Salivary Cystatin SN Binds to Phytic Acid In Vitro and Is a Predictor of Nonheme Iron Bioavailability with Phytic Acid Supplementation in a Proof of Concept Pilot Study

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ABSTRACT

Background: Acute phytic acid intake has been found to decrease iron bioavailability; however, repeated phytic acid consumption leads to iron absorption adaptation. Salivary proline-rich proteins (PRPs) have been shown to inhibit iron chelation to tannins and may mediate similar iron absorption adaptation with phytic acid intake.

Objectives: The objectives of this study were to determine whether salivary proteins bind to phytic acid in vitro, and to explore a proof of concept in a pilot study that examined the impact of 4-wk, daily phytic acid supplementation on individuals' iron status, bioavailability, and salivary PRP concentrations.

Methods: High-performance liquid chromatography (HPLC) and matrix-assisted laser desorption/ionization–time of flight were used to characterize in vitro salivary protein–phytic acid interactions. Nonanemic women ($n = 7$) consumed 350 mg phytic acid supplements 3 times daily for 4 wk, and meal challenges were employed to determine iron bioavailability, iron status, and salivary protein concentrations before and after supplementation periods. Enzyme-linked immunosorbent assay (ELISA) analysis of purified protein fractions and participant saliva identified proteins bound to phytic acid.

Results: In vitro salivary protein–phytic acid interaction identified cystatin SN, a non–proline rich salivary protein, as the specific bound protein to phytic acid. Iron bioavailability ($P = 0.32$), hemoglobin ($P = 0.72$), and serum ferritin ($P = 0.08$) concentrations were not reduced from week 0 to week 4 after phytic acid supplementation. Basic PRPs and cystatin SN concentrations were positively correlated with iron bioavailability at week 4.

Conclusions: Overall, results suggest that phytic acid binds to the non-PRP cystatin SN and that salivary protein production may improve iron bioavailability with phytic acid consumption. *Curr Dev Nutr* 2019;3:nzz057.

Introduction

Phytic acid is the major phosphorous storage compound found in plants, including grains and legumes (1), which are commonly consumed in countries with high rates of iron deficiency. Phytic acid's propensity to bind to metal cations and proteins reduces their bioavailability (1), and phytic acid is thus denoted as an "antinutritional" factor when it forms a phytic acid–nutrient complex (denoted phytate). The amount of phytic acid consumed (2), the food matrix in which it is consumed (1), and food preparation (1) all determine its ultimate "antinutritional effect," but human single-meal studies have repeatedly found that phytic acid reduces iron bioavailability (3–5).

Despite conventional views that support phytic acid's contribution to marginal iron status, there are also studies that dispute this idea. The individual effect of phytic acid consumption on iron bioavailability has been found to be highly variable (6), and many individuals consuming diets rich in grains and legumes, and thus phytic acid content, maintain normal iron stores (7, 8), pointing to possible gaps in the understanding of phytates' antinutritional effects. Adaptation, or homeostatic maintenance of iron storage despite such antinutritional factor consumption, is one possible explanation. An experimental study showed that increased dietary phytic acid consumption over 8 wk improved iron absorption of a high-phytate meal compared with a week 0 meal and a low-phytate control in marginally iron-deficient women (9). Another study examining long-term consumption (10 wk) of high- and low-bioavailability diets in iron-replete men also suggested adaptation to low-bioavailability diets from week 0 to week 4, and authors suggested that single-meal bioavailability may have exaggerated long-term effects of antinutritional factors (10). In a 12-wk crossover study that divided women into high- (rich in heme protein and ascorbic acid) and low- (rich in grains, legumes, and fiber) bioavailability diets, only women consuming a low-bioavailability diet over time absorbed more nonheme iron from week 0 to week 4 with either a high- or low-bioavailability meal challenge, although improved iron storage was better correlated with a high-bioavailability diet (11). Despite studies highlighting possible adaptation to phytates, a physiological explanation for adaptation has not been proposed.

Saliva is the first defense mechanism of the alimentary tract to toxins and pathogens (12), and salivary proline-rich proteins (PRPs) in particular have been noted in sensory studies because of their ability to precipitate tannins (12, 13), another antinutritional factor, thereby reducing tannin–iron chelation, and creating the sensation of astringency. Production of PRPs when consuming tannins has been shown to improve protein (14) and iron availability (15, 16) in animal studies, whereas hamsters, which do not upregulate PRP synthesis in response to tannin consumption, have been reported to have poor growth outcomes when consuming tannins (17). In saliva, there are 6 main classes of salivary proteins that may reduce phytic acid–iron chelation: histatins, cystatins, statherins, acidic PRPs (aPRPs), basic PRPs (bPRPs), and glycosylated PRPs (gPRPs) (13). Statherin and aPRPs are recognized for their ability to regulate oral calcium (18, 19). Elevated concentrations of salivary cystatins have been linked to bitter sensation acceptance (20, 21) and function to inhibit cysteine proteases (22). The bPRPs are thought to protect against the negative effects of polyphenols (23), whereas the function of gPRPs (12) is not well characterized. Although PRP binding to tannins has been characterized previously (24–28), PRP–phytate interaction has not. Phytates may not directly interact with salivary PRPs, but it is important to establish whether phytates directly interact with salivary proteins, whether salivary protein concentrations can be upregulated by phytate consumption, and whether there are specific proteins that bind with phytates, which often coexist in tannin-rich foods. The primary objectives of this study were to determine *1*) whether salivary proteins interact with phytic acid in vitro, and to identify potentially bound proteins. Secondary objectives were to determine *2*) whether in vivo iron bioavailability, or markers of iron status, are affected with repeated phytic acid consumption, *3*) whether phytic acid consumption changes salivary protein concentrations over time, and *4*) whether salivary

proteins are associated with improved iron bioavailability during phytic acid supplementation as proofs of concept in a small pilot study. Post hoc study objectives included determining whether cystatin SN was correlated with improvements in iron bioavailability during the clinical study duration.

Methods

Study activities were divided into 3 phases. In phase I, to address our primary objective, in vitro phytic acid–salivary protein interaction assays were designed to determine whether these interactions occurred. In phase II, a proof of concept pilot study was designed to measure iron bioavailability with repeated phytic acid consumption over time, as well as salivary protein concentrations with phytic acid consumption to address our secondary objectives. In phase III, in vitro and proof of concept pilot study salivary protein samples were analyzed via ELISA for confirmation of phytic acid–protein interactions as a post hoc analysis.

Experiments to investigate in vitro phytic acid-salivary protein interactions
Salivary PRP measurement.

High performance liquid chromatography parameters and equipment. All reagents were analytical grade. Acetonitrile, trifluoroacetic acid (TFA), and high-performance liquid chromatography (HPLC) grade water were purchased from Fisher Scientific.

