

*Impact of a (poly)phenol-rich extract from the brown algae *Ascophyllum nodosum* on DNA damage and antioxidant activity in an overweight or obese population: a randomized controlled trial*

Article

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1 **Impact of a (poly)phenol-rich extract from the brown algae *Ascophyllum nodosum* on**  
2 **DNA damage and antioxidant activity in an overweight/obese population**

3

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25

26 Running head: Seaweed phenolics, *Ascophyllum nodosum*, inflammation, DNA damage, C-  
27 reactive protein (CRP)

28

29 Abbreviations used: SPE, seaweed (poly)phenol extract; CRP, C-reactive protein; CVD,  
30 cardiovascular disease; ROS, reactive oxygen species; TNF- $\alpha$ , tumour necrosis factor alpha;  
31 COX, cyclooxygenase; UUREC, University of Ulster Research Ethics Committee; WISP,  
32 Weighed Intake Software Program; TF, tissue factor; TOC, Total oxidative capacity; TMB,  
33 tetramethylbenzidine; IL, interleukin; IFN, interferon; TNF, tumour necrosis factor; UHPLC-  
34 HRMS: ultra-high performance liquid chromatography-high resolution-mass spectrometry;  
35 VIP: variable of importance in projection; OPLS-DA: Orthogonal Partial Least Square  
36 Discriminant Analysis.

37

38 This trial was registered at [clinicaltrials.gov](https://clinicaltrials.gov) as NCT02295878.

39

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51 **ABSTRACT**

52 **Background:** Epidemiological evidence suggests a diet rich in (poly)phenols has beneficial  
53 effects on many chronic diseases. A rich source of (poly)phenols can be found in brown  
54 seaweed.

55 **Objective:** The aim of this study was to investigate the bioavailability and effect of a  
56 seaweed (poly)phenol extract from *Ascophyllum nodosum* on DNA damage, oxidative stress,  
57 and inflammation *in vivo*.

58 **Design:** A randomised double-blind placebo-controlled crossover trial was conducted in 80  
59 participants aged 30-65 years with a BMI  $\geq 25$ kg/m<sup>2</sup>. The participants consumed either a 400  
60 mg capsule containing 100 mg of seaweed (poly)phenol and 300 mg maltodextrin or a 400 mg  
61 maltodextrin placebo control capsule daily for an 8-week period. Bioactivity was assessed  
62 with a panel of blood-based markers including lymphocyte DNA damage, plasma oxidant  
63 capacity, C-reactive protein and inflammatory cytokines. To explore the bioavailability of  
64 seaweed phenolics, an untargeted metabolomics analysis of urine and plasma samples  
65 following seaweed consumption was determined by UHPLC-HR-MS.

66 **Results:** Consumption of the seaweed (poly)phenols resulted in a modest decrease DNA  
67 damage but only in a subset of the total population who were obese. There were no significant  
68 changes in CRP, antioxidant status, inflammatory cytokines or isoprostanes. We identified  
69 phlorotannin metabolites including pyrogallol/phloroglucinol-sulfate, hydroxytrifurahol A-  
70 glucuronide, dioxinodehydroeckol-glucuronide, diphlorethol sulfates, C-O-C dimers of  
71 phloroglucinol sulfate, C-O-C dimers of phloroglucinol and diphlorethol sulfate are considered  
72 potential biomarkers of seaweed consumption.

73 **Conclusion:** To the best of our knowledge, our work represents the first comprehensive  
74 study in human participants investigating the bioactivity and bioavailability of seaweed  
75 (poly)phenolics. There was a modest improvement in DNA damage but only in the obese subset

76 of the total population, and several biomarkers of seaweed consumption, further studies  
77 involving stratification according to weight are required to elucidate these findings.

78

79 *Key words:* Seaweed, Phenolic compounds, Inflammation, DNA damage, Oxidative stress,  
80 bioavailability

81

## 82 INTRODUCTION

83 Diets rich in plant-derived foods protect may against chronic degenerative diseases,  
84 including cardiovascular disease (CVD), effects attributable in part to highly bioactive  
85 (poly)phenolic compounds contained therein (1-3). Fruits and vegetables are a well-known  
86 source of (poly)phenols but a less familiar source rich in (poly)phenolic compounds is brown  
87 algae which uniquely contains phlorotannins (4) for example *Ascophyllum nodosum*, a brown  
88 alga common to the British Isles, that is rich in phlorotannin (5). Phlorotannins are oligomers  
89 of phloroglucinol whose concentration in seaweed is affected by numerous factors including  
90 plant size and age, water salinity, nutrient and heavy metal content, and light intensity changes  
91 (6-8).

92 Phlorotannins, and more commonly brown seaweed extracts, beneficially effect a range of  
93 biological processes including modulation of inflammation; reduction of oxidative stress and  
94 improvement in cardiovascular function (9-11). However, the evidence base depends heavily  
95 upon cell and small animal models with few studies involving humans (12-13). Moreover,  
96 species relevance also becomes an issue; in Southeast Asia, *Ecklonia* and *Eisenia* are  
97 considered commercially important seaweed species, while from a European perspective  
98 *Ascophyllum nodosum* is of interest as it is one of only a few commercially sustainable seaweed  
99 species.

100 Phlorotannin-rich extracts from brown seaweeds have been shown to be effective in  
101 controlling inflammation *via* a number of pathways including inhibition of pro-inflammatory  
102 cytokines including tumour necrosis factor (TNF)- $\alpha$  and interleukins (IL)-1 $\beta$  and IL-6 (14).  
103 Extracts from *Ascophyllum nodosum* and other Furoid species have shown efficacy in  
104 mitigating the effects of oxidative stress by playing an inhibitory role in the generation of  
105 reactive oxygen species (ROS); in preventing DNA damage and also in stimulating the  
106 production of glutathione in affected cells (15-19). Our initial *in vitro* (15) and acute *in vivo*

107 (20) studies on the *Ascophyllum nodosum* extract(s) demonstrated antioxidant and anti-  
108 inflammatory activity, suggesting that the potential antioxidant and anti-inflammatory benefits  
109 from longer term consumption of *Ascophyllum nodosum*-derived (poly)phenols was worthy of  
110 further investigation *in vivo*.

111 A few human and *in vitro* studies have been conducted in relation to the bioactivity of  
112 phlorotannin rich extracts from Fucooid species. Clinical studies have been used to evaluate the  
113 safe consumption of some of these extracts including a (poly)phenol-enriched extract of the  
114 brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus* and its effects of on glycaemic  
115 response to sucrose (21). However, to the best of our knowledge, the current investigation is  
116 the first clinical study aimed to specifically address the effect of a phlorotannin-rich extract  
117 from *Ascophyllum nodosum* on oxidative damage to DNA, plasma antioxidant capacity,  
118 inflammatory responses and chronic, low level inflammation *in vivo*.

119

## 120 **PARTICIPANTS AND METHODS**

### 121 **Seaweed material**

122 Fresh *Ascophyllum nodosum* was supplied by The Hebridean Seaweed Company, Isle of  
123 Lewis, Scotland in March 2011. The seaweed biomass was harvested by hand to ensure quality,  
124 cleaned of any contaminating sand and fouling organisms and then shipped refrigerated to the  
125 processing facility (CEVA) in France where it was immediately chopped and frozen.

### 126 **Preparation of food-grade seaweed extracts and capsule**

127 A novel (poly)phenol-rich seaweed extract from *Ascophyllum nodosum* was produced by  
128 CEVA (France) using food grade solvent based (ethanol:water, 60:40) extraction system that  
129 was specifically developed for use with fresh or frozen *Ascophyllum nodosum*. Approximately  
130 half of the produced extract was then fractionated using tangential flow ultra-filtration to  
131 produce further extracts of varying molecular weight range and with varying (poly)phenol



132 content. A standardised blended (poly)phenol-rich *Ascophyllum nodosum* extract was  
133 formulated by CEVA (**Table 1**) using 175 mg of extract and 50 mg of high molecular weight  
134 fraction (>10 kDa cut off) for use in the current study, so as to maximise the seaweed  
135 (poly)phenol content (>100 mg per day) available from the extraction of fresh or frozen  
136 *Ascophyllum nodosum* against the need to minimise the level of iodine to within accepted  
137 regulatory guidelines (<500 µg per day), potential for heavy metal contamination was also  
138 assessed (Table 1). Maltodextrin (175 mg) was added to the capsule formulation as an  
139 excipient. Blending was carried out at the food grade CEVA facilities in France. Samples of  
140 400 mg of the *Ascophyllum* (poly)phenol rich blend (SPE) or a placebo which contained 400  
141 mg maltodextrin of and no seaweed (poly)phenols were packed into identical white, opaque,  
142 vegetarian capsules by Irish Seaweeds, Belfast, UK and identically sized and match capsules  
143 used for the clinical study. The food grade seaweed capsule was characterized by NP-HPLC  
144 and LC-MS analysis and has been reported previously (20) (see supplemental figure 2 & 3).  
145 Phlorotannins were quantified using the Folin-Ciocalteu Method (22) using phloroglucinol as  
146 the standard. In brief, 1 mL of suitability diluted sample was reacted with 1.00 mL of 40 %  
147 Folin Ciocalteu reagent for 5 min, and then made alkaline with the addition of 1.00 ml of 100  
148 g/L Na<sub>2</sub>CO<sub>3</sub>. Absorbance was read at 730 nm after the solution had developed for 1 h at room  
149 temperature. Phloroglucinol dihydrate (0-30 mg/L) was used as the standard and was treated  
150 in the same way as samples.

