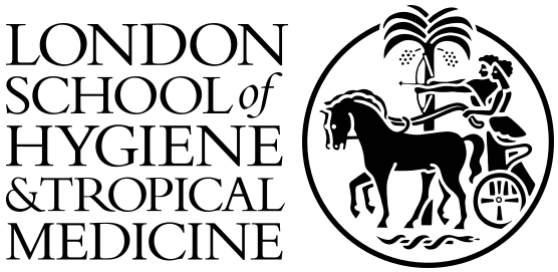

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Effects of long-term consumption of broccoli sprouts on inflammatory markers in overweight subjects

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Keywords

Glucosinolates; *Brassica oleracea*; Sulphoraphane; IL-6; Bioavailability; Inflammation

ABSTRACT

Background & aims: Broccoli sprouts represent an interesting choice of healthy food product as they are rich in glucosinolates and their cognate bioactive metabolites, isothiocyanates able to counteract the negative effects of diverse pathologies. As obesity is linked to an inflammatory component, the aim of the study was to evaluate the anti-inflammatory action of broccoli sprouts in overweight adult subjects.

Methods: An *in vivo* controlled study was performed in 40 healthy overweight subjects (ClinicalTrials.gov ID NCT 03390855). Treatment phase consisted on the consumption of broccoli sprouts (30 g/day) during 10 weeks and the follow-up phase of 10 weeks of normal diet without consumption of these broccoli sprouts. Anthropometric parameters as body fat mass, body weight, and BMI were determined. Inflammation status was assessed by measuring levels of TNF-α, IL-6, IL-1β and C-reactive protein.

Results: IL-6 levels significantly decreased (mean values from 4.76 pg/mL to 2.11 pg/mL with 70 days of broccoli consumption, $p < 0.001$) and during control phase the inflammatory levels were maintained at low grade (mean values from 1.20 pg/mL to 2.66 pg/mL, $p < 0.001$). C-reactive protein significantly decreased as well.

Conclusions: This study represents an advance in intervention studies as the broccoli sprouts were included in a daily dietary pattern in quantities that reflect a real consumption. Further studies are necessary to elucidate the role of this healthy rich and nutritious food product, but these promising results support the current evidence on the healthy properties of *Brassica* varieties.

INTRODUCTION

Nowadays there is an increasing demand by consumers on healthy food products prepared in convenient forms, simple to use and not containing additives. In this sense, broccoli sprouts (*Brassica oleraceae var. italica*) are a good option, as they are the main dietary source of glucosinolates, nitrogen-sulfur compounds, and phenolic derivatives (flavonoid glycosides, and hydroxycinnamic acids), vitamins A, C, E, K and minerals [1]. Glucosinolates are derivatives from amino acids and particularly abundant in *Brassica* species and are believed to be responsible of the biological effects attributed to these vegetables. Cultivar conditions can affect and improve the biosynthesis of these secondary metabolites in the plant, favouring their storage in the sprouts. Broccoli sprouts contain 10- to 50-fold higher concentrations of glucosinolates than the mature broccoli [2].

Isothiocyanates are degradation products from glucosinolates formed by the hydrolytic action of myrosinase vegetable enzyme when the vegetable structure is crushed, e.g. during mastication process. Hydrolysis can also be performed by gastrointestinal enzymes during digestion; the metabolic conversion of glucosinolates to isothiocyanates is a crucial step for the benefits observed with the consumption of *Brassica* vegetables [3]. Most of these reported effects are linked to anti-cancer properties and epidemiological studies have evidenced a decrease in the risk of cancer with the intake of cruciferous foods [4, 5]. Human clinical studies have mainly focused on these antitumoral activities, with mechanisms including the upregulation of phase II detoxification enzymes, as well as the direct action on cell cycle, causing apoptosis of cancer cells [6] [7]. There is less evidence on the anti-inflammatory properties of cruciferous vegetables in humans.