Acidified saliva sample preparation. Frozen salivary samples from an individual donor, and later, from clinical trial participants, were thawed in a refrigerator overnight. Before sample analysis, we verified consistency in chromatogram output with duplicate samples by HPLC. Samples were analyzed for qualitative protein characteristics, and peak consistency allowed for samples to be analyzed in a single run. For PRP extraction, 900 µL of saliva was mixed with 10 µL of 10% TFA in water, centrifuged for 5 min at 5223 × g, and the supernatant was filtered through a 0.2-µm polyvinylidene fluoride (PVDF) syringe filter as described previously (29–31). Before samples were analyzed, it was verified by investigators with HPLC analysis that there was no PRP peak loss with use of syringe filters. The supernatant was then analyzed by HPLC as described previously (29–31).

In vitro phytate-PRP testing.

Matrix-assisted laser desorption/ionization–time of flight and HPLC determination of protein–phytate binding. Acidic saliva PRP fractions were prepared, and phytic acid (inositol hexaphosphate, ACOS Organics) was added to samples at concentrations of 0.000512 (denoted physiological, equivalent to 2:1 phytic acid supplement drink: saliva concentration, pH 4.5), 0.00512 (10:1, pH 2.3), or 0.0512 (100:1, pH 1) mg/100 µL saliva and compared with an acidic saliva sample (buffered to 100 × physiological saliva–phytic acid sample pH of 1 with TFA), or a saliva-only control. All samples were prepared to equivalent sample dilutions by adding aliquots from a single saliva sample. After preparation, samples were shaken for 30 s and allowed to sit at room temperature (20°C) for 5 min. Samples were spotted on matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF)/TOF in several fractions: *1*) whole extracted saliva, *2*) washed

supernatant from samples centrifuged at 5223 × g for 5 min, and *3)* pellet digestion of insoluble phytic acid–salivary pellets formed during interaction. In pellet digestion, 2,2,2-trifluoroethanol was used to disassociate aggregated hydrophobic proteins before spotting samples to MALDI-TOF/TOF. Pellets were then digested with trypsin (Promega, Trypsin Gold) to verify that protein was bound in phytic acid salts.

To reduce MALDI signal loss from phytic acid interference and quantify protein losses with phytic acid interaction, acidic saliva PRP fractions were prepared at lower concentrations with phytic acid concentrations of 0.000512 (physiological, matching clinical study exposure), 0.000256 (0.5×), or 0.000064 (0.125×) mg/100 μL saliva, and a control saliva sample (no additions), to equivalent sample dilutions and buffered to the pH of the physiological phytic acid supplement (pH 5.5). Samples were vortexed for 30 s, then allowed to sit at room temperature (20°C) for 5 min, and finally centrifuged for 5 min at 5223 × g. The supernatant was collected, filtered with a 0.2-μg PVDF syringe filter, and immediately run on HPLC. The remaining precipitate pellet was digested with addition of 10 μL wheat phytase (Sigma Aldrich) in hydrochloric acid–buffered distilled water (62 mg/mL phytase in water; pH 5.5) at 20°C for 5 min, filtered with a 0.2-μg PVDF syringe filter, and run on HPLC. Chromatograms were analyzed for peak reductions at 214 nm, and HPLC-purified peak-loss fractions from phytic acid and control samples were collected from control saliva for tryptic digestion and MALDI-TOF/TOF analysis. A phytase standard was run on HPLC to assess peak changes in the phytase sample created by the enzyme or enzyme impurities. Protein peaks recovered from phytase digestion were also collected and run on MALDI-TOF/TOF for identification.

Tryptic digestion. Pellets and HPLC fractions of interest were subjected to in-solution tryptic digestion. Trypsin (Promega, Trypsin Gold) was added to HPLC purified peaks and phytate pellets at a 1:200 trypsin:protein ratio. Samples were digested in 50 mM ammonium bicarbonate overnight and subsequently spotted to MALDI after acidification with 1% TFA.

MALDI-TOF analysis. Intact mass and in-solution trypsin digestion were analyzed using a MALDI-TOF mass spectrometer, (Bruker Daltonics Ultraflex III) in linear mode for intact proteins and reflectron mode for peptides, in the Biotechnology/Proteomics Core Facility at Kansas State University. Intact proteins were spotted with 20 mg/mL sinapinic acid in 1:1 0.1% TFA/acetonitrile. Peptide digest samples were spotted with 2,5 dihydroxybenzoic acid (50 mg/mL) in 1:1 0.1% TFA/acetonitrile. Digested mass spectra were matched against a SwissProt database for proteins within the intact mass range using mMass software (http://www.mmass.org).

Proof of concept pilot study

This trial is registered at ClinicalTrials.gov (identifier NCT03030703).

Sample size.

A paired t-test sample size calculation (SAS studio version 3.6) determined that 4 participants would be needed to detect a change in incremental area under the curve (iAUC) of 41%, with a pooled SD of 141 found from results in a similarly designed study (9). Sample

size calculation was found to be statistically significant, with power of 0.87, and at an *α*-level of 0.05. Post hoc, we calculated that our recruited sample size (*n* = 7) was powered for a conservative range of findings from the aforementioned study (9), including the maximum SD observed in both groups at study end (SD = 186 with AUC 41% change, *n* = 6), and the minimum significant absorption difference found in the study from week 1 to 8 (SD = 141, AUC change 29%, *n* = 6).

Inclusion/exclusion.

The study protocol was approved by the Institutional Review Board at Kansas State University (#8121). Details of eligibility, recruitment, enrollment, and retention for this pilot study are described elsewhere (32). Participants were premenopausal women, aged 18–35 y, who were not obese (BMI ≤30.0 kg/m^2), had no history of oral or gastrointestinal disease, were moderate (≤1 drink per day) or non–alcohol consumers, and non–tobacco users. Participants were nonanemic (hemoglobin >12 g/L) to reduce the risk associated with potential iron losses resulting from the study. Iron absorption has been significantly changed after antinutritional supplementation in nonanemic, iron-replete individuals previously (32, 33). Participants were compensated for completing study activities. In total, 7 participants were enrolled in, and completed, the study.

Study conditions.

The study consisted of week 0 and week 4 meal challenges, with 4 wk of daily phytic acid supplementation in between. Four-week supplementation periods were chosen as completed previously (32, 34) to assess for iron status changes with inhibited iron absorption at each meal, and to allow for time to adapt to phytic acid. Each participant consumed 350-mg phytic acid supplements (inositol hexaphosphate, ACOS organics), the same dose as provided in a previous 8-week trial (9). Supplements were prepared weekly by an outside researcher; participants returned weekly to pick up supplements and were questioned about supplement adherence, and supplement bottles were checked for total supplement consumption. Supplements were provided in an opaque bottle, formulated with a noncaloric sweetener/flavoring (Mio Original) to improve palatability, and were consumed as liquid supplement 3 times daily for 4 wk with meals.

