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Effect of lactoferrin on taste and smell abnormalities induced by chemotherapy: A proteome analysis

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1 **Effect of lactoferrin on taste and smell abnormalities induced by chemotherapy:**

2 **A proteome analysis**

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24 **Abstract**

25 Cancer patients receiving chemotherapy often experience taste and smell abnormalities (TSA).
26 To date, the underlying molecular mechnisms of this frequent side-effect have not been
27 determined and effective treatments are not available. This study assessed the feasibility of
28 lactoferrin (LF) supplementation as a treatment for TSA and investigate the related mechanisms
29 through salivary proteome analysis. Nineteen cancer patients with establishedTSA following
30 chemotherapy administration were enrolled in this study. Cancer patients and additional 12
31 healthy subjects took LF supplements, 3 tablets per day (250 mg/tablet), for 30 days. Saliva was
32 collected at three timepoints: baseline, 30-day LF supplementation, and 30-day post-LF
33 supplementation. Patient's TSA level, salivary proteome, and salivary minerals at each LF
34 treatment stage were analyzed. High TSA level was associated with high concentration of
35 salivary Fe and loss of critical salivary immune proteins. LF supplementation significantly
36 decreased the concentration of salivary Fe ($P = 0.025$), increased the abundance ($P < 0.05$) of
37 salivary α-amylase and Zn-α-2-GP, and led to an overall increase of expression (≥ 2-fold changes)
38 of immune proteins including immunoglobulin heavy chain, annexin A1, and proteinase inhibitor.
39 Abundance of α-amylase and SPLUNC2 were further increased ($P < 0.05$) at 30-day post-LF
40 supplementation in cancer patients. At the same time, total TSA score was significantly reduced
41 ($P < 0.001$) in chemotherapy patients. This study demonstrated the feasibility of developing
42 lactoferrin supplementation as a treatment to reduce TSA caused by chemotherapy and improve
43 cancer patient's oral immunity.

44

45 **Keywords** Chemotherapy; Lactoferrin; Taste and smell abnormalities; Immune proteins; Saliva

46

47 **Introduction**

48 Many cancer patients receive chemotherapy as treatment during the course of their
49 disease. A secondary effect of this treatment is taste and smell abnormalities (TSA), experienced
50 by a large proportion of this population.[1,2] Bernhardson et al.[3] surveyed 518 patients following ≥
51 6 weeks of chemotherapy and 75% of these patients described taste and smell changes during
52 chemotherapy. The most common TSA symptom described by cancer patients is the perception
53 of a persistent metallic flavor and/or aftertaste with or without food intake.[4] TSA commonly
54 occurs during active chemotherapy administration, and can last several hours, several weeks, or
55 even several months after the completion of the treatments.[5,6] As a consequence, affected
56 patients may suffer poor appetite, weight loss, depression, and diminished nutrition, all of which
57 are detrimental to clinical rehabilitation.[1,3]

58 Although TSA is widespread and a frequent complaint of cancer patients, there are no
59 established therapies that reliably prevent or treat this problem. In a systematic review of the
60 literature,[6] the authors found only 26 articles addressing metallic taste in chemotherapy patients,
61 illustrating a paucity of information to address this frequent side effect. A dietary supplement of
62 synsepalum dulcifum, which is known as “miracle fruit”, has been demonstrated to temporarily
63 (20-30 min) mask the metallic taste in 5 out of 8 patients receiving chemotherapy.[7] A more
64 effective and longer lasting therapy is urgently needed to improve the prolonged and recurring
65 metallic taste abnormality described by cancer patients.

66 Recently, iron-binding proteins that effectively reduce iron-induced metallic flavor are
67 garnering attention. In our previous study, post-rinse of lactoferrin solution (10.4 mg/L) in the
68 mouth significantly decreased ($P < 0.05$) metallic flavor stimulated by ferrous sulfate solution (1
69 mg/L) in 53 healthy subjects.[8] Thus, we hypothesized that LF may be used to treat

70 chemotherapy-induced TSA, which most commonly presents as a metallic taste abnormality. In
71 addition, accumulated evidence has indicated that chemotherapy affects the integrity of the
72 immune system and destroys salivary peroxidase system.[9] Lactoferrin is well-known as a first-
73 line defense in the human body and induces host immune-modulatory activation.[10] Effects of oral
74 LF supplementation on immunity-related gene expression in the small intestine have been well
75 studied.[11,12] However, influence of LF supplementation on immune proteome, especially salivary
76 proteome in chemotherapy patients has not been reported yet.

77 The objective of this study is to assess the feasibility of LF supplementation as a treatment
78 for TSA induced by chemotherapy. In addition, our study investigated the molecular etiology of
79 TSA in chemotherapy patients and the treatment mechnism of LF supplementation by analyzing
80 salivary protein profiles through two-dimensional electrophoresis analysis.

81 Materials and methods

82 Human subjects

83 This study was approved by the Institutional Review Board at the Wake Forest Baptist
84 Medical Center (CCCWFU 98112) and Virginia Tech (IRB 14-880). The study was registered at
85 clinicaltrials.gov as NCT01596634. Nineteen cancer patients (8 females, ages 45-79 years with
86 median age of 65), who had developed self-reported TSA after receiving chemotherapy, were
87 recruited by treating oncologists at the Wake Forest Baptist Comprehensive Cancer Center for
88 this study. Eligible cancer patients had normal taste perception before the development of cancer
89 and were currently undergoing chemotherapy at the time of enrollment. Any dose or schedule of
90 chemotherapy administration was allowed as long as patients had self-reported TSA. Exclusion
91 criteria included difficulty in producing abundant saliva, HIV-positive test result, pregnant or
92 breastfeeding, milk/iron allergy, active oral infection, or active mucositis. A wide variety of

93 cancer types were represented in the patients enrolled on this study, including colorectal (6),
94 breast (4), brain (3), pancreatic (2), lymphoma (2), mycosis fungoides (1), and myeloma (1). All
95 19 cancer patients received LF supplementation and serial TSA assessements. A pre-salivary
96 proteome analysis was conducted on all patients. Twelve patients whose saliva showed
97 repeatable and stable protein profiles in two-dimensional electrophoresis gels were chosen for
98 proteome and mineral analysis.

99 Additionally, twenty healthy subjects were originally recruited for this study as normal
100 controls from the local community (New River Valley region, and students, faculty and staff of
101 Virginia Tech) by Virginia Tech researchers. Six of the healthy subjects were withdrawn from
102 the study and two was exluded due to poor repeatability and stability of salivary protein
103 composition shown on 2-D image. Therefore, 12 healthy subjects (six females) with age ranging
104 from 45-71 years (median age of 59) were ultimately enrolled. Enrolled healthy subjects had
105 normal taste perception, no chronic oral or general health problems, no milk/iron allergy, were
106 non-smokers, and were neither pregnant nor breastfeeding.