A non-communicable disease of high prevalence in Western societies is obesity. Nowadays it is assumed that obesity is a condition characterized by a chronic low-grade inflammation status. Levels of pro-inflammatory mediators as IL-6, TNF-α and C-reactive protein are increased with the higher visceral adiposity and all of them are risk factors for cardiovascular diseases, metabolic syndrome and diabetes [8]. The permanent low-grade of inflammation is a response of the organism to the imbalance in the nutrient due to energy excess and it is manifested in the development of insulin resistance and type 2 diabetes [9]. Dietary interventions able to reduce the inflammatory conditions linked to obesity may improve health conditions of overweight population.

The hypothesis of our research is that broccoli sprouts are able to reduce the inflammatory status in overweight subjects due to their content in phytochemicals, mainly glucosinolates. Hence, the aim of the study is to evaluate the changes in markers of inflammation in overweight subjects after 10-week ingestion of broccoli sprouts as well as to assess the bioavailability of glucosinolates to correlate the changes observed.

MATERIAL AND METHODS

Study design

We performed an interventional follow-up study to evaluate the effect of the daily consumption of broccoli sprouts during 10 weeks (70 days).

The study was performed according with the Helsinki Declaration of Human Studies and approved by the Ethical Committee of the Catholic University of Murcia as well as the Bioethics Sub-Committee of the CSIC' Department of Ethics for the AGL-2013-46247-P project registered also at ClinicalTrials.gov ID NCT 03390855. Volunteers (n=40; 21 men, 19 women) were recruited in the Catholic University of Murcia (UCAM) and all of them were informed on the characteristics of the study and they

signed the written-informed consent. The inclusion criteria were: BMI within the overweight range according to the World Health Organization criteria (24.9-29.9 kg/m^2), aged 35-55 years, taking no vitamins, supplements or medication during the previous two months; no-smoking. The exclusion criteria were: diagnosed diseases as hypertension and cardiovascular pathologies, diabetes, hepatic, gastrointestinal and renal diseases, as well as the intake of drugs related to these pathologies, vegetarian diet, pregnancy or breastfeeding. Dietetic and life style habits were recorded from all participants.

There were no drop-outs during the whole period of study and no adverse effects were reported due to the broccoli sprout ingestion. Physical parameters of the volunteer at the beginning of the study are listed in **Table 1**.

One week before the beginning of the intervention period, subjects were asked to avoid the consumption of *Brassica* vegetables (broccoli, radish, cauliflower, Brussel sprouts, mustards, among others) and their derived products, and to follow a well-balanced diet (based on Mediterranean diet), with no other food restriction criteria. These dietary instructions were maintained during the entire period of study. Besides, they were requested to record any sign of adverse effect, illness or deviation of the experimental diet. The subjects maintained their usual lifestyles during the study.

On the first day, participants were given the portions of fresh broccoli sprouts to be taken for the whole week (7 trays of broccoli sprouts of 30 g each) and each week they had an appointment to provide them the fresh products. The intervention consisted on a 10-week period which included daily consumption of a portion (30 g) of raw, fresh broccoli sprouts. This amount is consistent with a half- serving [11]. Subjects were instructed to ingest 1 tray per day and to keep the trays refrigerated (4º C) at home. The intake of the broccoli sprouts was included in their normal daily diet and no specific

time of consumption was established, with the only limitation of avoid cooking of the sprouts and to consume them fresh. Cooking procedures can affect the content of glucosinolates as well as their bioavailability [12, 13] and therefore some recipes were provided to the participants to facilitate the intake of the sprouts without affecting the phytochemical composition and absorption. We gave instructions to the volunteer of not cooking broccoli sprouts but of consuming them in raw manner. They included the sprouts in vegetable salads, cold pasta salads or in cold sandwiches with different combinations of the following ingredients: cheese, ham, tomato, lettuce, grilled pork or grilled vegetables (in both cases the sprouts should be added after the grill and once the ingredient was lukewarm). It could also be included in burgers, as the "Californian style burger", which ingredients are broccoli sprouts, bacon, avocado, tomato, burger and bread. Other recipes include elaboration of "Gazpacho" (Spanish cold soup made with vegetables as tomato, cucumber, green pepper, onion, oil, vinegar and salt), or mixed with smashed potatoes with melted cheese, or mixed with bread and spreadable cheese. In all cases it was important to use in cold or warm temperatures so that the glucosinolates were preserved.