Phytic acid meal challenges.

Meal challenges followed a format outlined elsewhere (32) at week 0 and week 4 of the studies. Briefly, premeal saliva was collected by passive drool into cryovials, and samples were immediately placed into freezer storage (−80°C). An intravenous catheter was placed, and 2 separate samples were collected in 5-mL serum separator and 3-mL EDTA evacuated tubes to measure serum iron (by spectrophotometry), C-reactive protein (CRP; by nephelometry, sensitivity 0.2 mg/dL), ferritin (by immunoassay, sensitivity 0.1 ng/ml), and whole-blood hemoglobin concentrations (by electronic cell cytometry) (32). After blood draw, a phytic acid challenge meal was consumed with the phytic acid supplement assigned for daily consumption. Meals consisted of the liquid phytic acid supplement, a 95-g bagel with 12 g sugar-free strawberry jam (half sprinkled with 15 mg anhydrous ferrous sulfate, (34) and half with 75 mg ascorbic acid) (9, 35), and a 90-g banana. The molar ratio of the phytic acid supplement to the anhydrous ferrous

sulphate was 0.0054. Salivary samples were taken 15 min following the challenge meal, and blood samples were drawn at 180 and 240 min postmeal in a 3–time point draw to complete serum iron curves as described previously (32). Three-point serum iron curves were found to be consistent with full iron curves proposed previously (35) in our previous work (32). After study activities were completed, blood and serum were sent to Quest Diagnostics for analysis within 24 h (32). Serum iron data were used to calculate percentage of maximum iron recovery and incremental AUC for iron bioavailability analysis. Calculations were completed as described by us (32) and others (35).

Astringency testing.
After the peripheral intravenous line was removed at week 0 and week 4, participants were asked to complete an astringency test (36, 37) as described previously (32) in order to evaluate *1*) whether PRP binding to phytic acid would change astringency and *2*) whether astringency would change with repeated phytic acid consumption. Previous work has described astringency as a result of PRP–tannin binding (13, 29). Each participant was given 4 different concentrations of 10 mL alum powder in distilled water (0.03, 0.07, 1.5, and 2.5 mg/dL) to sip in randomized order.

Dietary analysis.
At the beginning of week 2 of the study, participants were emailed a unique username and password to complete 24-h dietary recalls on 2 weekdays and 1 weekend day on the Automated Self-Administered 24-Hour Recall (ASA24). Details of all dietary analysis have been described previously (32). Food intake logs were downloaded from the ASA24 website for manual calculation of dietary proanthocyanidins, polyphenols, and phytic acid. Proanthocyanidin and polyphenols were calculated to control for other dietary factors that might affect salivary protein concentrations in participants. During this process, a research assistant reviewed all dietary data for each participant, information kept in an electronic spreadsheet (Microsoft Excel). Food items were referenced from the USDA, transferred to an electronic spreadsheet, and total proanthyocyanidin (38) amounts were calculated and summated for each recall. Individual meal recalls were added into the Nutrition Data System for Research (University of Minnesota) post hoc to quantify phytic acid meal content. From these summations, group averages were calculated.

Post hoc enzyme-linked immunosorbent assay confirmation of protein and protein-phytate binding
To confirm MALDI-HPLC findings, peak loss fractions from in vitro phytic acid and control samples were tested for presence and absence of cystatin SN in samples, respectively, using an enzyme-linked immunosorbent assay (ELISA) kit (Raybiotech). Samples were prepared according to manufacturer instructions by adding the instructed sample amount and read on a 96-well plate reader (Biotek Synergy HT) at 450 nm immediately after adding stop solution. Participant saliva from the clinical arm of this study was analyzed to quantify cystatin SN before and after phytic acid supplementation at week 0 and week 4. One participant, whose iron bioavailability data were not obtained due to blood lysis in blood draws at 180 and 240 min, was excluded from analysis. Separately, salivary phytic acid samples

obtained after phytic acid consumption (stimulated saliva, chosen because it has higher greater salivary protein concentrations than fasting saliva) were interacted with phytic acid in vitro to verify phytate formation with cystatin SN. During this experiment, phytic acid was added to saliva obtained after meal consumption at a concentration of 0.00512 mg/100 µL saliva (physiological). Samples were shaken and allowed to incubate for 5 min, saliva was centrifuged at 5223 × g for 5 min, and then the supernatant was spotted onto ELISA 96-well plates.

Statistical analysis
Data were analyzed using SAS statistical software (SAS Studio version 3.6), statistical significance was set at $P < 0.05$, and data are presented as mean $\pm$ SD. Before analysis, all data were analyzed for normality and homogeneity of data in Q-Q plots. Ferritin and CRP values were nonnormal, log transformed, and determined to be normal before analysis. Log-transformed variables were included in stepwise variable selection in adjusted model building (below). All log-transformed data were back-transformed for presentation of results. To look for differences from the mean in individual dietary intake, week 0 demographic and nutritional intake data were analyzed by ANOVA.

Hematological outcomes analysis.
Unadjusted percentage of maximum iron absorption, ferritin, and hemoglobin were analyzed by linear regression at week 0 and week 4 to answer our second primary research question, which asked whether iron bioavailability or status would be affected by repeated phytic acid consumption. Multiple regression was used to adjust outcomes (percentage maximum iron absorption, incremental serum iron AUC, hemoglobin, ferritin) for repeated (participant) and random covariates after stepwise selection for variables. Final outcomes were also adjusted for week 0 outcomes and covariates in the regressive model. Significant differences for all outcomes were determined using pairwise comparison after ANOVA.

Astringency and salivary protein outcomes analysis.
Our third primary research question asked whether changes in salivary protein concentrations were induced by phytic acid consumption over time, and whether there were correlations between salivary proteins and improved iron bioavailability before and after phytic acid supplementation. To answer these questions, proline-rich proteins were divided into type by retention times as described previously (13, 29–32). Salivary proteins were analyzed by multiple factor ANOVA including covariates from regression analysis at week 0 or week 4; Pearson's product-moment correlations were used to analyze correlations between iron absorption, incremental AUC for serum iron, astringency ratings, and PRP types. Changes in participant cystatin SN concentrations were analyzed at week 0 and week 4 by ANOVA, and effect sizes were calculated using the equations:

Hedges' g (unbiased estimator of Cohen's d) (32)

$$= M1 - M2/\textbf{Spooled}$$

Where $S_p = \sqrt{\frac{(ne-1)Se2+(nc-1)sc2}{ne+nc-2}}$ *and M1/M2 were the mean of experimental and control groups.*

The 95% CIs were calculated using the equation:

$$g^* \pm 1.96\,(\sigma g^*)$$

$$\sigma g^* = \left(\frac{ne + nc}{nenc} + \frac{g^*}{2\,(ne + nc)}\right) \text{ and } g^* = g\left(1 - \frac{3}{4\,(ne + nc) - 9}\right)$$

Astringency perception, connections to salivary protein concentrations, and iron bioavailability

We determined whether astringency perception was changed from week 0 to week 4 of phytic acid supplementation using Chi-square testing and Fisher's exact tests. Correlations between salivary protein concentrations, iron bioavailability and astringency were analyzed by Pearson's product-moment correlations.