107 **Lactoferrin treatment**

108 Enrolled cancer patients (n=12) and healthy subjects (n=12) were provided with
109 lactoferrin tablets, with directions to take one tablet three times a day for 30 days. Lactoferrin
110 tablets (250 mg/tablet) used in this study were purchased from Jarrow Formulas Inc. (Los
111 Angeles, California). Lactoferrin (LF) supplementation was continued for 30 days, followed by a
112 30-day washout period. Data collection occurred before LF supplementation (baseline),
113 following 30-days of LF supplementation, and 30 days after the completion of LF
114 supplementation (30-day post-LF supplementation).

115 **Salivary collection**

116 Saliva samples were collected at each stage of LF treatment for all subjects. Participants
117 were required not to consume any food or beverage (except water) at least one hour prior to
118 saliva collection. Before sample collection, participants were instructed to rinse their mouth with
119 purified drinking water (Kroger®). After a 1-minute rest, they sipped 2 mL of purified drinking
120 water as the control sample, swished it around their mouth for 15-20 sec. Without swallowing,
121 participants expectorated saliva into a clean sample collection tube until approximately 4 mL of
122 saliva was collected. Collected saliva samples were immediately frozen and stored at -80°C until
123 analysis.

124 **TSA assessment**

125 We used a taste and smell questionnaire (Appendix) that has been previously used to
126 evaluate TSA in AIDS patients,[13] to assess TSA of the cancer patients in our study. The
127 assessment was conducted prior to saliva collection. In the taste section, cancer participants were
128 asked to rate their individual taste changes as "insignificant", "mild", "moderate", "severe", or
129 "incapacitating" during LF treatment, and rate their taste abnormality when experiencing salt,
130 sweet, sour, and bitter. This tool yields a taste score (0-10) based on subject's responses to nine
131 questions addressing changes to the sense of taste. One point was added for each reported taste
132 complaint and additional one point would be added if a rating was "severe" or "incapacitating".
133 Similarly, a smell complaint score (0-6) was generated by adding one point for a positive
134 response to each of five questions addressing self-perceived changes to the senses of smell. Two
135 points were assigned to a severity rating of "severe" or "incapacitating" for the severity of the
136 smell abnormality question. The total abnormality score (0-16) was calculated by adding the
137 taste and smell abnormality scores.

138 **Salivary proteome analysis**

139 Twelve of the cancer patients and 12 healthy subjects were selected for salivary proteome
140 analysis. Selection criterion included salivary proteins which were able to be clearly separated on
141 2-DE gels, number of missing proteins spots between duplicate gels was below 10, and
142 correlation coefficients (calculated by PDQuest® software) of protein spots between gels of each
143 group (healthy and cancer) was above 0.85. Saliva samples were analyzed by two-dimensional
144 electrophoresis (2-DE) as previously described.[14] Thawed samples were mixed thoroughly by
145 vortex mixer followed by centrifugation at 18500 ×g (4 °C) for 15 min to reduce viscosity and
146 remove debris.

147 Proteins in each saliva sample were precipitated by adding a solution containing 10%
148 trichloroacetic acid (TCA)/90% acetone/20mM dithiothreitol (DTT) at twice the volume of
149 saliva sample, and chilled overnight at -20 °C.[15] The next day, the samples were placed in a
150 chilled centrifuge (18500 ×g, 4°C) for 15 min to pelletize the protein. The pellet was washed a
151 second time using a 20 mM DTT/acetone wash and spun in the chilled centrifuge (18500 × g,
152 4°C) for 15 min to pellet the protein once again.

153 The protein pellet was then resuspended in the 2D cell lysis buffer containing 9 M urea, 2%
154 CHAPS (w/v), 50 mM DTT, 0.5% IPG buffer (v/v), and 0.01% bromophenol blue. Saliva protein
155 concentration was determined by 2D-Quant Kit (GE Healthcare, Pittsburgh, PA) following the
156 protocol given in the brochure and using BSA as a standard. Each saliva sample was loaded (20
157 µg protein) on a 11cm immobilized pH gradient strip (pH 3-11NL, GE Healthcare, Pittsburgh,
158 PA), and carried out by GE Healthcare Ettan IPGphor 3 Cell (GE Healthcare, Pittsburgh, PA).
159 Sample strips were equilibrated with a two-step process: (1) rinsed with equilibration buffer
160 containing 6 M urea, 4 % SDS (w/v), 0.375 M Tris-HCl (pH 8.8), 20 % glycerol (v/v), and 130
161 mM DTT for 15 min, (2) rinsed with equilibration buffer containing 6 M urea, 4 % SDS (w/v),

162 0.375 M Tris-HCl (pH 8.8), 20 % glycerol (v/v), and 130 mM iodoacetic acid (IAA) for 15 min.
163 Then, sample strips were transferred into 11 cm Criterion Precast 12.5 % Polyacrylamide Gels
164 (BIO-RAD, Hercules, CA). The gels were run at 35 mA for 15 min and then at 70 mA until the
165 dye front ran out of the gels. Gels were stained using Flamingo™ Fluorescent Gel Stain (BIO-
166 RAD, Hercules, CA) following the manufacturer's instructions or stained by silver staining
167 (PlusOne Silver Staining kit; GE Healthcare). Gel images were scanned by Molecular FX Imager
168 (BIO-RAD, Hercules, CA).

169 **In-gel trypsin digestion and mass spectrometry identification**

170 Selected spots were excised from 2-DE gels by hand with spot picker. Protein digestion
171 was carried out by adding 0.065 μg of trypsin and incubated on ice for 15 min, then followed by
172 incubation at 37 °C overnight. The next day, 1 μL of each digest was transferred to a freshly-
173 polished MALDI plate and covered with freshly-prepared matrix containing 4 mg/mL α-cyano-
174 4-hydroxycinnamic acid, 50 % CH_3CN, 0.1 % TFA (v/v), 0.1 % formic acid (v/v), and 5 mM
175 $(NH_4)Cl$. Protein identification was performed by an Applied Biosystems 4800 MALDI-
176 TOF/TOF (matrix-assisted laser desorption ionization-time-of-flight/ time-of-flight) mass
177 spectrometer (AB Scienx, Framingham, MA), which was based on peptide fingerprint mass
178 mapping (using MS spectra) and peptide fragmentation mapping (using MS/MS spectra). The
179 MASCOT search engine software (Matrix Science, Boston, MA) was used to identify proteins
180 from the National Center Biotechnology Information nonredundant Homo sapiens amino acid
181 sequence database. The parameters for searching and identifying matches were adjusted as
182 follows: enzyme of trypsin, 1 missed cleavage, fixed modifications of carbamidomethyl (C),
183 variable modifications of oxidation (M), peptide mass tolerance: ± 0.5 Da, fragment mass
184 tolerance: ± 0.5 Da, mass tolerance of 30 ppm, peptide charge of 1+ and monoisotopic.

