.05	75.33 ± 18.11
Serum P (mg/dl)	7.60 ± 0.91	5.38 ± 0.81	5.25 ± 0.89	5.64 ± 1.02	7.55 ± 0.75
Calcium-phosphate product	63.82 ± 10.01	45.68 ± 6.45	44.30 ± 7.99	47.75 ± 9.89	64.71 ± 8.03

<sup>a</sup>Data are means ± SD.



**Figure 1.** Serum and salivary PO<sub>4</sub> levels (mg/dl) at baseline (T0), at 7 d (week 1) and 14 d (week 2) of chewing gum use, and at 14 d (week 4) and 30 d (week 6) after chewing gum discontinuation.

0.00001) and by 25.2% at week 4 ( $P < 0.0001$ ) and fully reversed to baseline at week 6 (Table 3), following the serum PO<sub>4</sub> trend.

**DISCUSSION**

Saliva is a good indicator of the plasma levels of various substances, particularly toxic materials.<sup>16</sup> We previously reported that increased serum PO<sub>4</sub> levels are reflected in increased salivary PO<sub>4</sub> content in patients with CKD and HD patients.<sup>8,9</sup> Despite undergoing dialysis three times a week and being regularly treated with sevelamer hydrochloride for at least 12 mo at study entry and before chewing gum use, our HD patients had a mean salivary PO<sub>4</sub> level of 73.21 ± 19.19 mg/dl and a mean serum PO<sub>4</sub> level of 7.6 ± 0.9 mg/dl, thus remaining at high risk for cardiovascular disease and cardiac mortality. Daily salivary secretion volumes range between 500 and 700 ml,<sup>16</sup> and even higher rates have been reported in healthy populations,<sup>17</sup> whereas low salivary flow rate has been documented in HD patients with diabetes.<sup>18</sup> Our HD patients neither had diabetes nor presented signs or symptoms of autonomic neuropathy. Moreover, dry mouth symptoms and Sjögren syndrome were exclusion criteria, and the Saxon test showed a normal salivary secretion among the enrolled patients. In our HD patients, taking into account the high salivary PO<sub>4</sub> content with sevelamer treatment only (baseline values) and a daily salivary production of 500 ml, a conservative estimate of at least 366 mg of bioavailable PO<sub>4</sub> daily entering the gastrointes-

tinal fluid could be proposed, which forecasts an additional weekly intestinal PO<sub>4</sub> load of at least 2500 mg. This estimate is comparable to the still poorly understood dietary PO<sub>4</sub> burden from food additives.<sup>10</sup> In HD patients at high risk for cardiac mortality, such a salivary PO<sub>4</sub> contribution could partially explain the unsatisfactory control of hyperphosphatemia exerted by the currently available non-calcium- and metal-free PO<sub>4</sub> binders administered at meals, with the intent to restrict the intestinal absorption of PO<sub>4</sub> generated from food intake. In this preliminary study, we observed an approximate average of 2-mg/dl reduction in serum PO<sub>4</sub> after 15 d of salivary PO<sub>4</sub> binding during fasting periods, as an add-on to sevelamer treatment.

Our observations are preliminary and require confirmation in a randomized, double-blind, placebo-controlled clinical study on a larger HD population, in which fecal PO<sub>4</sub> elimination also needs to be determined. Nevertheless, the reduction observed in serum PO<sub>4</sub> by adding to sevelamer salivary PO<sub>4</sub> binding during fasting periods is worthy of note. Although chitosan loaded into the chewing gum seems to affect salivary PO<sub>4</sub> binding into the mouth acutely, the delayed recovery in serum PO<sub>4</sub> merits further studies. It could be suggested that chitosan, once solubilized into the stomach at acidic pH, could be retained onto the gastrointestinal mucosa for a prolonged time (estimated by some authors to be in the order of 17 to 40 h, depending on pH and the manufacturing process of chitosan),<sup>13</sup> thereby exerting further PO<sub>4</sub> binding. The daily removal of an additional PO<sub>4</sub> load from gastrointestinal fluids could support sevelamer treatment. It could be inferred that our observations simply mirror increased adherence to the medically advised diet and sevelamer treatment during the study. The short study duration, the provision and accounting for pills, and counseling for both sevelamer and chewing gum use seem to indicate that counseling was sufficient to support adherence. We agree with Haynes *et al.*<sup>19</sup> on the concept that adherence, measured with a pill count, may not be a reliable method to ensure compliance to treatment. What is more effective is the measurement of serum PO<sub>4</sub> before, during, and after treatment. To this end, diet and sevelamer hydrochloride do not seem to explain the salivary and serum PO<sub>4</sub> reduction observed by adding on the chewing sessions during fasting periods for 14 d. This being the case, a similar trend in the recovery of salivary and serum baseline values should have been