Results

In vitro phytic acid salivary protein interaction
HPLC analysis of phytic acid-saliva interactions.
To establish whether there was potential for meaningful mediation of iron-phytic acid chelation by salivary proteins, a single volunteer was chosen prior to study activities, and their saliva was used for interaction experiments with phytic acid at various concentrations. The single donor's salivary characteristics showed typical qualitative protein peaks described previously (29), and with salivary samples collected previously (32), and in this pilot study. HPLC results from nonpelleted salivary supernatant extracted after interaction showed progressive peak reduction at 40.3 minutes, suggesting preferential binding of phytic acid to this HPLC fraction (**Figure 1**). We later verified these peak changes from pilot participants in HPLC analysis.
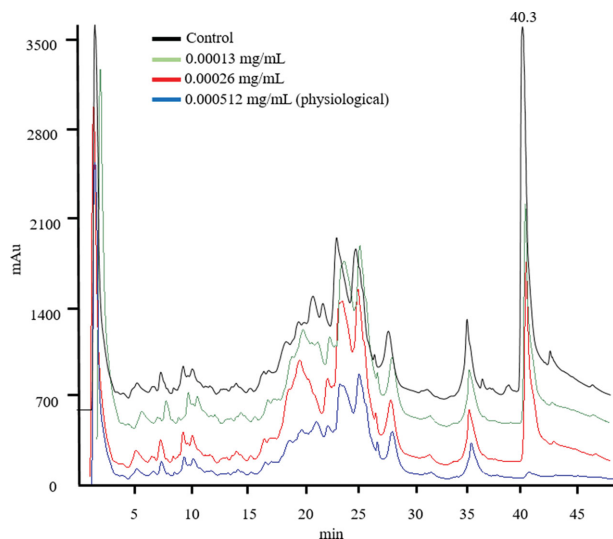

FIGURE 1   In vitro phytic acid–salivary interactions. Progressive peak reductions were seen at 40.3 min, with phytic acid concentration interaction independent of other peak reductions. At physiological doses (equivalent to phytic acid supplement in clinical study), there was total peak loss at 40.3 min.

Saliva-phytic acid pellet MALDI outcomes.
Phytic acid-saliva pellets analysis resulted in peaks like those from purified protein fractions. Pellets were dissolved with tryptic digestion, indicating that the protein precipitated phytic acid. MALDI-TOF results found in vitro phytic acid supernatant sample peak reductions compared with control or acidified saliva (**Figure 2**). This lost fraction was recovered by tryptic digestion of MALDI-spots, also suggesting that phytic acid was directly precipitating specific protein fractions, rather than nonspecific precipitation of proteins due to pH reductions during experiments.

In vitro pellet tryptic digestion and purified protein fraction analysis.
In vitro tryptic digestion of the phytic acid-salivary pellet dissolved the complex, suggesting that proline-mediated bonds were not pellet components, and thus the protein identified on HPLC was not likely a proline-rich protein. MALDI spots of purified whole saliva and in vitro phytic acid fractions collected at 40.3 minutes recovered the same protein peaks as were identified on MALDI after tryptic pellet digestion, suggesting that HPLC peak reductions were the same as MALDI pellet components (Figure 2). To match peptide components to proteins, in vitro phytic acid sample peaks were removed from control samples before searching the database. The best matches were for cystatins S, SN & SA fragments. All cystatins without signal peptides were mapped to the data, the match with the best fit was for cystatin SN (49.6%).

Proof of concept pilot study
Participant demographics.
Participant ages ranged from 20–35, average age of participants was 26.2 ± 1.2 yrs. All participants were occasional (2–3 drinks/month) or moderate (2–3 drinks per week) alcohol consumers. Aside from one participant, who was vegan, and took vitamin B_{12} supplements, no participants took vitamin or mineral supplements during the study period. Average BMI of participants was 25.4 ± 5.8 (range 19.7–29.8). Participant weights (kg) did not significantly change between week 0 (59.3 ± 9.1 kg) and week 4 (60.3 ± 9.4 kg, $P = 0.93$).

Participant dietary intake.
There was good compliance with the study protocol based on sample bottles returned and adherence questions asked of participants at weekly supplement pickup times. Mean caloric intake, calculated by using Atwater factors, during the study was 2107 ± 672.8 kcal/d, 13.7 ± 3.2% of average caloric intake was from protein, 35.1 ± 12.1% and 51.2 ± 20.2% came from fat and carbohydrates, respectively (**Table 1**). Average meat consumption ranged from 0 to 5.7 oz/d. Average ascorbic acid intake exceeded the United States Recommended Daily Allowance (RDA) by 15%, iron intake was on average 93% of the RDA. The average daily phytic acid intake was 863.8 ± 812.8 mg/d (range 199.8–2388 mg/d), phytic acid supplementation more than doubled the typical dietary phytic acid intake for 5 of the 7 participants.