185 **Salivary minerals analysis**

186 Thawed saliva samples (500 μL) were diluted with 4.25 mL deionized water followed by
187 digesting with 250 μL trace metals grade nitric acid (TraceMetal™ Grade, Fisher, St. Louis, MO)
188 at room temperature, which resulted in a final dilution ratio at 1:10 (v/v). Reagent blank was
189 prepared by adding 250 μL nitric acid into 4.75 mL deionized water. Concentration of salivary
190 minerals including iron, magnesium, potassium, copper, zinc, sodium, calcium, phosphorus,
191 sulphur, and chloride of each diluted saliva sample were measured by emission spectroscopy
192 using Inductively Coupled Plasma (ICP) technique (Thermo Electronic Corporation, X-Series
193 ICP-MS, Waltham, MA).[16,17]

194 **Statistical analysis**

195 Differential expression of salivary proteins between 2D gels were analyzed by
196 PDQuest® software v.7.3.1 (Bio-Rad, Hercules, CA). Intensity of proteins was determined as the
197 percentage of total valid spots volume on respective gels. Protein spots that had at least 2-fold
198 change in intensity were considered as differences among treatments in each replicated group. P
199 value was calculated based on Wilcoxon test and $P < 0.05$ was used as cutoff for significance.
200 Differences of abnormality score among different stages of LF treatment were analyzed by one-
201 way ANOVA (analysis of variance) followed by Tukey's test ($\alpha = 0.05$). Concentrations of
202 salivary minerals between healthy and cancer subjects at each stage of LF treatment were
203 analyzed by student's t-test ($\alpha = 0.05$). Changes of salivary minerals between each stage of LF
204 treatment were analyzed by paired t-test ($\alpha = 0.05$) for both healthy and cancer subjects.
205 Statistical analysis was performed by statistical software programs JMP® Pro 13.0.0.

206 **Results**

207 **Taste and smell abnormalities**

208 TSA scores of cancer patients (n=12) at each stage of LF treatment are shown in Fig.1.
209 Compared with baseline, taste abnormality scores ($P = 0.0197$), smell abnormality scores ($P =$
210 0.0110), and total abnormality scores ($P = 0.0006$) were significantly reduced for cancer patients
211 at 30-day post-LF supplementation. A decreasing TSA score implies improved taste and smell
212 function. Although there was no significant change ($P > 0.05$) of taste/smell/total abnormality
213 score at 30-day LF supplementation, a decreasing trend in patients' abnormality score was
214 observed throughout the intake of LF supplements.

215 **Salivary proteome**

216 Based on repeatability and stability of salivary proteins shown on 2-D image, twelve of
217 the cancer patients and 12 healthy subjects were subjected to salivary proteome analysis. Saliva
218 of each human subject was loaded on each 2-DE gel at equal amount of protein (20 μg) for 2-DE
219 analysis. After in-gel image analysis, 102 salivary protein spots with expression differences in
220 intensity were found in all comparisons (cancer patient/healthy subjects, pre/post -LF
221 supplementation). Based on their fold change (≥ 2-fold change) and abundance, 47 protein spots
222 were further excised and analyzed by MALDI-TOF-TOF mass spectrometry. Identified protein
223 spots were marked on each 2-DE image with consistent spot ID number (Table 1). The reference
224 whole salivary proteome maps from representative healthy subjects and cancer patients are
225 shown in Fig. 2. Fold changes of differentially expressed proteins in saliva of healthy subjects
226 and cancer patients at each stage of LF treatment are shown in Table 2.

227 *Healthy subjects*

228 LF supplementation led to a significant increase (Wilcoxon test, $P < 0.05$) in intensity of
229 zinc-alpha-2-glycoprotein (Zn-α-2-GP) (spot 8,13,14,15) and prolactin-inducible protein (PIP)
230 (spot 16-21) in saliva of healthy subjects (Fig. 2b, Table 2). Abundance of these salivary proteins
231 then significantly decreased ($P < 0.05$) without consistent intake of lactoferrin tablets (Fig. 1c).
232 There was no significant increase ($P = 0.068$) in intensity of salivary lactoferrin (spot 35) in
233 healthy subjects along with LF supplementation.

234 Human whole saliva proteome showed variation between individuals in previous
235 studies,[16,17,18] especially the relative location and intensity of low-abundance salivary proteins.
236 Although this variation was also found in our study, low-abundance salivary proteins [pH 5.5-8.5,
237 MW (molecular weight) 25-75 kDa] in healthy subjects showed an overall increase (intensity ≥
238 2-fold change) in response to LF supplementation (Fig. 2b). The up-regulated protein spots
239 included CAVI (spot 33,34), α-amylase (spot 24,36,37,40,41,42,43), and immunoglobulin heavy
240 chain (spot 28,29), as shown in Table 2. However, most of the up-regulated proteins did not
241 retain their intensity in saliva after the termination of LF supplementation (Fig. 2c). Furthermore,
242 we found that after termination of LF intake for 30 days, composition of low-abundance salivary
243 proteins was changed as the expression of α-amylase (spot 22,23,24,38,39,44,45) was
244 significantly increased ($P < 0.05$) and the intensity of CAVI (spot 5,6,7,32,33,34) was decreased
245 ($P < 0.05$) (Table 2).

246 *Cancer patients*

247 Compared with healthy subjects, cancer patients showed significantly lower ($P < 0.05$)
248 intensity of salivary α-amylase (spot 51, spot 52,53), Zn-α-2-GP (spot 8,13-15), PIP (spot 16-21),
249 and low-abundance proteins (pH 5.5-8.5, MW 25-75 kDa) at baseline. LF supplementation
250 significantly increased ($P < 0.05$) the intensity of salivary Zn-α-2-GP (spot 8,15) and α-amylase

251 (spot 52,53) compared with baseline (Fig. 2e). Low-abundance salivary proteins spots presented
252 an overall increase in expression (≥ 2-fold change) along with LF supplementation, including
253 immunoglobulin heavy chain (spot 29), annexin A1 (spot 48), proteinase inhibitor (spot 22), and
254 α-amylase (spot 23,24,49) (Table 2). A post-LF supplementation effect was observed, in which
255 the intensity of α-amylase (spot 37,39,44,45,49,52,53) and SPLUNC2 (spot 9) were further
256 increased ($P < 0.05$) as shown in Fig. 2f. At the same time, cancer patients' taste ($P = 0.0389$)
257 and total abnormality scores ($P = 0.0025$) were significantly decreased compared with baseline
258 (Fig. 1). There was no significant increase ($P = 0.058$) in intensity of salivary lactoferrin (spot 35)
259 along with LF supplementation.