After the intervention period, a follow-up recovery period for all subjects continued for other 90 days with no ingestion of broccoli sprouts.

Fasting blood samples and 24-h urine samples were taken on day 0 (D0: just before starting the intervention), day70 (D70: end of intervention period), day 90 (D90: 20 days after end of intervention) and 160 (D160: 90 days after end of intervention). Blood samples were collected from each subject by venipuncture from the antecubital vein; 3 mL were placed in heparin tubes and centrifuged at 10000 rpm for 10 min at 4ºC. Plasma was aliquoted and stored at -80ºC until analysis. Analysis were performed once each period was finished and in the same batch to minimize analytical variations. The

144 total volume of the 24h-urine was recorded to calculate the absolute amounts of the
145 compounds and metabolites excreted in the study period and aliquots were frozen at -
146 80ºC for further analysis. Body weight and percentage of fat mass were measured as
147 well and BMI calculated in each sampling time point.

148 **Broccoli sprouts**

149 Raw, fresh broccoli sprouts (*Brassica oleracea* var. *italica*) were supplied by
150 Aquaporins&Ingredients, S.L (Alcantarilla, Murcia, Spain). The sprouts were
151 biostimulated with methyl jasmonate 250 µM, for 4 days previous to delivery, in order
152 to increase up to 2-fold levels the production of bioactive compounds, according to a
153 protocol previously validated [10]. In that study we performed some tests on elicitation
154 and seed priming to enrich the broccoli sprouts in glucosinolates. We used the elicitor
155 methyl jasmonate (MeJA) by priming the seeds as well as by spraying daily over the
156 cotyledons from day 4 to 7 of germination. We observed that MeJA at concentrations of
157 250 µM act as stressor in the plant and enhances the biosynthesis of the phytochemicals
158 glucosinolates. Compared to control plants without MeJA treatment, the content of
159 compounds as the aliphatic glucosinolate glucoraphanin was enhanced up to a 70 % and
160 similar increases were observed with glucoiberin or glucobrassicin. In this way, we
161 improved the content of these health-promoting compounds. Other nutritional facts did
162 not change with these treatments.

163 Three trays of sprouts were collected once a week during the study, frozen and
164 lyophilized prior to analysis on glucosinolates and isothiocyanates, as previously
165 described [2]. The phytochemicals (glucosinolates) provided by the broccoli sprouts are
166 summarized in **Table 2**.

167 *Biochemical analysis*

Markers of inflammation as IL-6, C-reactive protein, IL-1β and TNF-α in plasma were determined in our laboratory using high-sensitivity ELISA kits. ichroma^{TM} hsCRP kits were purchased from Boditech Meed Inc.'s. Human IL-6 ELISA high sensitivity kits were from BioVendor. Human IL-1β high sensitivity ELISA kits and human TNF-α high sensitivity ELISA kits were acquired from IBL International GmbH Instrumental.

Analysis of glucosinolates and isothiocyanates in urine and plasma

Levels of glucosinolates, isothiocyanates and their metabolites (GRA, IB, SFN, SFN-GSH, SFN-NAC, SFN-CYS, I3C, 3,3-DIM) were measured in urine by a rapid, sensitive and high throughput UHPLC-QqQ-MS/MS [14]. All LC-MS grade solvents were obtained from J.T. Baker (Phillipsburg, New Jersey, USA). The standards of Sulphoraphane, SFN-glutathione, SFN-cysteine and SFN-N-acetylcisteine (SFN, SFN-GSH, SFN-CYS, SFN-NAC, respectively) and Iberin (IB), Indole-3-carbinol (I3C) and 3,3-Diindolyl-methane (DIM_3_3) were from SantaCruz Biotech (CA, USA). Glucoraphanin (GRA) was obtained from Phytoplan (Diehm & Neuberger GmbH, Heildelberg, Germany).