151

## 152 **Ethics and participants**

153 Ethical approval was received from the Ulster University Research Ethics Committee  
154 (UUREC). All participants gave written informed consent. Participants were recruited between  
155 May 2011 and August 2011 from Ulster University and the surrounding area. The intervention

156 study ran between August 2011 and February 2012. The study was registered at  
157 [clinicaltrials.gov](http://clinicaltrials.gov) as NCT02295878.

158 The study was conducted in 80 participants (age range 30-65 years). All participants were  
159 apparently healthy, non-smoking,  $BMI \geq 25$  kg/m<sup>2</sup>, omnivores, who did not habitually use  
160 vitamin or mineral supplements, as determined using a pre-screening health and lifestyle  
161 questionnaire. Pregnant and lactating women, vegetarians and vegans and lactose intolerant  
162 individuals were excluded from the study, as were those with chronic medical complications  
163 such as diabetes; cardiovascular diseases; autoimmune/inflammatory disorders or those who  
164 had chronic medication use including anti-inflammatory agents.

165

## 166 **Study design**

167 The study was a 24-week randomised, double-blind, placebo-controlled crossover trial.  
168 After obtaining consent, participants were randomly assigned, in blocks of four using a  
169 random-number generator ([www.randomization.com](http://www.randomization.com)), to either the intervention or the control.  
170 In total, eighty participants were randomised to 2 groups of 40, each starting on either a 400  
171 mg seaweed (poly)phenol extract (SPE) capsule containing 100mg of (poly)phenols or a 400  
172 mg maltodextrin placebo control capsule (Avebe MD14P) daily for an 8-week period. The  
173 participants were supplied with all capsules in weekly labelled capsule boxes at the beginning  
174 of each phase, which was interspersed by an 8-week washout phase. During the washout phase,  
175 the participants were asked to maintain their habitual diet. Participants were asked to bring any  
176 unconsumed capsules to their study appointment at the end of each treatment phase.  
177 Participants were also contacted weekly by the study researcher to encourage compliance and  
178 to discuss any difficulties they were experiencing.

179

180

## 181 **Blood and urine sample collection**

182 Fasting blood samples were collected before and after each phase (week 0, week 8, week  
183 16, and week 24) by venepuncture into EDTA, serum or sodium heparin-containing tubes, as  
184 required. All blood samples were processed on ice. Lymphocytes were isolated by using  
185 Histopaque-1077, according to the manufacturer's instructions (Sigma Diagnostics, St Louis,  
186 MO), and plasma samples were prepared by centrifugation at 1000 x g for 10 min at 4°C. Serum  
187 samples were allowed to clot for 30 min at room temperature and then were centrifuged at 2000  
188 x g for 10 min at 4°C. Whole blood from sodium-heparin treated tubes was prepared according  
189 to the manufacturer's instructions (BD Bioscience Fast Immune Cytokine System) and 24-hour  
190 urine samples were prepared by centrifugation at 1000 x g for 10 min at 4°C. Whole blood,  
191 plasma, serum and urine samples were immediately stored at -80°C, whereas lymphocytes were  
192 stored frozen in liquid nitrogen. All biological measurements were carried out at the end of the  
193 intervention in batches containing equal numbers of active and control phase samples in each  
194 batch, and the researchers were blinded to these samples during analyses. A 24hr urine  
195 collection occurred at each time point, volume and pH was measured. The urine sample was  
196 mixed and 2x 14ml aliquots removed, centrifuged at 3000rpm for 10 minutes, supernatant was  
197 stored -80°C until use.

198

## 199 **Questionnaire assessments**

200 All participants completed a health and lifestyle questionnaire assessing their alcohol intake  
201 and physical activity levels, as well as a validated 4-day food diary at the mid-point during  
202 each treatment phase (active/placebo) of the study. Data on type of food and corresponding  
203 weight was entered into a food analysis database (WISP, Weighed Intake Software Program;  
204 Tinuviel Software, Warrington, U.K.) by two independent researchers and the dietary  
205 composition calculated.

206

**207 DNA damage in peripheral blood mononuclear cells**

208 Peripheral blood lymphocytes, previously isolated and stored in liquid nitrogen, were  
209 thawed and screened for basal single strand breaks (SBs) in DNA using the single cell gel  
210 electrophoresis (Comet) assay (23) and adapted by Gill *et al.* (24). Spontaneous DNA SBs are  
211 associated with an altered cell function spontaneous DNA SBs are considered appropriate for  
212 the substantiation of health claims in the context of protection against generic DNA  
213 damage (25). In addition, resistance to induced DNA damage (SB) was measured in  
214 lymphocytes subjected to increased oxidative insult *ex vivo* by pre-treating lymphocytes with  
215 150 $\mu$ mol H<sub>2</sub>O<sub>2</sub>/L for 5 min at 4°C, before the measurement of SBs. The mean (percentage  
216 DNA in tail) was calculated from 50 cells per gel (each sample in triplicate) and the mean of  
217 each set of data were used in the statistical analysis.

218

**219 Plasma total oxidative capacity**

220 Total oxidative capacity (TOC) measures total peroxide levels in plasma, by the reaction of  
221 endogenous peroxides with peroxidases, using tetramethylbenzidine (TMB) as the  
222 chromogenic substrate (26). The blue colour of TMB turns to yellow after addition of the stop  
223 solution and can be measured photometrically at 450 nm. For the assay protocol, 10  $\mu$ l of  
224 standard (freshly-prepared hydrogen peroxide, 0-1 mmol/L) and samples were incubated with  
225 200  $\mu$ l of the reaction mixture consisting of a reaction buffer (phosphate-citrate buffer 0.05M,  
226 pH 5.0), TMB solution (1 mg/ml), and peroxidase (>2500U/ml) in a proportion of 100:10:1 in  
227 uncoated microtiter plates and incubated at room temperature for 15 min. 50  $\mu$ l of stop solution  
228 (2MH<sub>2</sub>SO<sub>4</sub>) were added into all wells and the absorbance at 450 nm was measured using a  
229 microplate reader (GENIOS Tecan). Hydrogen peroxide standard solutions (0-1 mmol/L) for  
230 calibration curve were freshly prepared before use.

231

**232 Lipid profile and serum C-reactive protein**

233 Plasma total cholesterol, HDL cholesterol and triglycerides were measured on an Instrument  
234 Laboratory (ILAB) 600 (Warrington, UK) autoanalyzer using commercial kits (Roche  
235 diagnostics, Lewis, UK) according to kit manufacturer's protocols. Plasma LDL cholesterol  
236 was calculated using the Friedewald formula (27).

237 C-reactive protein, an acute phase protein synthesized by the liver in response to  
238 inflammatory stimuli, especially the cytokine interleukin (IL)-6, was determined on an ILAB  
239 600 autoanalyser using a *quantex* CRP Ultra-Sensitive commercial kit (0.4-18.3  $\mu\text{g}/\text{dl}$ ) in  
240 accordance with manufacturer's instructions.

241

**242 Measurement of inflammatory markers**

243 Intracellular cytokine levels in lymphocyte and monocyte populations and tissue factor (TF)  
244 expression were assessed using a whole blood labelling method that utilises flow-cytometry  
245 (Fast Immune Cytokine System, BD Biosciences) in accordance with manufacturer's  
246 instructions for all participants at all time-points. The method was used to measure intracellular  
247 IL-1 $\beta$ , IL-2, IL-6, IL-10, IL-12, interferon (IFN)- $\gamma$  and tumour necrosis factor (TNF)- $\alpha$   
248 expression in mononuclear cells. Briefly, whole blood was incubated with either  
249 lipopolysaccharide or phorbol 12-myristate 13-acetate to activate monocytes and lymphocytes,  
250 respectively. Cells were labelled with the appropriate cell surface antibody and cytokine-  
251 specific antibody and analysed on a Gallios flow cytometer (Beckman Coulter). The number  
252 and percentage of each cell type expressing the cytokine, as well as the mean channel  
253 fluorescence was recorded. The cytokine profiles were examined by ratio of TNF- $\alpha$  to IL-10,  
254 IL-1 $\beta$  to IL-10, IL-6 to IL-10 and CRP to IL-10, according to Laird et al. (29).

255 Isoprostanes have been established as chemically stable, highly specific and reliable  
256 biomarkers of *in vivo* oxidative stress, and were measured in frozen serum samples using a  
257 commercial 8-Isoprostane EIA Kit (Item no. 516351, Cayman Chemicals). This assay is based  
258 on the competition between 8-isoprostane and an 8-isoprostane acetylcholinesterase (Ache)  
259 conjugate (8-Isoprostane Tracer) for a limited number of 8-isoprostane-specific rabbit  
260 antiserum binding sites consequently 8-isoprostane concentrations are measured as a function  
261 of turbidity (absorbance).