Hematological indices and iron absorption.
    Unadjusted iron outcomes. Due to hemolysis in 1 sample drawn at 240 min on week 4 measurement, the sample size of the iAUC and percentage maximum iron absorption was reduced to $n = 6$ for weeks 0 and 4, as well as week 0 and 4 salivary correlation
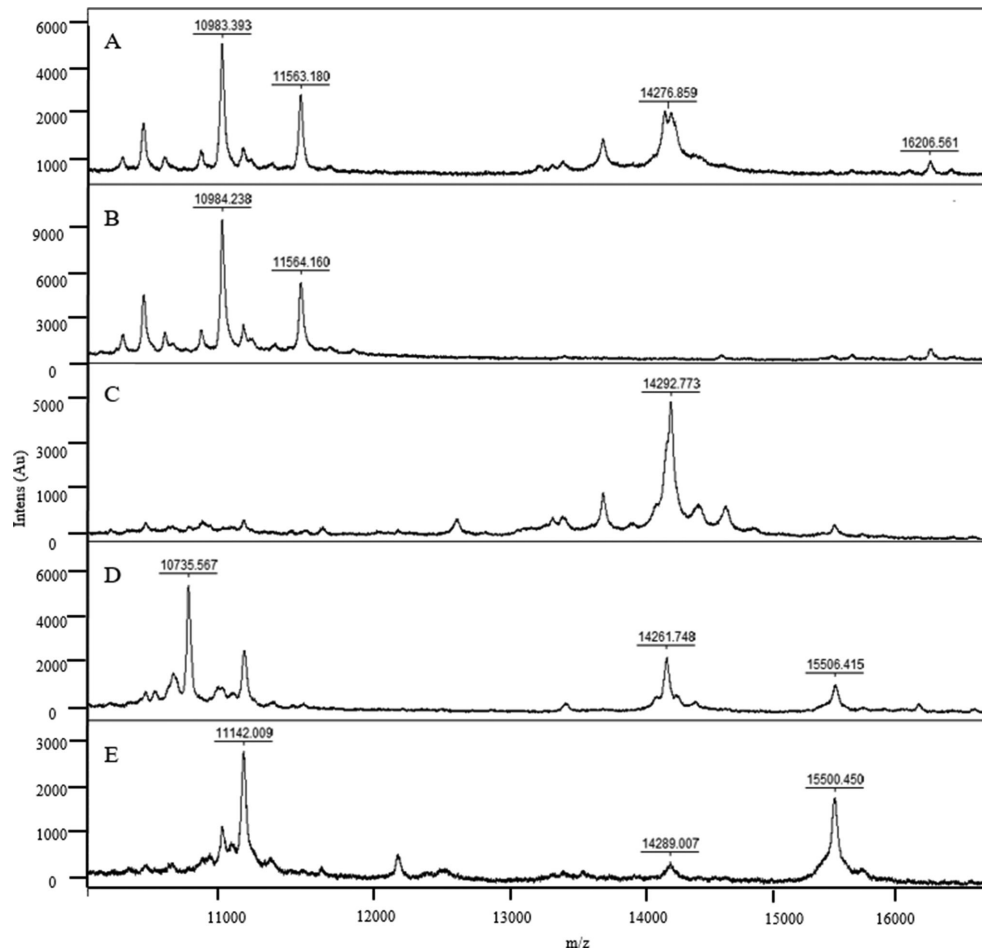
FIGURE 2 The MALDI-TOF whole saliva, phytic acid–interacted supernatant purified protein peak, and phytate pellet digest. The mAu values are correlated with signal strength, rather than concentration with MALDI-TOF analysis. Salivary pellet trypsin digest and purified peak MALDI-TOF results: From the purified peaks collected at 40.3 min, less peak loss was seen in the control purified protein peak (A, pH 4.5) than in the purified protein peak from saliva–physiological phytic acid (0.000512 mg/100 μL saliva) interaction (B, pH 4.5) samples. The peak lost after phytic acid addition to saliva is recovered in 0.000512 and 0.0512 mg/100 μL saliva phytic acid–saliva pellet samples subjected to tryptic digestion (C, pH 4.5, and D, pH 2.3, respectively). This peak was not recovered in the pellet sample acidified with TFA (E, pH 1), suggesting selectivity of phytic acid to this protein. MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; TFA, trifluoroacetic acid.

calculations. Iron absorption was not significantly different from week 0 to week 4 by either percentage maximum iron absorption or iAUC for serum iron (**Table 2**, **Figure 3**). There were no significant differences in hemoglobin, ferritin, or CRP from week 0 to week 4 of the supplementation period both with and without the subject who was not included in the iAUC. To present all relevant results for iron status changes found in the study, results for hemoglobin, ferritin, and CRP are included with the subject excluded in iAUC and percentage maximum iron absorption ($n = 7$, CRP, ferritin, hemoglobin).

Hematological covariate identification and adjustment. To test the impact of dietary and individual physiological differences (iron status, anthropometric, salivary protein) on iron bioavailability and status, we employed stepwise regression analysis to establish significant covariates to build an adjusted model for hematological outcomes. Covariates that were significantly associated with serum iron percentage maxium iron absorption included dietary fat and proanthocyanidin trimer

intake, 0.07 mg/dL astringency rating, aPRP, and cystatin concentration (**Table 3**). Significant covariates associated with iAUC included hemoglobin and proanthocyanidin monomer intake. Ferritin and hemoglobin were both associated with CRP as a covariate; however, hemoglobin was associated with 0.2 mg/dL (highest) astringency rating and bPRP, whereas ferritin was associated with total calorie, dietary protein intake, and total PRP concentrations. Significant covariates for each outcome measure were added to the linear regression for adjusted outcomes analysis. There were no significant differences between week 0 and week 4 percentage maximum iron absorption, hemoglobin, or serum ferritin after adjusted outcomes analysis.

Correlations between salivary protein subtypes, iron absorption, and dietary phytic acid intake.

Week 4 iron bioavailability was significantly positively correlated with bPRP concentration ($r = 0.819$; $P = 0.02$, **Table 4**). Cystatin, statherin, aPRP, and gPRP concentrations were not significantly correlated at

TABLE 1 Participant macronutrient, select micronutrient, proanthocyanidin, and phytic acid intake during the study duration[1] ($n = 7$)

Outcome	Totals
Caloric intake, kcal/d	2107.7 ± 672.8
Protein, g/d	72.4 ± 15.6
Fat, g/d	82.2 ± 28.6
Carbohydrates, g/d	279.2 ± 109.0
Meat, oz/d	3 ± 1.8
Sugar, g/d	132.2 ± 88.4
Fiber, g/d	24.7 ± 13
Iron, mg/d	16.8 ± 7.1
Ascorbic acid, mg/d	69.5 ± 54.2
Zinc, mg/d	11.3 ± 3.2
Copper, mg/d	1.5 ± 0.66
Total proanthocyanidin intake, mg/d	89.1 ± 45.5
Total phytic acid intake, mg/d	863.8 ± 812.8

[1]Values are means ± SDs.

week 0 or week 4 (Table 4). Dietary phytic acid intake was significantly positively correlated with bPRP ($r = 0.89$, $P = 0.007$, data not shown), and cystatin ($r = 0.79$, $P = 0.03$) concentration at week 0. Phytic acid intake was not significantly correlated with percentage maximum iron absorption ($r = 0.71$, $P = 0.11$) or iAUC for serum iron ($r = 0.77$, $P = 0.07$) at week 0.

Astringency testing.
There were no significant differences in astringency ratings from week 0 to week 4 of the study ($P > 0.18$). At week 0, there was a significant positive correlation between the 0.07-mg/dL (middle) astringency rating and iron bioavailability among participants ($r = 0.996$, $P < 0.001$, **Table 5**). Correlations at higher astringency ratings were not significant. At week 4, greater astringency perception ratings with phytic acid consumption were significantly negatively correlated with iron absorption in all but the lowest concentration of alum powder, suggesting that lower ratings of astringency were associated with better iron absorption (Table 5).