260 **Salivary minerals**

261 As expected, LF supplementation significantly decreased ($P = 0.025$) the concentration
262 of salivary Fe from 0.20 ± 0.05 mg/L to 0.07 ± 0.04 mg/L in cancer patients (n=12). In addition,
263 the decreased salivary Fe content was maintained at 0.08 ± 0.06 mg/L in cancer patients even
264 after 30 days without consistent LF supplement intake, which was still significantly lower ($P =$
265 0.032) than salivary Fe content at baseline (Figure 3b). There was no significant difference ($P >$
266 0.05) in concentration of salivary minerals between baseline and 30-day post-LF
267 supplementation. However, compared with 30-day LF supplementation, a post-LF effect on
268 minerals was observed, including significantly increased concentrations of salivary P ($P =$
269 0.0216), S ($P = 0.0451$), K ($P = 0.0313$), Ca ($P = 0.0242$), and Mg ($P = 0.0025$) in cancer
270 patients. For healthy subjects (n=12), LF treatment at each stage did not significantly influence
271 ($P > 0.05$) the concentration of any tested salivary minerals.

272 Compared with healthy subjects, cancer patients showed significantly higher
273 concentration of salivary Na ($P = 0.013$) and Fe ($P = 0.033$) at baseline (Figure 3a and 3b). This

274 significant difference, however, was eliminated following LF supplementation. In contrast,
275 concentrations of salivary Mg ($P < 0.001$), P ($P = 0.021$), K ($P = 0.011$), Ca ($P = 0.002$), S ($P =$
276 0.003) in cancer patients increased at 30-day post-LF supplementation compared with healthy
277 subjects.

278 **Disccussion**

279 **Production of Taste and Smell Abnormalities**

280 In this study, concentrations of salivary Fe in cancer patients were significantly higher (P
281 $= 0.033$) than those in healthy subjects at baseline. According to Toyokuni's study,[19] disease
282 pathology such as cancer is usually associated with the release of unbound and reactive forms of
283 iron (Fe^{2+}), which might result in metallic taste abnormality. In addition, high Fe concentration
284 in cancer patients' saliva not only produces metallic taste, it also is highly associated with
285 neurodegenerative changes that commonly results in sensory disorders, such as taste (sweet, sour,
286 salty, bitter) impairment found in Parkinson's disease.[20,21] In addition, chemotherapeutic agents
287 such as procarbazine lead to an increase in reactive oxygen species,[22] which results in lipid
288 oxidation of oral epithelial cells that contributes to the production of carbonyls that causes
289 metallic taste.[23]

290 Another possible mechanism of taste/smell abnormality is the localized taste/smell
291 damage caused by chemotherapy.[24] Accumulated evidence has indicated that cytotoxic
292 chemotherapy agents not only kill cancer cells, but they also destroy the salivary peroxidase
293 system.[9] In our study, production of metallic taste in chemotherapy patients was associated with
294 the significant decrease of salivary α-amylase and immune proteins including CAVI, Zn-α-2-GP,
295 PIP, and immunoglobulin. Our result was in agreement with previous studies that patients with
296 taste disorders had lower abundance of Zn-α-2-GP, PIP, and CAVI in saliva.[25] In addition,

297 chemotherapy agents such as oxaliplatin are reported to cause peripheral sensitization and
298 destroy the sensory neurons that lead to neuropathic pain and TSA.[26,27]

299 **Effect of lactoferrin treatment on Taste and Smell Abnormalities**

300 *Lactoferrin*

301 Lactoferrin is produced by activated microglia and dopaminergic neurons around the
302 central nervous system, which contributes to the repair of neuropathological disorders.[28]
303 Injection of lactoferrin conjugates in a rat model of Alzheimer's disease further confirms a
304 potential neuroprotective effect of lactoferrin in neurodegeneration through metal-chelation
305 therapy.[20] The supply of lactoferrin in this study might assist in repairing and transmitting neural
306 signals to the central nervous system, which relieved TSA as a consequence.

307 Furthermore, serving as a metal chelator, lactoferrin may have been able to reduce
308 metallic taste abnormality by binding salivary Fe that was naturally higher in chemotherapy
309 patients' saliva. After transferring ferrous to ferritin, lactoferrin would be eliminated through
310 receptor-mediated endocytosis of phagocytic cells. Excess lactoferrin could also be removed
311 from the circulation through direct uptake by liver, then degraded and excreted into the urine by
312 the kidneys.[29] This might explain the non-significant ($P > 0.05$) increase of salivary lactoferrin
313 in all human subjects during LF supplementation in this study. In addition, lactoferrin might
314 decrease the metallic taste caused by lipid oxidation in oral epithelial cell through minimizing the
315 catalytic action of salivary Fe.

316 As a first-line immune defense protein, abundant lactoferrin in the body might trigger
317 innate protective mechanisms in mucosal immunity and in nonimmune mucosal defense,[30] which
318 stimulated the production of other associated salivary defense proteins such as immunoglobulin
319 A secretory chain (anti-bacteria and anti-viruses), α-amylase (anti- inflammation), annexin A1

320 (anti-inflammation), and prolactin-induced protein (anti-bacteria and anti-viruses).[31-34] Oral
321 infection and inflammation often result in taste disorders; ion transportation and the associated
322 afferent nerve of salt taste perception are acutely sensitive to inflammatory stimuli.[35]
323 Furthermore, accumulating evidence suggests that systemic peripheral inflammation may result
324 in exacerbation in several neurodegenerative diseases that commonly cause taste/smell
325 dysfunction, such as Parkinson's disease.[36] In a recent study, taste dysfunction in obesity has also
326 been proven to be a result of systemic inflammation.[37] Therefore, controlling of oral
327 inflammation should be an integral part of prevention of taste dysfunction and salivary immune
328 proteins are important in maintaining normal taste function.