262

### 263 **Processing of urine and plasma.**

264 Urine samples were defrosted, vortexed, centrifuged at 16110 g for 10 min at 5 °C, and  
265 passed through 0.45 µm filter discs prior to the analysis of 50 µL aliquots by UHPLC-HR-MS.  
266 The extraction of metabolites from the plasma samples has been carried out as described  
267 previously (28). Briefly, plasma samples were defrosted, vortexed and 400 µL aliquots were  
268 mixed with 10 µL of ascorbic acid (10%, v/v), and 980 µL of 1% formic acid in acetonitrile.  
269 One µg of rutin was added to the samples as internal standard for plasma extraction efficiency.  
270 The samples were then vortexed for 1 min and ultrasonicated for 10 min. After centrifugation  
271 at 16,110 g for 15 min, supernatants were reduced to dryness *in vacuo* using a concentrator  
272 plus (Eppendorf, Hamburg, Germany) and resuspended in 150 µL of distilled water containing  
273 1% formic acid and 50 µL of methanol, which was then centrifuged at 16,100 g for 10 min and  
274 10 µL aliquots of the supernatant analysed by UHPLC-HRMS. The recoveries values of the  
275 internal standard were of  $79 \pm 16\%$  (n=78).

276

### 277 **Non-targeted analysis of urine and plasma by UHPLC-HR-MS.**

278 Aliquots of selected plasma and urine samples were analysed using a Dionex Ultimate 3000  
279 RS UHPLC system comprising of a UHPLC pump, a PDA detector scanning from 200 to 600

280 nm, and an autosampler operating at 4 °C (Thermo Scientific). The HPLC conditions were  
281 previously described by Corona *et al.*, (20) with some modifications. Briefly, reverse phase  
282 separations were carried out using a 100 x 2.1 mm i.d. 1.8 µm Zorbax SB C18 (Agilent)  
283 maintained at 25 °C and eluted at a flow rate of 0.2 mL/min with a 50 min gradient of 3-70%  
284 of 0.1% acidic methanol in 0.1% aqueous formic acid. After passing through the flow cell of  
285 the PDA detector the column eluate was directed to an Exactive™ Orbitrap mass spectrometer  
286 fitted with a heated electrospray ionization probe (Thermo Scientific) operating in negative  
287 ionization mode. Analyses were based on scanning from 100 to 1000 m/z, with in-source  
288 collision-induced dissociation at 25.0 eV. The capillary temperature was 350 °C, the heater  
289 temperature was 150 °C, the sheath gas and the auxiliary gas flow rate were both 25 and 5  
290 units, respectively, and the sweep gas was 4 and the spray voltage was 3.00 kv. Data acquisition  
291 and processing were carried out using Xcalibur 3.0 software.

292 Untargeted analysis of the selected urine and plasma samples was performed using mass  
293 spectral data from the orbitrap analysis applied to the Compound Discoverer software (version  
294 2, Thermo Fisher Scientific Inc.). The Compound Discover application processes the raw data  
295 in called processing workflows that can be defined on the basis of the nature of the experimental  
296 setup. In our case, the workflow selected was 'untargeted metabolomics workflow' that includes  
297 retention time alignment, component detection, grouping, elemental composition prediction,  
298 gap filling, hide chemical background (using blanks), ID using mzCloud and ChemSpider and  
299 differential analysis. The parameters were adjusted to our experimental conditions. Samples  
300 were grouped and labelled according to our experimental design, either before or after  
301 supplementation of seaweed capsule, 4 The output, as peak areas for the detected peaks was  
302 used to develop a multivariate data analysis by Orthogonal Partial Least Square Discriminant  
303 Analysis (OPLS-DA). Analysis of seaweed phenolics in blood and urine was also undertaken

304 by HPLC-DAD analysis (reported in Supplemental Tables 4 & 5) in a manner consistent with  
305 previous studies (15,20).

306

### 307 **Power calculations and statistical analyses**

308 Power calculations were performed for the primary endpoint of the change in DNA damage  
309 in peripheral blood mononuclear cells. Based on data from a previous study (24), 72.6  
310 participants were needed to detect a 25% change in DNA damage in lymphocytes ( $\alpha$  0.05).

311 All values are expressed as mean  $\pm$  SD, unless otherwise specified. The mean values are  
312 reported for all participants (n=78) during both treatment phases (SPE, Maltodextrin).  
313 Significant associations ( $P > 0.05$ ) between outcome variables including DNA damage and  
314 possible cofounders (age, gender, BMI) were identified at baseline using bivariate correlations  
315 or independent t-tests, where appropriate. Therefore, data was also analysed by stratification  
316 of increasing risk, including overweight participants (n=42) and obese participants (n=36). All  
317 biochemical analysis was conducted in duplicates, unless otherwise stated, and the mean values  
318 taken as the final result. For all markers, the results are presented as treatment effects. This was  
319 undertaken by calculating individual differences between pre- and post- values for both control  
320 and treatment phases for each subject. Paired T-tests were then carried out on the difference  
321 scores (post-treatment value *minus* pre-treatment values) for both treatment phases (SPE and  
322 Maltodextrin). Significance level was set at  $P < 0.05$ . All statistical analyses were performed  
323 using the Statistical Package for the Social Sciences (SPSS) for Windows version 22.0 (SPSS  
324 Inc., Chicago, IL, USA).

325

### 326 **Multivariate Data Analysis**

327 Data were obtained as peak areas from the Compound Discover automatic integration software  
328 and consisted of 2194 and 3289 potential metabolites (or features) in urine and plasma samples,



329 respectively. Relative peak areas of the metabolites (normalized by the total urine excretion of  
330 each subject) obtained by UPLC-HRMS were imported into MATLAB R2015b (Mathworks,  
331 USA). PLS toolbox v.8.5 (Eigenvector, USA) and homemade scripts were used. Principal  
332 component analysis (PCA) and hierarchical cluster analysis (HCA) are the most widely used  
333 tools to explore similarities and patterns among samples where data grouping are unclear.  
334 Moreover, the orthogonal partial least squares discriminate analysis (OPLS-DA) method was  
335 performed as a typical supervised multivariate methodology used in metabolomics studies  
336 (30,31). Several data pre-processing transformations were performed and evaluated, such as  
337 Probabilistic Quota Normalization (PQN), Log transformation, mean centering, pareto scaling  
338 and auto-scaling. In our case, PQN and autoscaling were selected as pre-processing techniques  
339 to reach the lowest root mean square error (RMSE) in an iterative process. A re-sampling  
340 method, cross-validation k-fold cross validation (k=5) was also used to evaluate the number of  
341 latent variables (according to the lowest RMSE) and the prediction ability of our models. To  
342 reduce the impact of the random split of CV-participants, the mean values of the estimated  
343 results were obtained after 20 random 5-fold CV. Urine models provided successful  
344 classification results at the cross validation step and achieved good prediction parameters that  
345 can be explained by the area under the curve values (1.00 and 0.895). Based on the plasma data  
346 set and classes selected a discriminant model could not be developed. Moreover, the Variable  
347 Importance in Projection (VIP) is the widely known metric that is used to identify potential  
348 markers in metabolomics studies (32). VIP is a weighted sum of squares of the PLS weight  
349 which indicates the importance of the variable to the whole model. The cut off VIP value  
350 selected in our study was 2. The potential markers were extracted from the two models to  
351 compare the results obtained.

352

## 353 **RESULTS**

### 354 **Baseline characteristics**

355 Eighty participants (males n=39, females n=41) were enrolled on this 24-week randomised,  
356 double-blind, placebo-controlled crossover trial. The intervention was conducted as per the  
357 protocol and there were no adverse events associated with the intervention. The study  
358 population had a mean group age of  $42.7 \pm 7.1$  years and a mean BMI of  $30.2 \pm 3.9$  kg/m<sup>2</sup>. The  
359 study had an overall compliance of 97% with 78 participants completing the 24-week study;  
360 two participants withdrew from the study at the midpoint for personal reasons and compliance  
361 was not significantly different by treatment group or time period ( $P > 0.05$ ) (**Figure 1**). There  
362 were no significant differences between the participants in age and physical characteristics at  
363 the beginning of either treatment phase (**Table 2**).

364

### 365 **Habitual dietary intake**

366 Dietary analysis of habitual intake (midpoint) during both treatment phases (SPE,  
367 Maltodextrin) of the crossover trial is described in **Table 3**. There were no significant  
368 differences between treatment phases for any of the macronutrients or micronutrients analysed  
369 indicating that the seaweed phenolic extract did not affect the habitual food consumption  
370 patterns in the study population.