Urine and plasma samples were extracted using SPE Strata-X cartridges (33um Polymeric Strong Cation) following manufacturer's instructions (Phenomenex, Torrance, CA, USA). The cartridges were preconditioned with 2 mL of methanol and equilibrated with 2 mL of water:formic acid (98:2, v/v). Samples (400 µL) were diluted with 2 mL water:formic acid (98:2, v/v) and loaded into the column. SPE cartridges were washed with 2 mL water:formic acid (98:2, v/v). Elution of target metabolites was performed with 1 mL of methanol/formic acid (98:2, v/v). Samples eluted were dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, Massachusetts, USA). The extracts were reconstituted in 200 µL of mobile phases A/B (90:10, v/v) and filtered with PTFE 0.22 µm filters Chromatographic separation was carried out using a

ZORBAX Eclipse Plus C-18 (2.1 x 50 mm, 1.8 µm) (Agilent Technologies) and the mobile phases employed were: solvent A ammonium acetate, 13 mM (pH 4 with acetic acid) and solvent B acetonitrile/acetic acid (99.9:0.1 v/v) as previously described [15].Twenty microliters of each sample were acquired in a Agilent Technologies UHPLC-1290 Series coupled to a 6460 QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany). Compounds were identified and quantified using MRM transitions and positive or negative ESI mode for confirmation of the target analytes, compared to available external standards [14]. Standard curves were prepared freshly every day of analysis.

Statistical analysis

Continuous variables were summarised with means and standard deviations while qualitative variables were summarised with proportions. We estimated the relative change on the continuous biomarkers (weight, BMI, body fat, IL_6, C-reactive protein and 3,3-DIM), between the different periods (before and after Broccoli ingestion) using linear regression models. Additional models were run controlling for age and sex in case these variables were confounding the effect of the broccoli. We then compared the proportion of samples with detectable metabolites in sulphoraphane pathway (SFN, SFN-CYS and SFN-NAC) between visits. We studied the association between changes observed in body fat mass, IL6 and C-reactive protein (outcome variables) and changes in metabolites 3,3-DIM, SFN-NAC, SFN-CYS and SFN (explanatory variables) using linear regression models. Data were analysed using R (3.4.1. version) software package.

RESULTS

Bioactive compounds in broccoli sprouts

Broccoli sprouts from each of the 10 weeks of the study were characterized for their glucosinolate (GLS) contents (**Table 2**). Results are presented as the serving portion

(30g) consumed daily by the volunteers. The major aliphatic glucosinolate in broccoli sprout detected was glucoraphanin (GRA, 4-methyl-sulphinylbutyl glucosinolate) and the major indolic glucosinolate detected was neoglucobrassicin (NGB, 1-methoxy3-indolylmethyl glucosinolate). Total concentration of aliphatic glucosinolates was 80.50 mg/30 g f.w., equivalent to 6.22 µmol/g fresh weight or 65.47 µmol/g dry weight. This concentration was two-fold higher than indolic glucosinolates (40.62 mg/30 g f.w., equivalent to 2.88 µmol/g fresh weight or 30.32 µmol/g dry weight). Volunteers consumed an average of 51 mg (117 µmol) and 20 mg (42 µmol) of glucoraphanin and neoglucobrassicin, respectively, on a daily basis, during the 70 days of the dietary intervention.

Biological effects of broccoli sprout on markers of inflammation and body composition

Baseline characteristics of volunteer are described in **Table 1**. Changes on plasma concentration of biomarkers at different time points of the intervention are shown in **Table 3**. Day 0 and day 70 refers to the first and last days of the broccoli ingestion, respectively. The days 90 and 160 refers to 20 days and 90 days of follow-up upon the broccoli dosage period, respectively.

The evolution of mean values of continuous variables are shown in **Table 4**. The metabolite 3,3'-diindolylmethane (3,3-DIM) was included in the statistical analysis as it was detected in all volunteers, at concentrations higher than limit of quantification, hence, for statistical purposes, it was treated as a continuous variable. Evolution of ratios of continuous variables in each visit are shown in **Figure 1**.

No significant changes were observed in weight and BMI. By contrast, body fat mass slightly decreased significantly after 70 days of broccoli consumption (ratio = 0.947, P-value= 0.02586) and returned to basal levels at day 90, a state that was maintained until day 160 (P-value= 0.94899 y P-value=0.07644).