329 *Other immune proteins*

330 Zn-α-2-GP and PIP were reported to play an important role in mucosal immunity and
331 mucosal defense functions through high binding affinity with bacteria and proteins.[38] In this
332 study, intensity of Zn-α-2-GP and PIP were significantly lower in saliva of cancer patients who
333 developed TSA. Our result was in agreement with the observation that patients with taste
334 abnormality showed decreased expression of Zn-α-2-GP and PIP in saliva.[25] Thus, Zn-α-2-GP
335 and PIP might be critical immune proteins in maintaining normal taste function. We hypothesize
336 that Zn-α-2-GP and PIP might competitively bind with taste compounds or taste receptors
337 through their high binding affinity, thus reducing metallic perception in the oral cavity.

338 In this study, increased expression of low-abundance immune proteins was associated
339 with a lower taste/smell/total abnormality score. CAVI has long been recognized as a critical
340 protein that is responsible for the growth of taste buds.[39] Failure of CAVI synthesis in saliva was
341 associated with the development of taste bud abnormalities which resulted in the loss of taste
342 capacity such as dysosmia and dysgeusia.[39] In the current study, CAVI was absent in saliva of

343 most cancer patients at baseline. Our result suggests that metallic taste abnormality might be
344 caused by inhibition of taste bud growth or damage to taste buds induced by chemotherapy.
345 Alpha-amylase is well known as a digestive enzyme in saliva,[40] which degrades
346 carbohydrates into glucose/maltose and generates sweet taste perception. In this study, cancer
347 patients showed a significantly lower intensity of salivary α-amylase than healthy subjects. The
348 decreased expression of α-amylase in chemotherapy patients might result in a difficulty to fully
349 digest carbohydrates in foods and a relative increase the intensity of metallic taste.
350 To understand the universality of LF treatment on chemotherapy-induced taste and smell
351 dysfunction, patients with a variety of cancers receiving different chemotherapy regimens were
352 included in this study. Although individual factors of cancer patients may influence the effect of
353 LF treatment, such as personal eating behavior, diet, tobacco history, and supertaster
354 (hypersensitive) or non-taster status, this study has found a positive immune system response to
355 LF supplementation was common among cancer patients. Therefore, saliva would provide an
356 easily available and noninvasive method to determine useful bio-markers for the early detection
357 of TSA in high-risk patients.
358 *Salivary minerals*
359 Lactoferrin is well known as an iron-binding protein that sequesters overabundant iron in
360 order to quickly decrease the oxidative stress; LF transports and delivers iron to cells in all
361 organisms for utilization or storage. Lactoferrin binds and transfers iron ions through its
362 polypeptide folding pattern.[41] Apart from iron, lactoferrin is also capable of binding many other
363 metal ions such as Al^{3+}, Ca^{2+}, Na^{+}, K^{+}, Cu^{2+}, and Zn^{2+},[41] which explained the slight decline of
364 salivary minerals after 30 days of intake of LF supplements.

365 In this study, concentrations of salivary minerals including P, S, K, Ca, Mg, were
366 significantly increased (P<0.05) in cancer patients at 30-day post-LF supplementation. To the
367 best of our knowledge, influence of LF supplementation on salivary minerals has not been
368 studied yet and the mechanism of lactoferrin intake on changes of salivary minerals is not yet
369 clear. However, we reason that the large amount of ingested lactoferrin might combine with
370 certain salivary minerals, which caused the decrease of these minerals in concentration. In
371 addition, cancer patients showed significantly higher ($P < 0.05$) concentration of salivary
372 minerals than healthy subjects at baseline. The increase of salivary minerals in cancer patients
373 might be a direct result of the chemotherapeutic agents or result from the chemotherapy-induced
374 salivary disorder. Therefore, the increase of salivary minerals after termination of LF
375 supplementation might be due to the continued chemotherapy effect.
376 Although Mg, K, Ca are all metal cations, they do not produce metallic flavor because
377 these metal cations do not cause lipid oxidation. Ca, Mg Na, and K are associated with bitter
378 taste and have not been reported to produce metallic taste.[42,43,44,45] Furthermore, according to
379 previous studies, recognition threshold (taste sensitivity) of metallic flavor induced by ferrous
380 sulfate solution was weakened when adding minerals such as Ca and Mg.[46,47] Therefore, LF
381 treatment might relieve metallic taste by increasing the concentration of salivary Mg, S, Cl, K,
382 Ca while decreasing salivary Fe at the same time.
383 In conclusion, LF supplementation successfully reduced TSA caused by chemotherapy
384 and this effect lasted at least 30 days. To our knowledge, this study is the first to associate taste
385 abnormality with the salivary proteome. Furthermore, our proteomic analysis in this study was
386 the first to illustrate the molecular mechanism of LF supplementation on salivary immunity:
387 intake of lactoferrin increased overall expression of salivary immune proteins, which are

388 associated with taste bud growth, neutral signal transduction, and taste threshold recovery. Our
389 results suggest lactoferrin may be developed as an effective dietary supplement to treat TSA
390 caused by chemotherapy and increase the expression of salivary proteins. Results of this study
391 may pave the way for further clinical studies in patients with TSA caused by taste buds damage
392 (such as radiotherapy), innate and diseases-associated immune deficiency, and
393 neurodegeneration (e.g. Parkinson's disease).
394

395 **Conflict of interest**

396 The authors have stated that they have no conflicts of interest.
397

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406
407

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555

Table 1 Mass spectrometric identification of differentially expressed proteins in saliva of healthy subjects and cancer patients[1] during LF treatment.

Protein name	Spots[2] ID	Accession No.	Matched peptides	MW[3] (kDa)		PI[4]	
				theo.	obs.	theo.	obs.
α-Amylase, salivary	23,24,36,37,38 ,39,40,41,42,43 ,44,45,49,51,52,53	gi|178585	12	57.8	40.5 - 62.0	6.5	4.5 - 7.4
Annexin A1	48	gi|119582950	4	40.2	37.8	6.6	7.2
Carbonic anhydrase VI precursor	5,6,7,32,33,34	gi|112693294	4	40.3	35.4 - 39.6	6.6	6.5 - 7.6
Immunoglobulin heavy chain variable region	28,29	gi|122892400	10	40.5	37.6	6.6	6.1
Lactoferrin	35	gi|85700158	4	84.5	85.8	9.3	9.6
Prolactin-inducible protein	16,17,18,19,20 ,21	gi|4505821	6	9.1	8.9 - 14.5	5.3	4.2 - 5.4
Proteinase inhibitor	22	gi|52001472	2	43.5	44.6	6.1	6.2
Short palate, lung and nasal epithelium carcinoma-associated protein 2 (SPLUNC2)	9,10,11,12	gi|34395850	4	27.2	26.8 - 29.1	5.4	4.8 - 5.6
Transferrin precursor	1,2,3	gi|553788	4	76.8	81.5	8.4	7.2 - 8.2
Unidentified protein	50	-	-	-	33.4	-	6.6
Zinc-alpha-2-glycoprotein precursor	8,13,14,15,26,27	gi|4502337	10	33.9	35.4 - 44.0	5.5	5.1 - 5.6

[1]Saliva samples were collected from 12 healthy subjects and 12 cancer patients.
[2]Identified proteins using the National Center Biotechnology Information nonredundant *Homo sapiens* amino acid sequence database and compared with previous publications.
[3]Molecular weight of theoretical (theo.) and observed (obs.) values.
[4]Isoelectric point of theoretical (theo.) and observed (obs.) values.