ELISA confirmation of purified protein peak findings and participant cystatin SN concentrations
*Confirmation of cystatin SN–phytic acid interactions.*
To confirm cystatin SN–phytic acid interaction, HPLC fractions for control and in vitro phytic acid purified protein saliva samples collected at 40.3 min were assessed using a cystatin SN ELISA. Total cystatin in

TABLE 2 Week 0 and week 4 iron bioavailability, status, and inflammatory indicators[1]

	Week 0 Mean (95% CI)	Week 4 Mean (95% CI)
% max iron absorption[2]	12.8 (−9.1, 34.1)	8.3 (−15.1–31.4)
iAUC, µg/dL/hr[2]	2027 (1787, 2267)	1525 (1188, 1862)
Hemoglobin, g/dl	13.7 (13.4, 13.9)	13.4 (13.1, 13.6)
Ferritin, ng/mL	48.9 (40.1, 57.6)	45.6 (36.8, 54.3)
CRP, mg/dL	0.21 (−0.1, 0.49)	0.20 (−0.5, 0.94)

[1]$n = 6$–7; no significant differences between week 0 and week 4 ($P < 0.05$). CRP, C-reactive protein; iAUC, incremental area under the curve for serum iron.
[2]$n = 6$, hemolysis in serum iron sample in one participant prevented iAUC calculation.
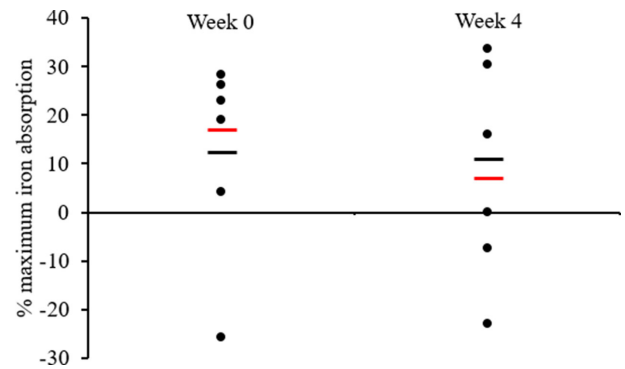
FIGURE 3 Iron bioavailability at week 0 and week 4 phytic acid postabsorptive meal challenges. Unadjusted (black) and adjusted (red) percentage maxium iron absorption at week 0 and week 4 of supplementation. There was a significant reduction in covariate-adjusted iron absorption from week 0 to week 4, $P = 0.02$. There were no significant differences in percentage maximum iron absorption from week 0 to week 4 in unadjusted, $P = 0.22$, models; $n = 6$, hemolysis in serum iron sample in 1 participant prevented iAUC calculation. iAUC, incremental area under the curve.

the control was 132 ng; 8 ng was recovered in the same fraction from 0.000512 mg/mL in vitro phytic acid interaction, suggesting that: *1)* cystatin SN was the protein identified on HPLC and MALDI, and *2)* that phytic acid interaction with saliva reduced cystatin SN in purified peak concentrations.

ELISA analysis of postmeal participant cystatin SN concentrations.
Postmeal participant cystatin SN concentrations were significantly reduced after in vitro interaction with phytic acid ($P < 0.001$), confirming purified protein ELISA results that cystatin SN was precipitated from saliva with phytic acid (**Figure 4**). Cystatin SN salivary concentration did not increase 15 min after meal consumption at week 0 ($R^2 = 0.89$; 95% CI: −0.55, 2.01; $P = 0.23$) and week 4 ($R^2 = 2.14$, 95% CI: −0.48, 2.10, $P = 0.19$) in measured participants. In participant salivary samples, fasting cystatin SN concentrations did not change with long-term phytic acid consumption ($R^2 = -0.14$, 95% CI: −1.39, 1.09). From HPLC results, cystatin SN concentration at week 0 was negatively correlated with iron absorption ($r = -0.977$, $P = 0.006$), but was positively correlated with iron absorption from week 0 to week 4 ($r = 0.996$, $P < 0.001$).

Cystatin SN concentrations were significantly negatively correlated with astringency ratings ($r = -0.82$, $P = 0.046$). There was no correlation between cystatin SN and bPRP at week 0 among participants ($r = -0.10$; $P = 0.86$), but there were positive correlations between them at week 4 ($r = 0.56$, $P = 0.07$, **Figure 5**).

Discussion

In vitro phytic acid saliva analysis
Our primary objective was to establish whether phytic acid would bind to salivary proteins, and identify them if so. To the best of our knowledge, this is the first study that has explored the effects of

TABLE 3 Covariate adjusted estimation of iron bioavailability and status due to phytic acid supplementation[1]

Variable	B	SE B	t	P
% max iron absorption[3]				
Constant	−95.3	3.82	−24.96	<0.0001
Week 0	4.59	1.4	3.31	0.02
Fat intake	0.68	0.04	19.04	<0.0001
Trimer PA	−2.95	0.20	−14.59	0.023
0.07 astringency	36.72	1.93	18.94	<0.0001
aPRP	−46.97	4.84	−9.71	0.0002
Cystatin	0.0005	0.0001	5.00	0.004
AUC serum iron, μg/dL/hr[2]				
Constant	815.0	583.4	1.4	0.196
Week 0	−68.12	64.2	−1.1	0.32
Hemoglobin	−60.6	44.9	−1.35	0.58
Monomer PA	16.35	3.1	5.35	0.0005
Hemoglobin, g/dL				
Constant	16.2	0.93	17.41	<0.0001
Week 0	0.11	0.30	0.37	0.72
0.2 astringency	−0.63	0.21	−3.01	0.017
bPRP	0.56	0.56	1.34	0.22
CRP	−1.58	0.65	−2.45	0.040
Ferritin, ng/dL				
Constant	97.7	27.1	2.49	0.037
Week 0	−5.09	6.91	−0.74	0.48
CRP	−66.3	14.79	−4.48	0.002
Kcal	−0.02	0.004	−3.68	0.006
Protein	−0.93	0.18	−3.77	0.006
Total PRP	174.02	35.2	2.67	0.028

[1] $n = 6$–7, significance: $P < 0.05$. aPRP, acidic proline-rich protein; bPRP, basic proline-rich protein; CRP, C-reactive protein; iAUC, incremental area under the curve for serum iron; PA, proanthocyanidin; PRP, proline-rich protein.
[2] $n = 6$, hemolysis in serum iron sample in one participant prevented iAUC calculation.

phytic acid supplementation on salivary proteins. PRPs did not bind to phytic acid. Instead, phytic acid formed complexes with another nonenzymatic salivary protein, cystatin SN. This finding was supported through tryptic and phytase digestion of phytate–saliva pellets formed after interaction, and co-analysis with supernatant fractions of whole and phytic acid–treated saliva. ELISA confirmation that cystatin SN in purified peak fractions and human participant saliva interacted in vitro with phytic acid suggests that this binding is consistent in a variety of participants. The findings that PRPs do not bind to phytic acid is an important one. Tannin–PRP binding is specific (24), and bonds may not dissociate during digestion (23). Because PRPs do not bind with phytic acid in addition to tannins, PRP-mediated protection against tannin–iron chelation may be viable in phytic acid– and tannin-rich foods.