371

### 372 **Effects of seaweed (poly)phenol extract on DNA damage**

373 The basal levels of DNA damage observed in the study were consistent with previous studies  
374 with a mean group average of  $6.72 \pm 2.48\%$  tail DNA (data not shown). In response to an  
375 oxidative challenge with  $150 \mu\text{M H}_2\text{O}_2$ , DNA damage was increased to an average of  
376 approximately  $34 \pm 7\%$  tail DNA in both the placebo and SPE phase in all participants as  
377 reported in **Table 4**. The 8-week intervention with a seaweed phenolic extract resulted in a  
378 significant reduction in basal DNA damage, as measured by the Comet assay, in obese

379 participants only (BMI >30 kg/m<sup>2</sup>), with a significant reduction (P=0.044) in basal DNA  
380 damage observed (**Table 4**). A significant reduction was not observed in the total population  
381 (n=78) nor in the overweight participants (n=42), either in terms of a challenge response or in  
382 basal levels. Additionally, consumption of seaweed phenolic extract also significantly reduced  
383 (P=0.009) basal DNA in males only with a mean group change value of  $-0.8 \pm 2.5$  % tail DNA  
384 (SPE) compared to  $0.9 \pm 2.8$ % tail DNA in the control. No significant effects were observed  
385 for females.

386

### 387 **Total oxidative capacity of seaweed phenolics**

388 **Figure 2** shows the total oxidative capacity (peroxide levels) in plasma samples from all  
389 participants (n=78), in overweight participants (n=42) and in obese participants (n=36)  
390 measured after the placebo and seaweed capsule intervention. There were no significant  
391 changes from baseline after either treatment phase (SPE, Maltodextrin) in all participants  
392 (n=78) nor in overweight (n=42) or obese (n=36) sub groups. However the consumption of  
393 seaweed phenolic extract also significantly reduced (P=0.018) TOC in females only with a  
394 mean group change value of  $-7.44 \pm 29.37$  (SPE) compared to  $4.33 \pm 22.36$  in the control. No  
395 significant effects were observed for males.

### 396 **Effects of seaweed (poly)phenol extract on blood lipids and CRP**

397 **Table 4** shows the pre- and post- values, as well as the percentage change, for both the  
398 placebo phase and the SPE phase for each blood lipid biomarker and CRP. An 8-week  
399 supplementation with seaweed (poly)phenol extract did not significantly affect any  
400 cardiovascular risk marker.

### 401 **Effects of seaweed (poly)phenol extract on inflammatory markers**

402 F<sub>2α</sub>-isoprostane levels at baseline were  $392 \pm 219$  pg/ml (data not shown). A high degree of  
403 inter-individual variation was observed, and the 8-week SPE intervention did not exhibit a

404 significant effect on this marker of oxidative stress (**Table 4**). There were no significant  
405 differences for any of the inflammatory cytokines measured in either treatment phase (SPE,  
406 Maltodextrin) for all of the participants (n=78), in the overweight participants (n=42) or in the  
407 obese participants (n=36) (**Figure 4**). Nor where significant effects observed in the cytokine  
408 profiles when examined by ratio of TNF- $\alpha$  to IL-10, IL-1 $\beta$  to IL-10, IL-6 to IL-10 and CRP to  
409 IL-10, according to Laird et al. (29)

#### 410 **Bioavailability of seaweed phenolics, untargeted Analysis**

411 Initially an unsupervised PCA model was carried out (**Figure 4A**) on the urine data from all  
412 78 participants. The first component of the PC1 vs PC2 scores plot obtained from the data set  
413 explained 23.14% of the total variance showing a clear trend among some of the participants  
414 compared to the rest. Moreover, a hierarchical cluster analysis was performed to verify this  
415 natural aggrupation pattern among the 78 participants suggesting, two clusters among all the  
416 participants (**Figure 4B**) which share, accounting for the inter-individual differences associated  
417 with differences in the urinary metabolite profiles. For instance, recent studies suggest that  
418 individuals can be clustered into distinct groups based on their gut microbiome composition,  
419 functional metabolism (33) or individual responses to obtain markers of a specific treatment,  
420 minimizing the inter-individual data that are not the target of the study. In order to elucidate  
421 the observed variability between these two groups, two supervised models were performed,  
422 one with the urinary profiles from all the participants before the seaweed consumption and  
423 another one with the urinary profiles from all the participants after seaweed consumption. Both  
424 supervised models verified differences between the two groups of individuals (Group 1 and  
425 Group 2) and provided successful classification results at the cross validation step and achieved  
426 in the pre-treatment and post-treatment samples 95% and 98% sensitivity (participants of the  
427 class of interest correctly assigned to their class) and 100% and 100% specificity (participants  
428 not belonging to the class of interest were correctly not assigned to that class), respectively

429 **(Supplemental Figure 1)**. It is noteworthy that even stratifying for BMI category, the  
430 multivariate analysis of the urine profiles did not show clear differentiation of the metabolome  
431 between obese or overweight individuals.

432 By using the preliminary information provided by PCA and HCA related to the stratification  
433 of the individuals into groups which share a common excretion profile before and after  
434 supplementation with seaweed capsules, two data sets were analysed. The individuals were  
435 stratified into two groups; Group 1 including 70 participants; and Group 2 including 8  
436 participants (S58, S60, S61, S71, S76, S78, S79, S83, all being overweight individuals). Two  
437 supervised OPLS-DA models were built to discriminate according to the seaweed treatment in  
438 both models (**Figure 5**). In our study we used two latent variables (LV) and the sensitivity and  
439 specificity values were set to 100% at the calibration step recognition ability of the two models.  
440 These analyses showed that the human urine metabolome in both groups of participants was  
441 modified after the 8-week supplementation with seaweed (poly)phenol extract compared to  
442 baseline urine metabolic profiles. Using the loadings plot and the variable importance in  
443 projection values (VIP) we ascertained important contributors between the modelled classes  
444 and therefore identified the compounds responsible for the difference in the urine metabolic  
445 profile before and after 8-weeks seaweed (poly)phenol extract consumption in both groups.  
446 VIP is the widely known metric that is used to identify potential markers (metabolites) in  
447 metabolomics studies. Further, the urine scores plot allowed the identification of those  
448 metabolites which appeared after the seaweed (poly)phenol extract intake (**Figure 5**) in both  
449 groups of participants.

450

451 The main contributors to metabolome differentiation in the urine before and after seaweed  
452 consumption in both groups of participants are described in Supplemental Table 1 and Table 2  
453 (Supplemental Material). Positive loading values (x axis) and higher VIP values (VIP>2.0)

454 were detected in urine after seaweed consumption and were the responsible of the observed  
455 differences between the participants classes (post ingestion vs non ingestion of seaweed  
456 (poly)phenol extract). Urine metabolites tentatively identified as pyrogallol/phloroglucinol  
457 sulfate, hydroxytrifurahol A-sulfate and dioxinodehydroeckol glucuronide are considered clear  
458 biomarkers of seaweed consumption in Group 1 while C-O-C dimer of phloroglucinol-sulfate,  
459 C-O-C-dimer of phloroglucinol, fucophloroethol-glucuronide, diphlorethol sulfates and  
460 dioxinodehydroeckol glucuronide are those biomarkers of seaweed consumption in Group 2.  
461 All of them have shown high values in the variable importance in the projection (values > 2)  
462 **(Table 5).**

463 Based upon the identified seaweed (poly)phenol metabolites, the total amount of seaweed  
464 metabolites excreted in urine varied noticeably between participant ranging from 0.001 to  
465 4.140 mmoles, with a group average of 1.29 +/- 0.88 mmoles. 25% of the population (19/78)  
466 appeared to be low excretors with urinary seaweed metabolites excretion less than 0.5 mmoles,  
467 55% of the population (43/78) were medium excretors with an urinary seaweed excretion  
468 between 0.5 and 2 mmoles, while 20% of the population (16/78) where high excretors with  
469 total seaweed metabolites concentrations >2 mmoles (see Supplemental Table 6 ).

470 The plasma metabolome demonstrated no clear differentiation for participants in either  
471 treatment phase (data not shown), nor with exploration of the data by PCA or HCA.  
472 Consequently no exposure biomarkers could be defined for plasma samples taking into account  
473 all the participants (n=78).

474

## 475 **DISCUSSION**

476 The association of seaweed consumption with reduced risk factors for CVD has been tested  
477 largely within *in vitro* or animal models, and only limited human data exists to substantiate the

478 proposed beneficial properties of seaweeds (12,13,34). To the best of our knowledge this trial  
479 is the first comprehensive study to investigate the *in vivo* bioactivity and bioavailability of  
480 seaweed (poly)phenolics on biomarkers of inflammation and oxidative stress in overweight  
481 and obese individuals. Participant retention rates were high (98%) and self-reported SPE  
482 capsule intake (97%) indicated that the intervention was implemented successfully in this  
483 group of participants. There was, however, no significant difference between the intervention  
484 (SPE capsule) and control (placebo capsule) phases for markers of oxidative stress, antioxidant  
485 status or inflammation in the population as a whole (n=78). Subset analysis, stratifying for BMI  
486 category, showed a significant albeit modest difference in DNA damage in the obese  
487 population (n=36). This is consistent with Park *et al.* (35) who tested astaxanthin, a natural  
488 carotenoid in the form of microalgae *Haematococcus pluvialis* in 14 healthy females (2 mg/d  
489 extract for 8 weeks) demonstrating a significant reduction in the oxidative damage marker  
490 plasma 8-hydroxy-2'-deoxyguanosine. Obese individuals are associated with increased DNA  
491 damage, higher oxidant status and increased oxidative damage to macromolecules a group and  
492 are at higher risk of chronic disease (36-39). In our study, an overweight/obese population (age  
493 30-65 years and a BMI >25 kg/m<sup>2</sup>) was shown to have significantly reduced basal levels of  
494 lymphocyte DNA damage (by 23%) but only in the obese population subset (n=36) following  
495 supplementation. Both seaweed-based intervention studies support data from *in vitro* studies  
496 indicating anti-genotoxic activity for seaweed extracts on a range of cell lines (40,41) including  
497 the SPE extract used in the study (15). Although a mechanism was not determined for the  
498 observed reduction in DNA damage in the obese group activate Nrf2-mediated cytoprotection  
499 may be involved, as seaweed (poly)phenols such as eckol are anti-genotoxic (42) and can  
500 activate Nrf2-mediated HO-1 induction (43,44) consistent with effects observed for  
501 (poly)phenols from terrestrial sources (45,46). Park *et al.* (35) also observed a significant  
502 reduction in plasma CRP levels, an acute marker of inflammation. Although in our study we