Table 2 Fold changes of differentially expressed proteins in saliva of healthy subjects and cancer patients[1] at each stage of LF treatment.

Protein	Spots ID	30-Day LF supplementation[2]		30-Day post-LF supplementation[3]	
		Healthy	Cancer	Healthy	Cancer
α-Amylase, salivary	23	2.1	4.6*	11.4**	2.9
	24	2.5	3.3*	12.6**	2.1
	36	4.7*	-	-2.1	-
	37	6.5*	-	-4.3*	3.8*
	38	-	-	10.1*	-
	39	-	-	15.0*	3.4*
	40	4.8*	-	-6.5*	-
	41	2.5	-	-5.4*	-
	42	4.2*	-	-4.1*	-
	43	2.4	-	-3.2*	-
	44	-	-	10.2**	8.1**
	45	-	-	11.5**	2.5*
	49	-	2.2	-	6.8*
	51	2.4	-2.1	1.4	-3.9
	52	2.5	7.9**	-2.5	5.6*
	53	3.4	9.5**	-2.4	4.4*
Annexin A1	48	-	2.1	-	4.7
Carbonic anhydrase VI precursor	5	-1.3	-	-5.4*	-
	6	-0.8	-1.1	-8.2*	0.8
	7	1.2	1.2	-8.5*	1.1
	32	1.5	-	-2.8*	-
	33	4.3*	-	-5.1*	-
	34	2.7*	-	-3.8*	-
Immunoglobulin heavy chain variable region	28	2.5*	2.4*	3.3	1.1
	29	2.4	1.2	5.8*	2.1
Lactoferrin	35	2.8	3.1	-0.5	-2.2
Prolactin-inducible proteins	16	0.8	-	-2.1*	-
	17	1.5	2.4	-3.5*	-1.4
	18	3.2*	3.8	-8.8*	-2.7
	19	9.6**	-	-5.5*	-

Protein	Spots ID	30-Day LF supplementation[2]		30-Day post-LF supplementation[3]	
		Healthy	Cancer	Healthy	Cancer
Prolactin-inducible proteins	20	2.1*	4.3	-5.9**	-3.1
	21	0.6	-	-2.2*	-2.1
Proteinase inhibitor	22	-3.4	4.5*	5.5*	-4.1
SPLUNC2	9	2.9	2.2	6.8	10.5*
	10	2.1	1.5	-1.9	2.2
	11	2.6	1.4	-2.4	-1.1
	12	2.1	1.4	-1.8	-1.2
Transferrin precursor	1	1.1	-	2.2	-
	2	1.2	-	4.1	-
	3	0.8	-	4.4	-
Unidentified protein	50	-	-	-	2.3
Zinc-alpha-2-glycoprotein precursor	8	12.8**	2.6*	-10.6**	2.1
	13	2.5*	1.1	-5.1*	1.5
	14	4.1*	0.8	-5.6*	1.8
	15	10.1**	2.8*	-11.5**	1.2
	26	-	-	-	2.3
	27	-	-	-	2.2

[1]Saliva samples were collected from 12 healthy subjects and 12 cancer patients.
[2]Fold change of each individual protein was compared to the same protein spot shown in baseline.
[3]Fold change of each individual protein was compared to the same protein spot shown in 30-day LF supplementation.
*Differences of protein expression between LF treatment stages reaching statistical significance indicated by a single ($P < 0.05$) or double asterisk ($P < 0.01$).

Figure 1. Taste, smell, and total abnormality scores of 12 cancer patients at baseline, 30-day LF supplementation, and 30-day post-LF supplementation.

Figure 2. Salivary proteome of the representative healthy subject and cancer patient, at baseline (a,d), 30-day LF supplementation (b,e), and 30-day post-LF supplementation (c,f). Gels were stained by fluorescent staining. Grouped spots that are shown on top of each gel picture were cut from silver-stained gel to provide a better visualization. Differentially expressed proteins (Wilcoxon test, $P < 0.05$) are circled on each 2-DE image with consistent spot ID number as listed in Table 3. Low-abundance protein spots concentrated between pH 5.5-8.5 and MW (molecular weight) 25-75 kDa are grouped in each 2-DE image. pI, isoelectric point; LF, lactoferrin.

Figure 3. Comparison of salivary minerals between healthy subjects (n=12) and cancer patients (n=12) at each stage of LF treatment for a) major minerals including Na, P, S, Cl, K, Ca, and b) minor minerals including Mg, Fe, Cu, Zn.

Figure 1.

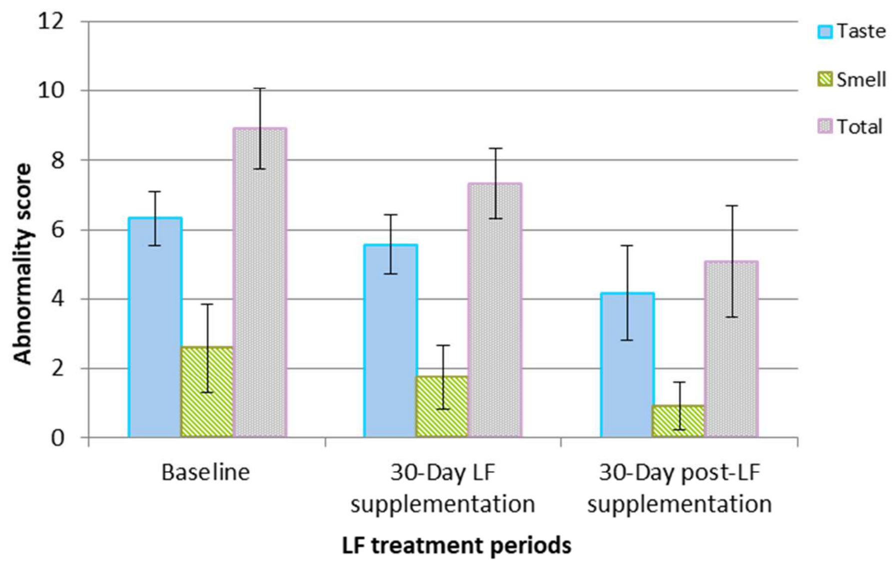


Figure 2.