TABLE 5 Correlations between astringency and percentage max iron absorption[1]

Dose	Week 0 Mean (95% CI), r	Week 4 Mean (95% CI), R
0.03 mg/dL	1 (0.7, 1.3), 0.0	1 (0.7, 1.4), 0.0
0.07 mg/dL	2 (1.6, 2.4), 0.996*	1.8 (1.3, 2.3), −0.959*
1.5 mg/dL	2.9 (2.3, 3.4), 0.492	3.0 (2.3, 3.7), −0.84*
2.5 mg/dL	4 (3.4, 4.6), 0.553	4.2 (3.5, 4.9), −0.768*

[1] 1 = not astringent, 5 = extremely astringent, $n = 6$. Dose: astringency concentration testing at weeks 0 and 4 of phytic acid supplementation. *$P < 0.05$. Hemolysis in serum iron sample in one participant prevented iAUC calculation; results for that participant's salivary outcomes have been removed. iAUC, incremental area under the curve for serum iron.

Unlike other cystatin proteins, cystatin SN is only found in saliva. Its primary purpose is inhibition of cysteine proteolysis, but it has been shown to be associated with enhanced tolerance of bitterness in infants and caffeine consumers (39, 40), which is consistent with our findings that higher cystatin SN concentrations were significantly correlated with lower astringency ratings. In addition, induction of S-type cystatins with capsaicin ingestion was found in rats, suggesting that these proteins may be stimulated with nonproteolytic oral irritants (41) like phytic acid.

Proof of concept pilot study
Hematological outcomes.
Similar studies have observed significant changes in ferritin (30), and hemoglobin (42) in nonanemic women consuming antinutritional factors over a period of 3–4 wk, respectively, suggesting that these markers of iron status can be changed with antinutritional factors. Although week 0 percentage maximum iron absorption was significantly greater than that for week 4 in the covariate adjusted model (Table 4), phytic acid supplementation did not change iron absorption, hemoglobin, or ferritin when consumed 3 times daily for 4 weeks in both covariate-adjusted and -nonadjusted models (Tables 3 and 4). Interestingly, although percentage maximum iron absorption was adjusted for several dietary and nondietary factors (Table 4), it was not adjusted for hemoglobin, which may explain predicted iron absorption differences between incremental AUC and serum iron concentrations (Table 4). Overall, these findings differ with those of a similar study that found improvement in bioavailability of a high-phytate meal after consumption of a phytate diet for 8 wk (9). However, it should be noted that there was great variation in dietary iron intake and an overall reduction in percentage maximum iron absorption and iAUC at week 4. Individual data show that these outcomes were truly variable among participants; 50% showed reductions in iron absorption over time, and 50% did not. Whereas increases in iron absorption might suggest
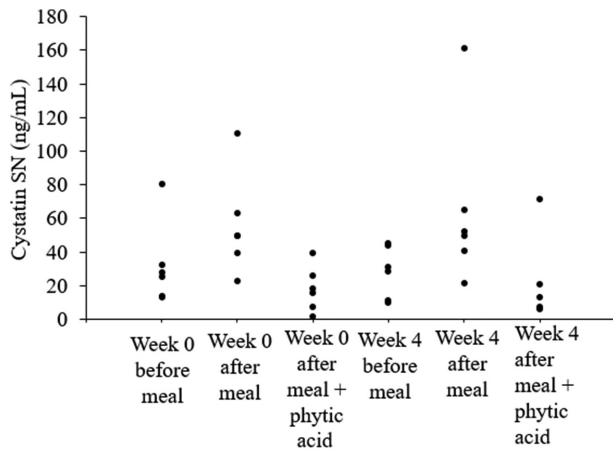
TABLE 4 Correlations between protein concentrations and percentage max iron absorption at week 0 and week 4 of phytic acid supplementation[1]

Week	bPRP r(P)	aPRP r(P)	gPRP r(P)	Statherin r(P)	Cystatin r(P)	Total r(P)
0	0.33 (0.46)	0.53 (0.23)	0.48 (0.28)	0.25 (0.59)	0.53 (0.23)	0.54 (0.21)
4	0.82* (0.02)	0.03 (0.95)	0.30 (0.52)	0.55 (0.20)	0.27 (0.56)	0.36 (0.43)

[1] $n = 6$; *$P < 0.05$. Hemolysis in serum iron sample in one participant prevented iAUC calculation; results for that participant's salivary outcomes have been removed. aPRP, acidic proline-rich protein; bPRP, basic proline-rich protein; gPRP, glycosylated proline-rich protein; iAUC, incremental area under the curve for serum iron.

FIGURE 4 Cystatin SN concentrations before and after phytic acid meal challenges at weeks 0 and 4. Cystatin SN ELISA results, each dot represents a study participant. Phytic acid in vitro interaction with saliva significantly reduced cystatin SN concentrations from saliva supernatant. There were no significant changes in cystatin SN concentrations before ($R^2 = -0.14$; 95% CI: -1.39, 1.09), or after ($R^2 = 0.24$; 95% CI -1.08, 1.40) meals from week 0 to week 4, $n = 6$, hemolysis in serum iron sample in one participant prevented iAUC calculation, results for that participant's salivary outcomes have been removed. iAUC, incremental area under the curve.

adaptation, or no changes suggest lack of adaptation, reductions in iron bioavailability noted in our participants could as likely be attributed to iron status (43) or inflammatory levels (44) as to worsening iron absorption due to phytic acid supplementation.

There is evidence that inulin improves iron bioavailability with phytic acid consumption over time (45), meaning that efficient adaptation may be due to combinations of homeostatic mechanisms, including changes in gut microbiota to resistant starch often found in phytate-rich foods. Our study examined the effect of phytic acid alone,
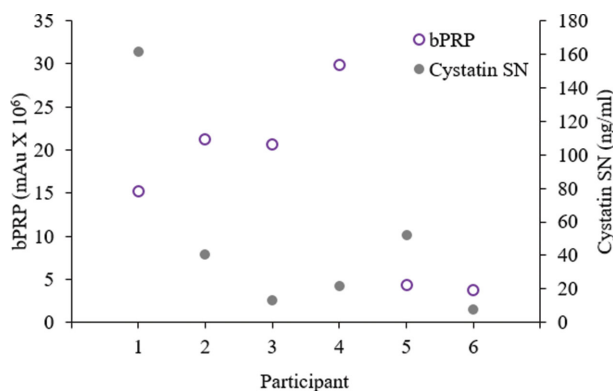

FIGURE 5 Comparison of week 4 bPRP and cystatin SN concentrations for individual participants and week 4 bPRP and cystatin SN for each participant. There is a positive correlation ($r = 0.56$), $P = 0.07$, between week 4 bPRP and cystatin SN concentrations. $n = 6$, hemolysis in serum iron sample in one participant prevented iAUC calculation, results for that participant's salivary outcomes have been removed. bPRP, basic proline rich protein; iAUC, incremental area under the curve.

which could explain differences between the present studies, which used liquid supplement, versus the aforementioned study, which used phytate-rich foods rich in fiber.