503 observed a 28% decrease in CRP levels in response to seaweed extract consumption, this  
504 change was not significant. However, a similar study investigating the effect of consuming  
505 *Palmaria palmata* (5 g/day) incorporated into bread found that it significantly increased CRP  
506 by 16%, suggesting that *P. palmata* stimulates inflammation rather than reducing it (47). Park  
507 *et al.* (35) reported that there was no difference in TNF and IL-2 concentrations, but plasma  
508 IFN- $\gamma$  and IL-6 increased on week 8 in participants given 8mg astaxanthin. Within this study,  
509 we similarly observed no significant changes in IL-2 or TNF, nor did we see an alteration in  
510 IFN- $\gamma$  or IL-6. The seaweed extract tested did not affect immune function (cell mediated and  
511 humoral immune responses as tested by the cytokine markers) following supplementation.

512

513 In order to evaluate the seaweed molecules responsible for its modest beneficial effects *in vivo*  
514 and the potential biological markers linked to seaweed consumption, an untargeted approach  
515 comparing the metabolic profiles between urine and plasma samples of 78 individuals who  
516 consume seaweed capsules have been carried out. As a first step, we have determined  
517 differences in the urine metabolic profiles between participants being able to stratify the  
518 individuals into groups which share common excretion metabolite profile before and after  
519 seaweed capsule supplementation. Urinary profiles of these two groups were statistically  
520 different between each other and the metabolites responsible for this discrimination have been  
521 selected as potential biomarkers of seaweed consumption (**Table 5**). Subsequently, the  
522 quantification of these potential biomarkers within all participants revealed substantial inter-  
523 individual variation in the concentration of seaweed metabolites excreted in urine by the  
524 participants ranging from 0.001 to 4.140 mmoles. Besides, the outcomes of the comprehensive  
525 multivariate analysis of the metabolite profiles shows, as expected, person-to-person variation  
526 in the 0-24h urinary excretion of seaweed (poly)phenols, probably due to differences in gut  
527 microbiota and no strict living conditions of the participants. As a consequence of these inter



528 individual differences, the 78 participants involved in this study can be grouped by their  
529 dissimilarity to metabolize seaweed (poly)phenols in two well established groups. For instance,  
530 group 1 of individuals is characterized by greater excretion of seaweed derived metabolites  
531 such as pyrogallol/phloroglucinol sulfate, hydroxytrifurahol A-sulfate and  
532 dioxinodehydroeckol; while participants belonging to group 2 C-O-C dimer of phloroglucinol-  
533 sulfate, C-O-C dimer of phloroglucinol, fucophloroethol-glucuronide, diphlorethol sulfates and  
534 dioxinodehydroeckol glucuronide in low concentrations. This inter-individual variation in the  
535 absorption of seaweed polyphenols has been observed in the bioavailability of dietary  
536 (poly)phenols such as orange juice, cranberry and pomegranate (48-50).

537 The results from the non-targeted approach confirmed the presence of seaweed phenolic  
538 metabolites, and can be divided into a) phase II sulphated and glucuronidated metabolites  
539 related to the targeted components described earlier by Corona *et al.* (20) (supplemental Tables  
540 4,5); arguably formed in the liver; b) an extended list of unknown compounds which may be  
541 potential breakdown products or metabolites of the original seaweed (poly)phenols catabolised  
542 by the colonic bacteria but further studies would be required to confirm the identity of these  
543 unknown compounds. Poor absorption of the high molecular weight phlorotannins in the upper  
544 gastrointestinal tract following acute consumption of our seaweed (poly)phenolic extract as  
545 reported (20) likely results in them reaching the colon and becoming subject to microbial  
546 fermentation to lower molecular weight derivatives; recently confirmed by *in vitro* gut  
547 modelling of SPE by colonic microbiota (15). The urinary metabolite profiles of seaweed  
548 phenolics from the low and high excretors clearly indicated a high inter-individual variation in  
549 metabolism similar to that observed in terrestrial plant phenolics, and no single common  
550 metabolite or pattern could be detected following seaweed consumption. It is possible that  
551 inter-individual variation in gut microbiota underlie these metabolic changes and were  
552 responsible for the observed differences. That said, the study of bioavailability of seaweed

553 polyphenolics remains challenging due to the high range of molecular weight compounds  
554 present, and their characterisation is complicated further by the lack of commercially available  
555 standards. Other limitations of the present study include a relatively short-term intervention,  
556 results cannot therefore be extrapolated to long-term chronic consumption. Participants in this  
557 study may not be representative of the general overweight population of Northern Ireland as  
558 they were mostly recruited from the University of Ulster staff and local residents. While  
559 lymphocyte DNA damage (spontaneous DNA SBs) is considered appropriate for the  
560 substantiation of EFSA health claims in the context of protection against generic DNA damage,  
561 a lesion specific enzyme such as (ENDO III) would have been required for a claim related to  
562 oxidative DNA damage (51). Finally, the study population number may have been too small to  
563 yield significant results in intercellular cytokines. These factors should be considered for the  
564 design of future studies in this area.

565 To conclude, to the best of our knowledge, this work represents the first comprehensive  
566 study represents the first comprehensive study involving 78 human participants investigating  
567 the bioavailability of seaweed (poly)phenolics. Consumption of SPE, decreased DNA damage  
568 albeit to a modest extent in obese individuals only; with no clear effects on clinical markers of  
569 inflammation. While untargeted analysis identified novel urinary biomarkers of seaweed  
570 consumption and highlighted a high degree of inter-person variation in the metabolism of  
571 seaweed phenolics. Future studies that address the ingestion of seaweed phenolics will need to  
572 consider and adjust for these parameters, including the importance of establishing an  
573 individual's capacity for metabolising (poly)phenol (52-54).

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577

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Table 1. Key components of phlorotannin rich basic seaweed extract, High Molecular Weight (HMW) seaweed extract fraction, and blend used for intervention capsules (SPE).

Extract component	Basic seaweed	HMW seaweed	Blend
	extract	extract fraction	(SPE capsule)
	mg/175mg of extract	mg/50mg of extract	mg/400mg capsule
Phlorotannin	61.25	46.05	107.3
Iodine	0.48	0.02	0.5
Maltodextrin <sup>1</sup>	0	0	175
Minerals	39.38	13	52.38
Fucoxanthin	0	36.5	36.5
Laminarin as glucose	10.68	1.75	12.43
Fuoidan as fucose	0	<0.001	<0.001
Mannitol	29.23	5.9	35.13
Inorganic arsenic	<0.001	<0.001	<0.001
Cadmium (LD 0.15mg/kg)	<LD	<LD	<LD
Mercury (LD 0.016mg/kg)	<LD	<LD	<LD
Lead (LD 1.1mg/kg)	<LD	<LD	<0.001
Tin (LD 1.7mg/kg)	<LD	<LD	<LD

<sup>1</sup>maltodextrin was added to the capsule formulation as an excipient.

Table 2. Baseline characteristics of the study population (n=80).

<b>Variable</b>	<b>Placebo</b>	<b>SPE</b>
	<b>treatment phase</b>	<b>treatment phase</b>
	<b>(n=80)</b>	<b>(n=80)</b>
Age (y)	42.8 ± 7.2	42.9 ± 7.1
Gender (M/W)	20/20	19/21
Height (m)	1.71 ± 0.08	1.72 ± 0.10
Weight (kg)	88.9 ± 14.1	89.1 ± 17.3
BMI (kg/m <sup>2</sup> )	30.3 ± 3.5	30.0 ± 4.4

No significant differences  $P>0.05$ , Paired T Test.