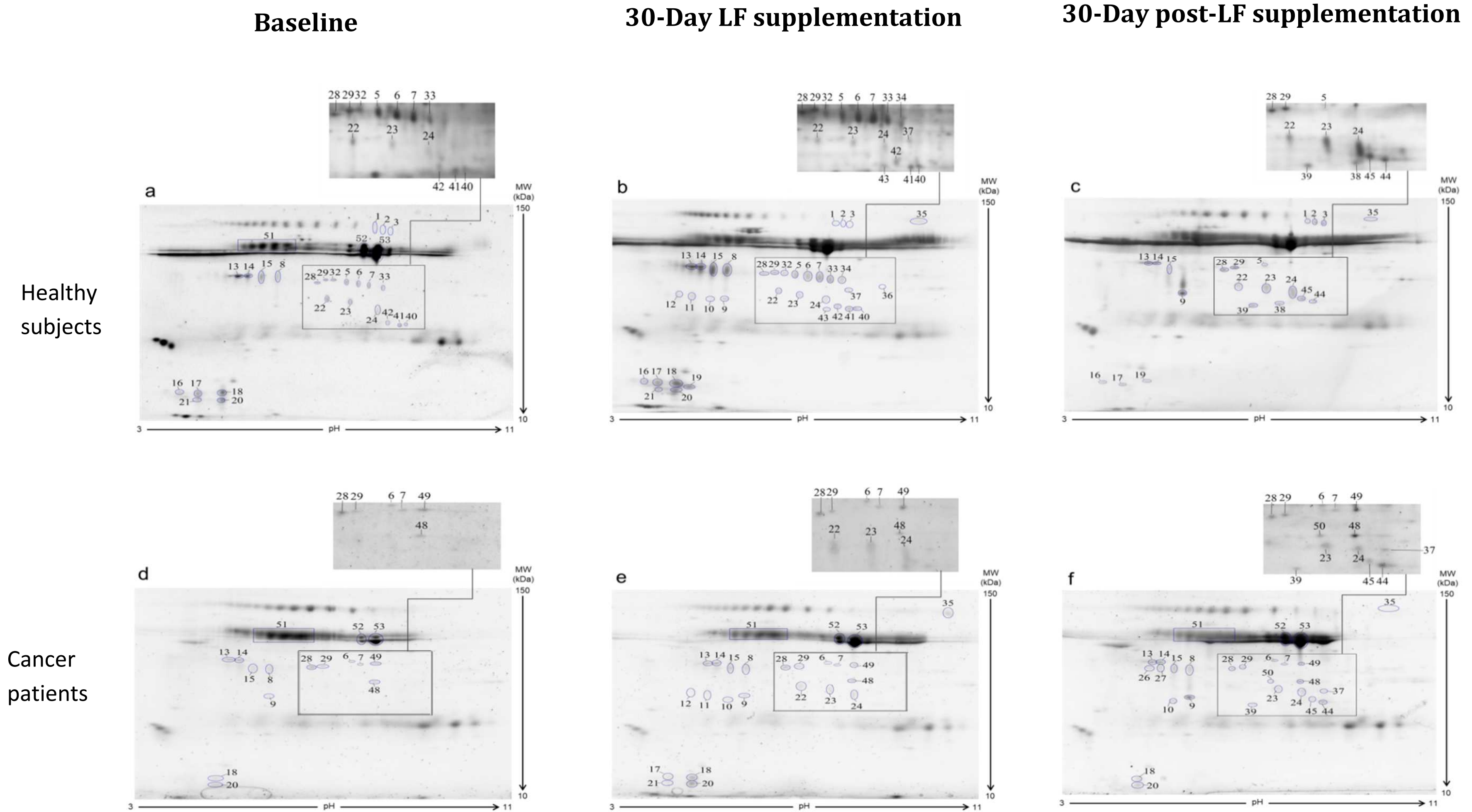


Figure 3a.

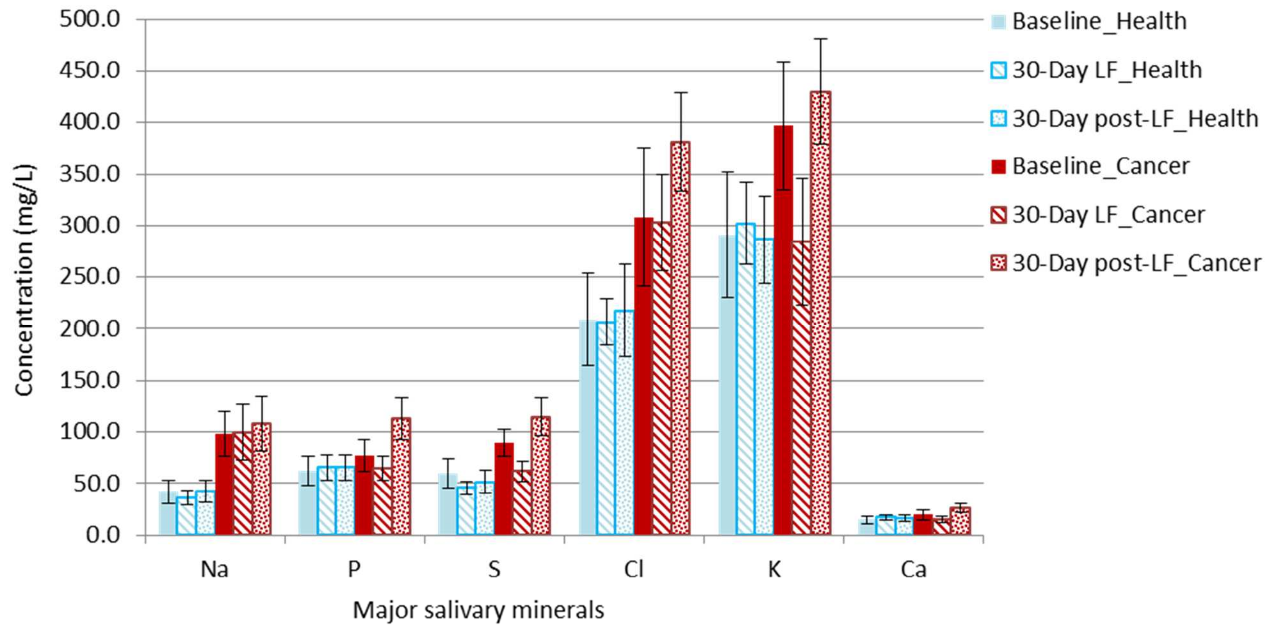
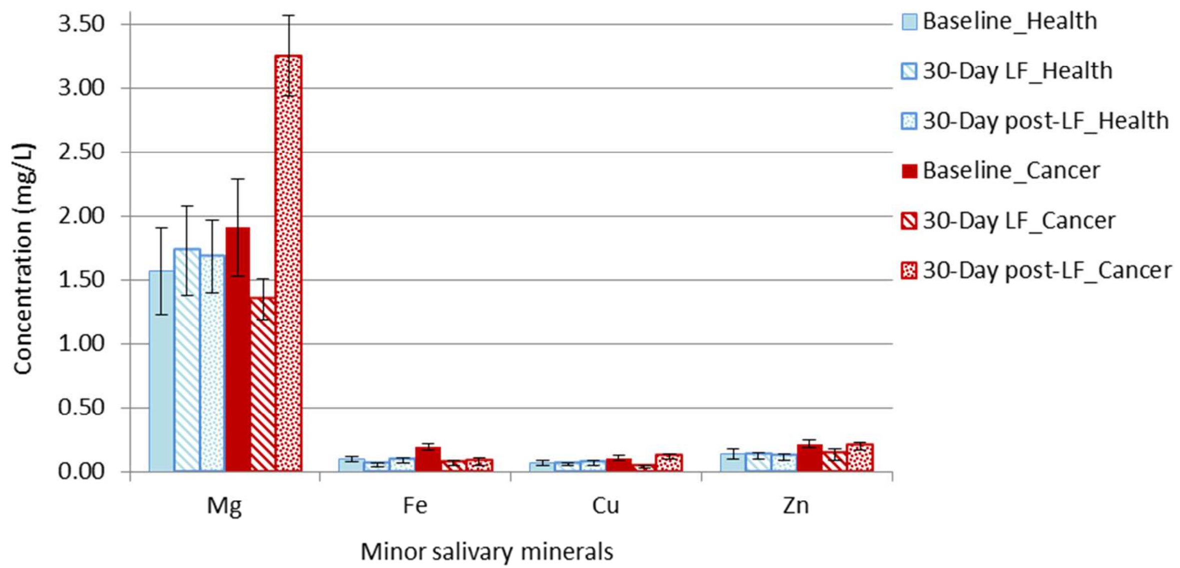