Contrary to findings in single-meal (3–5), and in vitro Caco-2 (46–48) models, we did not find that iron status, as indicated by ferritin or hemoglobin concentrations, was reduced with phytic acid supplementation over time. The current study findings are, however, consistent with results from multiple rat studies (45, 49–56). Despite similar findings, higher intestinal phytase activity should be considered in rat models (57). Findings from single-meal studies, and Caco-2 cell models, may not predict adaptive mechanisms employed by certain populations toward antinutritional factors like phytic acid over time (32, 58). In support of this assertion, in studies exploring long-term iron bioavailability in rat versus acute Caco-2 iron bioavailability, phytic acid inhibited iron bioavailability in in vitro, but not in vivo models (59, 60). The Caco-2 cell model may not accurately simulate nuances of digestion and absorption, such as salivary protein profiles and their effect on antinutritional factors and absorption.

Salivary protein concentrations correlations with iron bioavailability, and astringency.
There were no significant changes in salivary proteins over time, but although in vitro binding of PRPs with phytic acid did not occur, bPRP concentrations were significantly correlated with improved iron bioavailability at week 4 ($r = 0.819$, $P = 0.02$). Interestingly, bPRP concentrations significantly correlated with dietary phytic acid intake at week 0, suggesting that people who commonly consume phytic acid–rich foods may produce more bPRP than those who do not. These findings may suggest that repeated phytic acid consumption induces bPRP production.

Perhaps supporting this idea, astringency ratings at week 0 were positively correlated with iron bioavailability, whereas week 4 astringency ratings were negatively correlated. Previously, we found that bPRP concentrations were negatively correlated with astringency ratings as well; therefore, these findings are consistent with previous bPRP–iron absorption correlations (32). Combined, these data suggest that in individuals who do not typically consume phytic acid–rich diets, aPRP, gPRP, and total protein concentrations predict iron bioavailability in the short term, but bPRP concentration better predicts iron bioavailability overall.

Cystatin SN and iron bioavailability
There were no significant differences in cystatin SN concentration after daily phytic acid supplementation over 4 wk. Regardless, week 0 cystatin SN concentrations and iron absorption were negatively correlated ($r = -0.97$, $P = 0.006$). At week 0, cystatin SN concentrations were not correlated with bPRP concentrations, which suggests that cystatin SN may predict suboptimal iron bioavailability independent of bPRP concentrations in non–regular phytic acid consumers. It is possible that cystatin SN concentrations, in lieu of other salivary proteins or protective mechanisms, are inefficient in protecting against phytic acid–iron chelation. Thus, elevated cystatin SN concentrations may be a marker of inefficient phytic acid protection. We found that tryptic digestion easily destroyed cystatin SN–phytic acid complexes, raising questions regarding stability during digestion, which would likely be poor. It is possible that cystatin SN does not trigger nonsalivary

protective mechanisms to phytic acid as efficiently as other proteins. Higher cystatin SN concentrations at week 4 were positively correlated with bPRP concentrations, which may explain the significant positive relation between cystatin SN and iron bioavailability at week 4.

Limitations

This study was conducted in a small sample of participants from another study (32) that were willing to continue to participate in this pilot project. Due to the variability in iron absorption among participants, statistical power to detect significant findings was limited, including differences in cystatin SN concentrations from week 0 to week 4 of the study (effect size: 0.24; 95% CI:−1.08, 1.40). In addition, we used phytic acid, rather than food-source phytates, for the model. There is evidence that tannic acid may bind to salivary PRPs differently than condensed proanthocyanidins found in food (58), and it is reasonable to believe that phytates consumed in food may have different effects than those consumed in highly ionized, liquid form. Additionally, compared with a similar clinical trial (9), our participants' average week 0 phytic acid consumption was greater (863 mg versus 718 mg), more variable (199.8–2388 mg/d versus 548–941 mg/d), and increased during the study (week 4: 1913 versus 1190 mg/d), which may have affected bioavailability over time. Our study only lasted 4 wk, and although we anticipated that hemoglobin and ferritin would be impacted within this time frame given previous work in this time frame (61–64), other studies have used longer supplementation periods that have produced significantly improved iron absorption from study start to end (9). It may be that long-term phytic acid supplementation induces nonsalivary mechanisms not assessed with our study. It is also important to note that although cystatin SN concentrations did not change through the study, lack of measurement of salivary flow rate is a limitation in protein concentration quantification. Our study used a predetermined 2-mL salivary sample (which was obtained over varying time spans), and thus participants with higher or lower salivary flow rates may have had different total cystatin concentrations. Lastly, the current study was conducted in iron-sufficient, premenopausal women with a nutrient-replete and varied diet. It is important to note that within our study, iron intake was widely variable among participants, which may have impacted ferritin and hemoglobin outcomes. In Malawian children, a high-phytate diet resulted in increased zinc excretion in sick, but not well, children, highlighting the complexity of metabolism potentially based on inflammatory status (65). Findings should be investigated in other populations of interest, including children (39) and pregnant women (66), who have different salivary protein profiles than the current study population.

Future research

Future studies are needed to determine effects of salivary proteins on phytic acid–mediated reductions in iron bioavailability. In addition, more studies are needed to explore the nonchelating protective effects of bPRPs on iron bioavailability, and the potential effects of cystatins throughout the gastrointestinal tract, including potential interactions between salivary proteins and microbiota. Studies exploring consumption of foods with multiple antinutritional factors are needed to understand the interactions between compounds commonly co-consumed and salivary profiles, including secondary effects from salivary proteins on enterocyte-mediated iron bioavailability. Finally, further research is needed to understand the physiological changes during childhood development, pregnancy, and other vulnerable states to anemia on salivary protein production, iron absorption, and antinutritional factor impact.

Conclusions

To the best of our knowledge, this is the first time that salivary protein–phytic acid interactions have been investigated. Phytic acid does not specifically bind with PRPs, but does bind with cystatin SN, a non–proline rich salivary protein. Neither iron bioavailability nor status was significantly affected by 4 wk of phytic acid intake. Cystatin SN concentrations were significantly negatively correlated with iron absorption in subjects consuming phytic acid over time. This finding suggests that this protein is inefficient in protecting against phytic acid; however, increased salivary cystatin concentrations may allow for the identification of individuals who do not adapt to phytic acid. bPRP concentrations positively influenced iron absorption with phytic acid consumption. Overall, these pilot study findings indicate that repeated phytic acid consumption may not negatively influence iron bioavailability and status, and that salivary proteins may help protect against negative effects from phytic acid consumption. Larger and longer clinical studies are needed to confirm these pilot study findings.

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