Table 3. Habitual nutrient intake of subjects during intervention study

Variable intake	Placebo	SPE	% Change <sup>2</sup>	P value <sup>3</sup>
	treatment	treatment		
	phase	phase		
	(n=77) <sup>1</sup>	(n=77)		
Energy (kcal/d)	1949 ± 590 <sup>4</sup>	2057 ± 684	5	0.110
Protein (g/d)	79.4 ± 28.6	79.9 ± 25.3	0.5	0.782
Carbohydrate (g/d)	220.6 ± 72.1	231.3 ± 84.2	5	0.278
Total fat (g/d)	78.6 ± 26.5	85.2 ± 34.8	8	0.058
Saturated fat (g/d)	29.0 ± 10.2	30.7 ± 15.3	6	0.417
Monounsaturated fat (g/d)	24.4 ± 9.3	26.8 ± 12.4	9	0.112
Polyunsaturated fat (g/d)	12.1 ± 5.4	13.5 ± 6.4	10	0.059
Fibre (g/d) <sup>5</sup>	12.9 ± 6.2	13.3 ± 4.8	3	0.259
Vitamin C (mg/d)	71.8 ± 50.5	69.4 ± 47.1	-3	0.909
Vitamin E (mg/d)	7.17 ± 3.7	7.67 ± 4.0	7	0.279
Folate (µg/d)	223.9 ± 98.3	228.2 ± 85.5	2	0.370
Carotene (µg/d)	2821 ± 1900	2553 ± 1706	-11	0.185

<sup>1</sup>S66 did not complete a food diary in either phase. <sup>2</sup> Calculated from SPE phase – Placebo

phase. <sup>3</sup> Mean treatment group values were not significantly different between phases, P>0.05

(Wilcoxin signed rank test). <sup>4</sup> Mean ± SD. <sup>5</sup> Calculated using the Englyst method.

Table 4. Effects of seaweed polyphenol extract on lymphocyte DNA damage, CRP, blood lipids and F<sub>2</sub> isoprostanes

Blood marker	Total (n=78)				Overweight (n=42)				Obese (n=36)			
	Average baseline value	Placebo treatment effect	SPE treatment effect	P value	Average baseline value	Placebo treatment effect	SPE treatment effect	P value	Average baseline value	Placebo treatment effect	SPE treatment effect	P value
DNA damage – basal (% Tail)	6.72 ± 2.48	0.74 ± 2.86	-0.41 ± 3.13	0.350	6.59 ± 2.80	0.32 ± 2.14	0.57 ± 3.24	0.129	6.91 ± 2.00	1.81 ± 4.50	0.15 ± 2.93	<b>0.044</b>
DNA damage - H <sub>2</sub> O <sub>2</sub> (%Tail)	34.2 ± 7.00	-1.56 ± 6.60	-2.03 ± 6.40	0.390	35.2 ± 7.11	0.13 ± 2.19	0.76 ± 1.66	0.062	32.8 ± 6.69	1.26 ± 2.09	0.54 ± 1.54	0.111
CRP (mg/ml)	2.67 ± 3.9	0.01 ± 3.3	-0.83 ± 4.9	0.429	2.59 ± 4.69	0.00 ± 3.40	-1.32 ± 6.22	0.348	2.80 ± 2.56	-1.81 ± 11.82	0.72 ± 5.15	0.258
Cholesterol (mmol/l)	5.20 ± 0.77	-0.06 ± 0.57	-0.10 ± 0.57	0.256	5.18 ± 0.82	0.01 ± 0.55	-0.53 ± 0.54	0.201	5.24 ± 0.73	-0.13 ± 0.58	-0.15 ± 0.60	0.419
Triglycerides (mmol/l)	1.51 ± 0.94	0.01 ± 0.82	0.04 ± 0.96	0.385	1.34 ± 0.72	0.07 ± 0.79	-0.04 ± 0.48	0.278	1.75 ± 1.15	-0.06 ± 0.85	-0.02 ± 1.34	0.497
HDL (mmol/l)	1.37 ± 0.32	-0.01 ± 0.15	0.03 ± 0.15	0.187	1.46 ± 0.31	0.01 ± 0.16	-0.03 ± 0.15	0.150	1.25 ± 0.32	-0.04 ± 0.11	-0.04 ± 0.14	0.446
LDL (mmol/l)	3.16 ± 0.1	-0.08 ± 0.5	-0.06 ± 0.50	0.478	3.13 ± 0.69	-0.07 ± 0.57	0.01 ± 0.45	0.383	3.20 ± 0.67	-0.09 ± 0.56	-0.13 ± 0.56	0.412
F <sub>2</sub> Isoprostanes (pg/ml)	393 ± 220	-10 ± 182	-6 ± 138	0.374	342 ± 128	28 ± 152	-29 ± 108	0.130	439 ± 272	-44 ± 204	14 ± 158	0.283

Data is presented as treatment affects, calculated based on individual differences between pre- and post- values for both control and treatment phases for each subject. Paired T-tests were then carried out on the difference scores (post-treatment value *minus* pre-treatment values) between treatment (Seaweed phenolic extract capsule) and placebo control phase (maltodextrin). Significance level was set at P<0.05 (one-tailed T test).

**Table 5.** Phlorotannins metabolites tentatively identified in human urine samples from Group 1 (70 subjects) and Group 2 (8 subjects) after seaweed capsule consumption.

<b>ID</b>	<b>VIP value</b>	<b>Rt (min)</b>	<b>Experimental Molecular Weight</b>	<b>Predicted Formula</b>	<b>Metabolite putative identification</b>	<b>Ratio<sup>1</sup></b>
<b>Group 1 (72 subjects)</b>						
652	2.06	25.6	205.9881	C <sub>6</sub> H <sub>6</sub> O <sub>6</sub> S	Pyrogallol/phloroglucinol sulfate	1.5
800	2.28	25.8	205.9881	C <sub>6</sub> H <sub>6</sub> O <sub>6</sub> S	Pyrogallol/phloroglucinol sulfate	1.2
1472	3.86	24.9	205.9881	C <sub>6</sub> H <sub>6</sub> O <sub>6</sub> S	Pyrogallol/phloroglucinol sulfate	1.1
1352	4.26	25.6	486.1727	C <sub>26</sub> H <sub>30</sub> O <sub>7</sub> S	Hydroxytrifuhaol A-glucuronide	2.0
1453	3.83	25.4	486.1727	C <sub>26</sub> H <sub>30</sub> O <sub>7</sub> S	Hydroxytrifuhaol A-glucuronide	1.9
1458	4.23	25.3	486.1727	C <sub>26</sub> H <sub>30</sub> O <sub>7</sub> S	Hydroxytrifuhaol A-glucuronide	2.2
1483	4.39	25.3	486.1727	C <sub>22</sub> H <sub>30</sub> O <sub>12</sub>	Hydroxytrifuhaol A-glucuronide	2.2
1917	2.11	31.4	544.2881	C <sub>27</sub> H <sub>44</sub> O <sub>11</sub>	Dioxinodehydroeckol glucuronide	1.1
<b>Group 2 (8 subjects)</b>						
293	2.08	9.5	327.0951	C <sub>12</sub> H <sub>8</sub> O <sub>9</sub> S	C-O-C dimer of phloroglucinol-sulfate	1.2
702	2.20	24.1	248.0315	C <sub>12</sub> H <sub>8</sub> O <sub>6</sub>	C-O-C dimer of phloroglucinol	1.2
853	2.08	27.8	797.3186	C <sub>45</sub> H <sub>49</sub> O <sub>13</sub>	Fucophloroethol glucuronide	1.1
1293	2.28	34.2	330.1675	C <sub>12</sub> H <sub>10</sub> O <sub>9</sub> S	Diphlorethol sulfate	1.9
1356	2.31	34.1	330.1675	C <sub>12</sub> H <sub>10</sub> O <sub>9</sub> S	Diphlorethol sulfate	1.7
1633	2.07	35.1	544.2152	C <sub>27</sub> H <sub>44</sub> O <sub>11</sub>	Dioxinodehydroeckol glucuronide	1.2

ID, identification number. VIP (variable influence in projection) is a variable that summarizes the importance of X variables to the OPLS-DA model. Variables with values > 2 were the most influential in the model. All predicted formula derived with < 5 ppm mass accuracy data.

<sup>1</sup>Ratio: seaweed capsule consumption/placebo consumption.



**FIGURE 1.** CONSORT DIAGRAM. Progress of participants through the intervention study.

**FIGURE 2.** Comparison of the effects of SPE supplementation phase (■) with that of a placebo phase (□) on total oxidative capacity (TOC) in the total study population (n=78), in overweight (n=42), and in obese (n=36). All values were measured in duplicate expressed as mean  $\pm$  SD. TOC is represented as the mean of the individual difference values (after – before supplementation) in the SPE and placebo treatment phases.

**FIGURE 3.** Comparison of the effects of SPE supplementation phase (■) with that of a placebo phase (□) on cytokine levels in the total study population (n=78), in overweight (n=42), and in obese (n=36). All values were measured in duplicate expressed as mean  $\pm$  SD. Cytokines were measured as the mean of the individual difference value (after – before supplementation) in the SPE and placebo treatment phases. Change in mean treatment group values were not significantly different between treatment phases,  $P > 0.05$  (Paired T Test; one-tailed test). M.F.I.; Mean Fluorescence Intensity.

**FIGURE 4.** Principal Component Analysis (PCA) (A) and Hierarchical Cluster Analysis Cluster (HCA) (B) of urinary profiles before (◆) and after (■) seaweed consumption by 80 participants. The HCA was calculated based on Euclidean distances and the Ward hierarchical agglomerative method. The PC explained 23.14% of the total variance (PC-1 14.8% and PC-2 8.34%).