Figure 3b.



Appendix Smell and Taste Questionnaire

Please answer these questions based on your current perception of your sense of taste and smell. For those questions that require a comparison, please compare your current sense of taste and smell with your sense of taste and smell prior to developing cancer and receiving chemotherapy.

Taste Complaints: Please rate

Questions	Insignificant	Mild	Moderate	Severe	Incapacitating
I have noticed a change in my sense of taste					
A food tastes different than it used to					
I have a persistent bad taste in my mouth					
Drugs interfere with my sense of taste					
I would rate my abnormal sense of taste as					

Taste Complaints: Answer "yes" or "no"

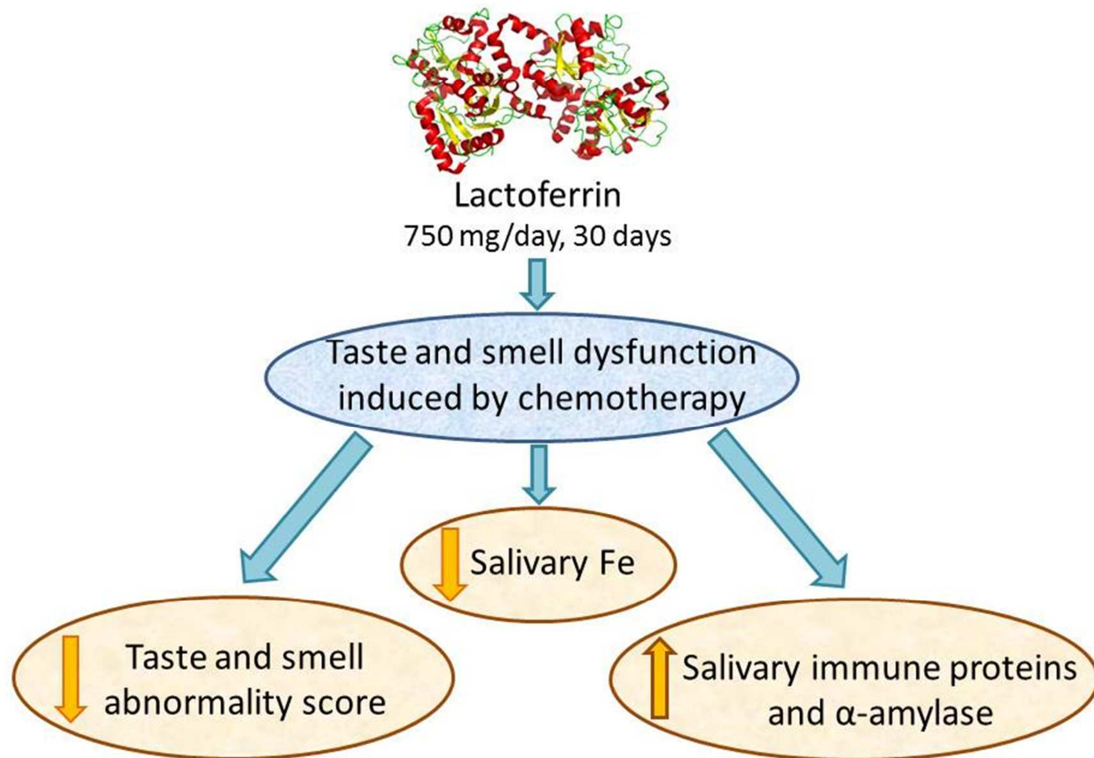
Questions	Yes	No	If "Yes" then:
I am experiencing an abnormal sensitivity to salt			Salt tastes: Stronger___ or Weaker ___
I am experiencing an abnormal sensitivity to sweet			Sweet tastes: Stronger ___ or Weaker ___
I am experiencing an abnormal sensitivity to sour			Sour tastes: Stronger ___ or Weaker ___
I am experiencing an abnormal sensitivity to bitter			Bitter tastes: Stronger ___ or Weaker ___

Smell Complaints: Please rate

Questions	Insignificant	Mild	Moderate	Severe	Incapacitating
I have noticed a change in my sense of smell					
A food smells different than it used to					
Specific drugs interfere with my sense of smell					
I would rate my abnormal sense of smell as					

Smell Complaints: Answer "yes" or "no"

Questions	Yes	No	If "Yes" then:
I have an abnormal sensitivity to odors			Odors are: Stronger___ or Weaker ___



Latoferrin supplementation significantly reduced taste and smell abnormality in chemotherapy patients and improved their oral immunity.