detected. Although salivary  $\text{PO}_4$  levels correlate with serum  $\text{PO}_4$  values in HD patients,<sup>9</sup> no significant variability was observed in patients' serum  $\text{PO}_4$  in the 6 mo before study entry, thereby supporting patients' compliance to diet and sevelamer use. Hyperphosphoric saliva seems to be an epiphenomenon in CKD and ESRD,<sup>8,9</sup> thus specifically suggesting local binding to affect  $\text{PO}_4$  balance positively. As a proof of concept, salivary  $\text{PO}_4$  immediately regained baseline values at chewing gum discontinuation, whereas an additional 2 wk was required for serum  $\text{PO}_4$  to regain baseline values progressively (week 6). If the dietary management of salivary  $\text{PO}_4$  by means of the chitosan-loaded chewing gum were irrelevant to the overall intestinal  $\text{PO}_4$  absorption and/or to sevelamer efficiency in  $\text{PO}_4$  intestinal binding, then no stepwise rises in serum  $\text{PO}_4$  should have occurred during follow-up.

Whatever the mechanism, this delayed recovery of  $\text{PO}_4$  may also explain the intriguing observation on PTH trend. Despite that serum PTH levels did not reach statistical significance, it paralleled serum  $\text{PO}_4$  throughout the study, including the serum  $\text{PO}_4$  level at chewing gum discontinuation. This clearly evident trend of serum PTH, even in the small cohort of patients of this study, deserves consideration. Hyperphosphatemia has a known direct influence on PTH synthesis and secretion, which seems to involve posttranscriptional mechanisms, leading to a prolonged PTH mRNA half-life and therefore PTH synthesis.<sup>20</sup> The  $\text{PO}_4$  reduction achieved during chitosan-loaded chewing gum treatment might well interact with PTH mRNA stability and increase transcript degradation, thereby reducing serum PTH level. This effect on PTH, suggested to be induced by  $\text{PO}_4$  restriction,<sup>21</sup> can be observed in our patients even after the discontinuation of chewing gum and in the presence of a serum  $\text{PO}_4$  levels even lower than at baseline. In our study, PTH also seemed to change along with serum  $\text{PO}_4$ , extending the positive effect of salivary  $\text{PO}_4$  binding to the overall  $\text{PO}_4$ -PTH relationship. Further studies of patients using chitosan loaded chewing gum for the dietary management of salivary  $\text{PO}_4$  during a longer treatment period, with a larger patient population, and a specific investigation into PTH at molecular level could clarify these aspects.

The exact mechanism of salivary  $\text{PO}_4$  binding exerted by the chitosan loaded into the chewing gum is under investigation, although the behavior of the three-layered gum, with the inner gum core acting as a slow delivery system for insoluble active principle ingredients (API) during a 60-min chewing session, has been previously described.<sup>22</sup> To this end, intensive developmental studies are ongoing.

In conclusion, the results of our study suggest that salivary  $\text{PO}_4$  binding could be a useful approach to the dietary management of serum  $\text{PO}_4$  level reduction in HD patients and that chewing sessions in fasting periods, as an add-on to phosphate binders at meals, lead to a better control of hyperphosphatemia. Given the importance of hyperphosphatemia in the high morbidity and mortality of patients with ESRD, the requirement to achieve neutral  $\text{PO}_4$  balance by means of different  $\text{PO}_4$  binders with different mechanisms of action could be helpful to improve patient compliance and reduce pill burden.

## CONCISE METHODS

### In Vitro Study of Chitosan's Phosphate-Binding Properties

The cationic biopolymer chitosan [poly(1-4-2-amino-2-deoxy- $\beta$ -D-glucosamine)] derives from chitin de-N-acetylation. The OH and  $\text{NH}_2$  polar groups in the structure act as electron donors and interact with inorganic salts. Solubility and viscosity (index of chitosan molecular weight) depend on deacetylation grade, chain length, and pH. The chemical and binding properties of chitosan have been extensively described.<sup>22</sup> Mimicking the human mouth environment at pH 6.5, water-insoluble low- and middle-viscosity polymers (Fluka, ref. 50494 and 28191, deacetylation degree 75 to 86%) were tested for their  $\text{PO}_4$ -binding capability according to published protocols (protocols A and B).<sup>12</sup> In protocols A and B, any detected quantitative difference in  $\text{PO}_4$  content in PBS relies on chitosan-bound  $\text{PO}_4$ . In protocol A,  $\text{PO}_4$  binding was expressed as the difference in  $\text{PO}_4$  amount between  $1 \times$  PBS and 2, 10, and 15% (wt/vol) chitosan-PBS solutions. After centrifugation, 0.5 ml of the supernatant in chitosan-PBS as well as the reference PBS were sampled for NMR analysis by Varian 500-MHz High-Resolution <sup>31</sup>P-NMR, with monodimensional spectra acquisition at 300°K. In protocol B, medium-viscosity chitosan was dissolved into 0.2 M acetic acid to give a 10 and 20% (wt/vol) solution at pH 4.2. The solutions were subsequently stirred at 50°C for approximately 2 h to obtain a homogeneous solution and then filtered to remove air bubbles. The final solutions were added to 0.0931 g of PBS powder and then frozen at  $-20^\circ\text{C}$  to allow phase separation. A total of 0.5 ml of the liquid phase as well as of the reference PBS was analyzed by NMR.