**FIGURE 5** A) OPLS-DA scores and B) loadings of the urine samples belong to group of participants 1 (70 participants). C) OPLS-DA scores and D) loading of urine samples belong to group of participants 2 (8 participants) before (◆) and after (■) seaweed ingestion. (Circles shown in the graph represent a confidence of 95%). LV1: latent variable 1; LV 2: latent variable 2. The cut off VIP value selected to be 2. For VIP scores identification see **Table 5** and **Supplemental Table 1**.



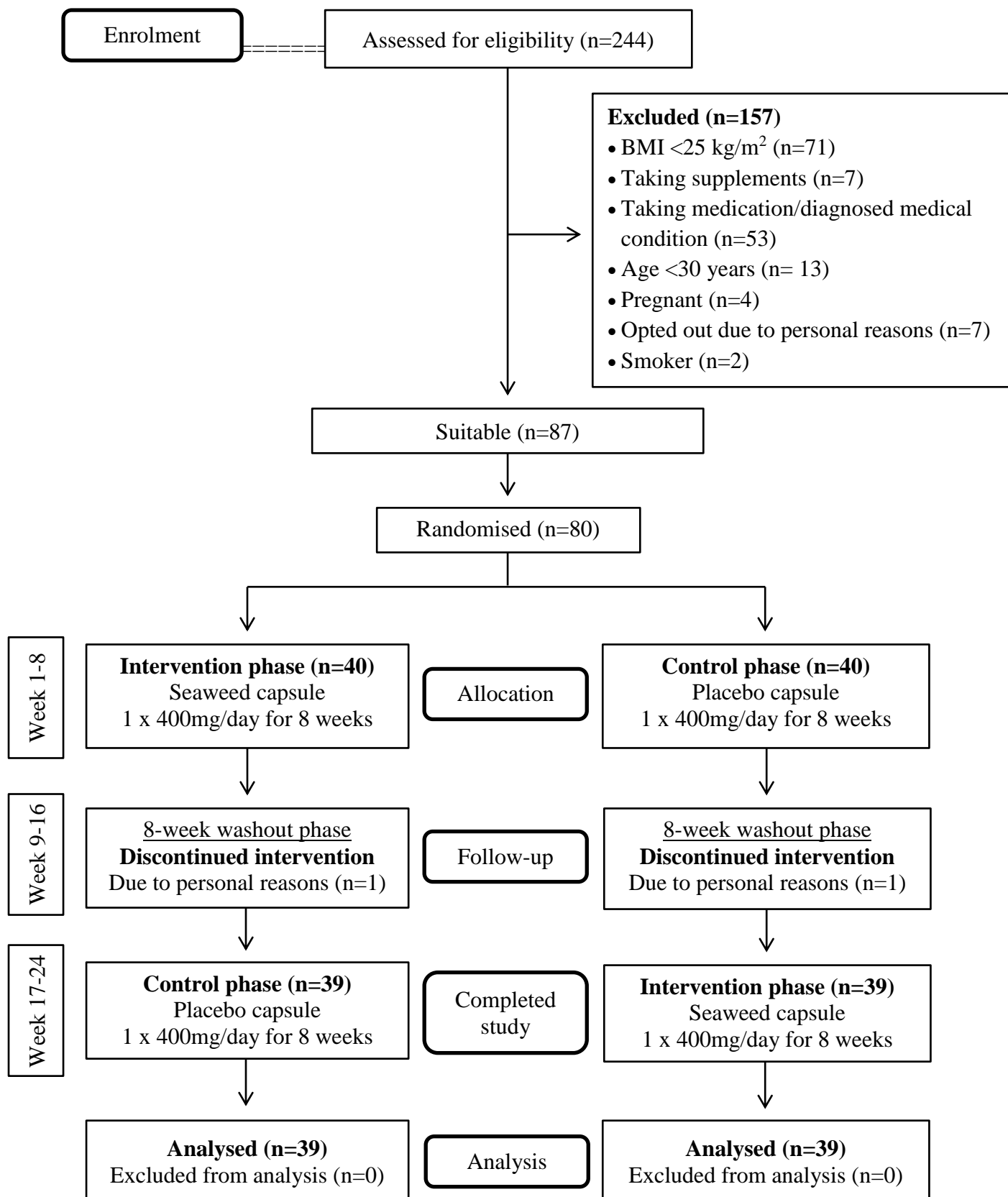


FIGURE 1

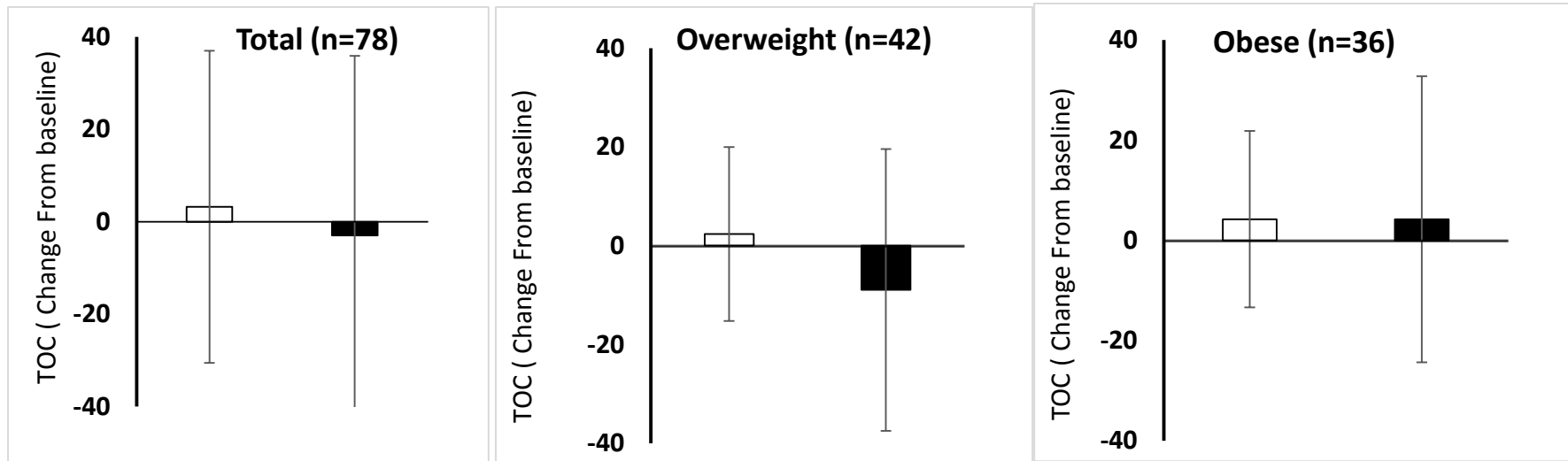


FIGURE 2.

FIGURE 3.

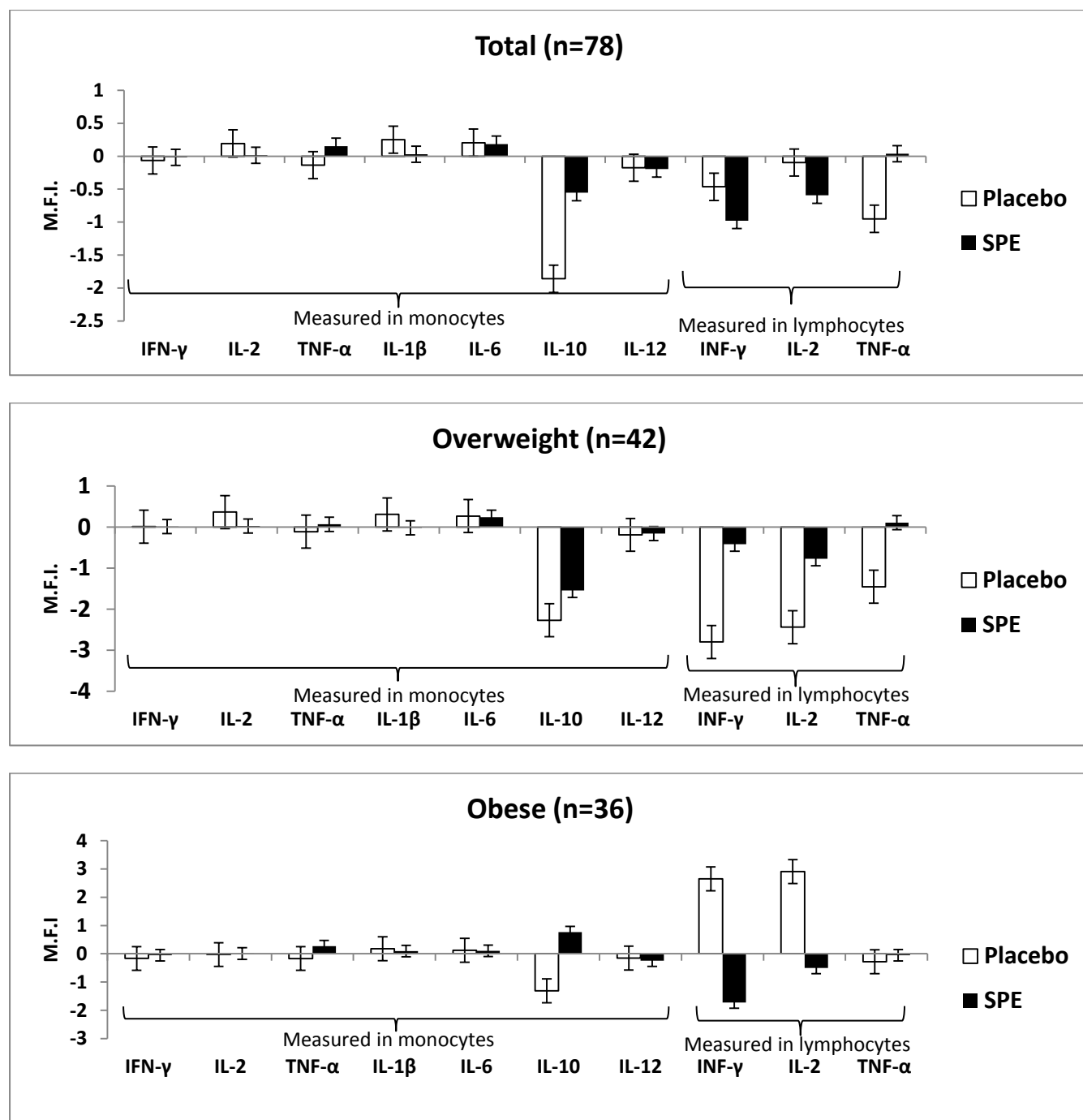
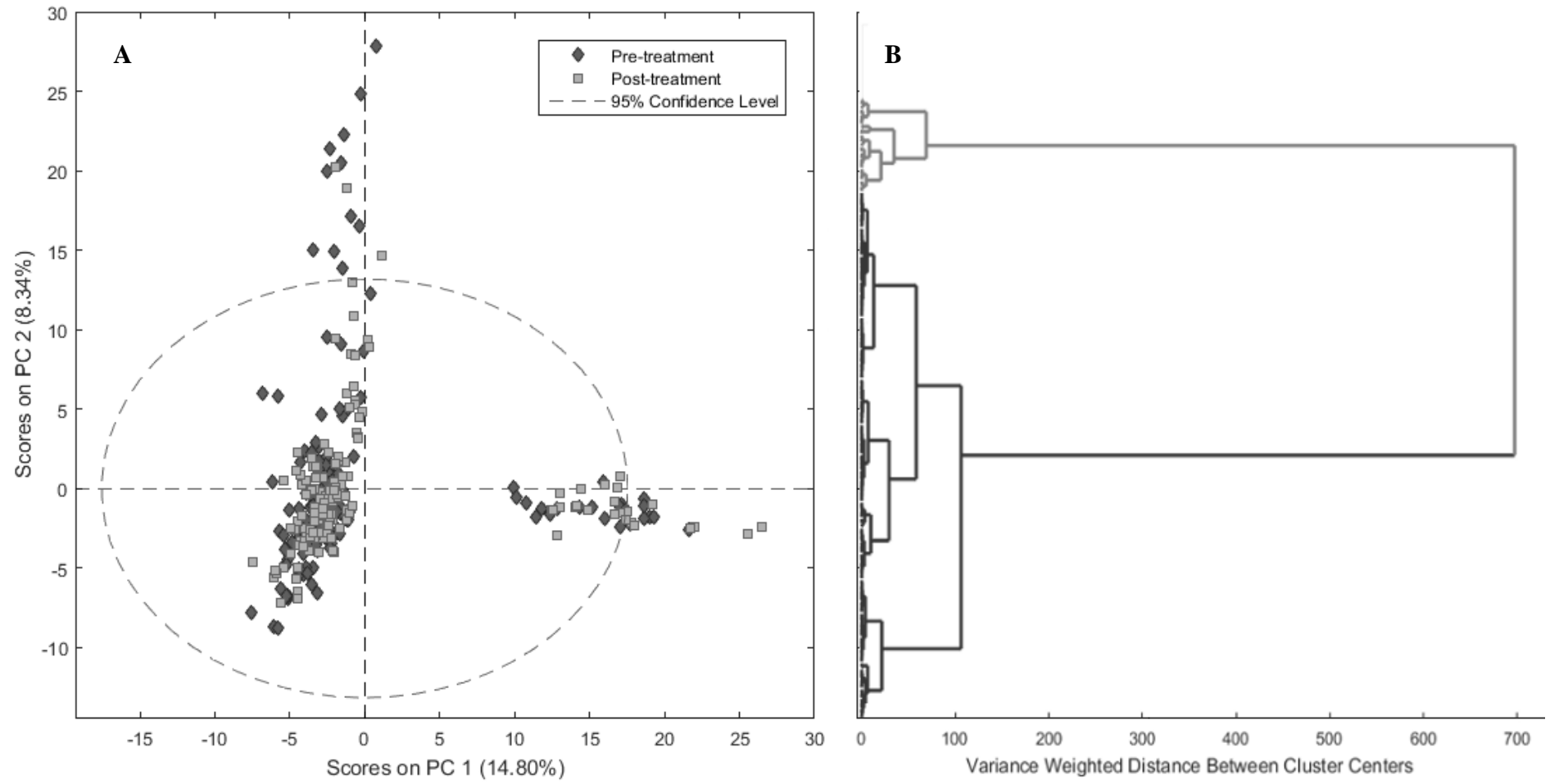


FIGURE 3.

**FIGURE 4.**

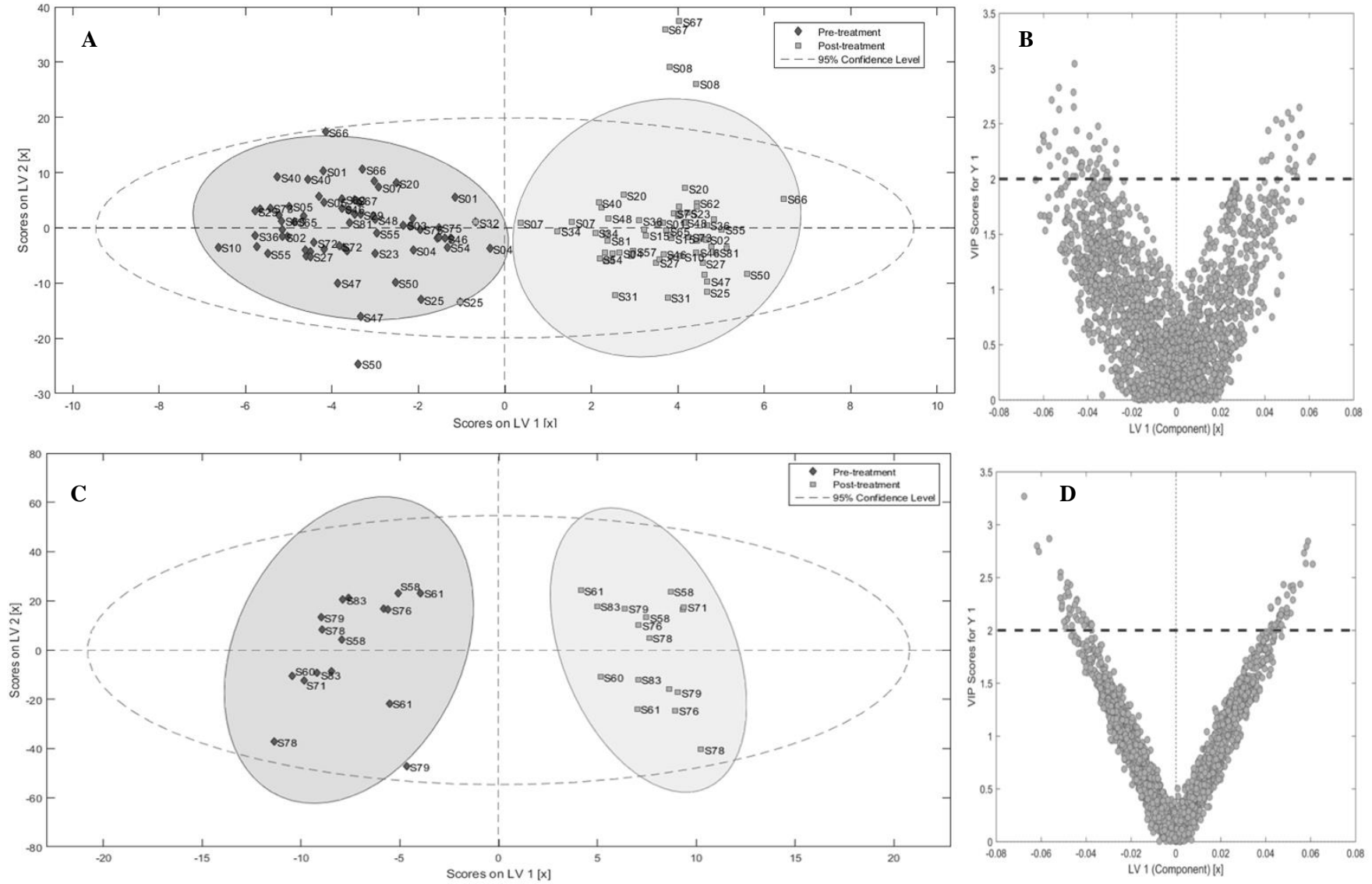


FIGURE 5