### Chewing Gum

The 20-mg chitosan-loaded chewing gum was manufactured as cold compressed three-layered tablets, 1.7 g in weight. The patented tablet (WO2004/073691, EP 1594478) is composed of two external layers allowing cold compression of the inner butyl rubber core. Before compression, the inner core was premixed with excipients and medium-viscosity, food-grade chitosan powder. Acting as a delivery system, the chewing gum has been noted for its fast release of soluble API from the external layers and slow release of insoluble API over a 60-min chewing session.<sup>23</sup>

### HD Patient Recruitment and Study Design

After ethics committee approval, HD patients at the Dialysis Unit of Papardo Hospital, Messina, were provided with detailed information on the aim and methods of the clinical test. According to our previous protocols,<sup>8,9</sup> exclusion criteria were diagnosis of acute infections, malignancy, ongoing inflammatory processes, dry mouth symptoms, Sjögren syndrome, vegetative nervous system alterations, and autonomic diabetic neuropathy. Thirteen long-term HD patients, receiving bicarbonate dialysis using polysulphone dialyzers for 210 to 240 min three times a week during a period of not less than 12 mo (with single-pool KT/V ratio expressed as mean  $\pm$  SD of KT/V determinations performed every month for all patients of  $1.39 \pm 0.15$ ) gave their informed consent (refer to Table 2 for age, gender, dialytic age, and serum  $\text{PO}_4$  level in the 6 m before recruitment). All of them were regularly treated with sevelamer hydrochloride at meals, at dosages of

3200 to 4800 mg/d. Sevelamer treatment was continued during and after the study. The observational period covered 6 wk, as follows. The 13 HD patients were initially evaluated for salivary PO<sub>4</sub>, serum PO<sub>4</sub>, serum calcium phosphate product, and PTH levels (week 0). Salivary PO<sub>4</sub> determination was performed before the dialysis session and 30 min after a mouth-washing with deionized water. Salivary samples were obtained by direct suction of a fixed 2 ml of salivary volume from the mouth's vestibule, using an automatic pipette. All patients were checked for normal salivary function using the Saxon test, as described previously.<sup>8,9</sup> Blood samples for serum PO<sub>4</sub>, serum calcium phosphate product, and PTH level evaluation were also collected before starting the HD sessions. In weeks 1 and 2, the 20-mg chitosan-loaded chewing gum was added to sevelamer hydrochloride. The 13 HD patients were instructed to chew the gum two times a day during fasting periods (*i.e.*, between meals) for 60 min and to spit it out at the end of the chewing session. At the end of week 2, the chewing gum was withdrawn, whereas dialysis and sevelamer hydrochloride were regularly maintained for all of the remaining observational period. Salivary PO<sub>4</sub>, serum PO<sub>4</sub>, and PTH data were collected at weeks 4 and 6 (*i.e.*, 15 and 30 d) after chewing gum discontinuation. Serum and salivary PO<sub>4</sub> levels were determined after centrifugation of the sample by a spectrophotometric assay using a flex reagent cartridge (Dade Behring, Newark, NJ). Serum PTH concentrations were evaluated using the Immulite 2000 intact PTH, solid-phase, two-site chemiluminescent enzyme-labeled immunometric assay (Diagnostic Product Corp., Los Angeles, CA), with normal values up to 53 pg/ml.

### Statistical Analysis

Data are presented as means ± SD. One-way ANOVA was used for univariate and multivariate repeated-measures analysis using Statgraphics statistical software (Statgraphics, Herndon, VA). Pairwise comparisons between the baseline values and values detected during chewing gum use (weeks 1 and 2) and values detected after chewing gum withdrawal (weeks 4 and 6) were carried out when ANOVA was found to be significant. Multiple-comparison type I error rate adjustment was based on a two-sided Bonferroni criterion, in which the experimental type I error rate was ≤0.05. Model goodness of fit was evaluated by standard residual diagnostic procedures. A logarithmic transformation of data was performed, when necessary.

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### DISCLOSURES

M.C.C. is R&D Project Leader at CM&D Pharma Limited, UK.

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