Plasma interleukine-6 (IL-6) concentrations decreased significantly (by 38 %) after 70 days of broccoli ingestion as well, respect to basal value (ratio=0.381, P-value < 0.00001). Moreover, these lower levels continue to significantly decrease after 20 days of ceasing broccoli ingestion (ratio = 0.195, P-value< 0.00001). At 90 days of the follow up period (day 160), levels returned somewhat but without returning to baseline values (ratio=0.472, P-value= 0.00000). **Figure 2** illustrates how the changes in IL-6, during broccoli intake, depends on baseline values. The negative slope of the regression line indicates that volunteer with higher concentrations at baseline tend to lose more concentration of this biomarker. Decreases in C-reactive protein were also observed, during broccoli ingestion period (ratio = 0.592, P-value= 0.00915). Shortly in the follow-up period, levels returned to baseline conditions (P-value=0.92162 and P-value=0.72756, at 90 and 160 days, respectively).

These results did not substantially change when we repeated the regression models adjusting for age and sex. TNFα and IL-1β were detected in a small number of samples and most of them below the limit of quantification, hence no valid conclusions can be inferred and data have not been considered for statistical purposes.

Bioavailability and metabolism of glucosinolates and isothiocyanates

Some glucosinolates and isothiocyanates, as glucoraphanin, glucoiberin, iberin, glucoerucin, erucin and glucobrassicin were absent in the urine samples. Indole-3-carbinol was detected only after broccoli ingestion in low quantities and in 50 % of samples. In contrast, the metabolite 3,3'-diindolylmethane (3,3-DIM) was detected and quantified in all volunteers and for statistical purposes it was treated as a continuous variable. It increased significantly during broccoli ingestion (ratio = 1.947, P-value < 0.00001). Shortly in the follow-up period, levels returned to baseline conditions (P-value=0.10484 and P-value=0.12312, at 90 and 160 days, respectively).

Metabolites from sulphoraphane pathway are present in 24 h-urine samples (**Table 3**); the metabolite at higher amount was SFN-NAC (mean concentration 2.0301 µM, corresponding to 3.21 µmol/24 h), whereas SFN was the compound with the lowest excretion (0.543 µM, corresponding to 0.77 µmol/24 h). The sum of SFN, SFN-NAC and SFN-CYS was ~ 5.11 µmol/24 h. Considering an amount of GRA of 117 µmol by serving, a 4 % on average was metabolized through mercapturic acid pathway.

Figure 3 shows the proportion of individuals in which the metabolites have been detected and quantified at each visit and **Figure 4** shows the differences of these proportions from baseline with their confidence intervals. The percentage of individuals where SFN-NAC is detected increases significantly during broccoli intervention (45% increase; P-value = 0.00001). Afterwards, the percentage diminishes although it is statistically different from baseline (32.5 % difference; P-value = 0.00303). At 160 days, no significant differences from baseline are observed (P-value = 0.07139). Similar behaviour is detected with SFN-CYS and SFN. Percentages increased during broccoli ingestion (67.5 % increases in SFN-CYS P-value = 0.0000; 82.5 % increases in SFN; P-value < 0.0001). Afterwards, the percentages of individuals detected decreased to baseline conditions (P-value = 0.43858 and P=value 0.26355 for SFN-CYS and SFN, respectively). This behaviour is maintained for the longer period at 160 days (P-value = 0.29330 and P=value 0.73532, respectively). SFN-GSH was detected in very few samples of volunteer during broccoli ingestion (data not included), hence, it has not been considered for statistical purposes.

The decrease in IL-6 levels was significantly related to the increase in 24 h-urine SFN levels (p=0.03319). In case of C-reactive protein, the decrease was significantly related to the increases in 24 h-urine SFN-NAC (p=0.04783) and SFN-CYS (p=0.04116). (Supp. Table-6).

293

294

295 **DISCUSSION**

296 We conducted a human intervention study to test whether regular consumption of
297 broccoli sprouts improves inflammatory biomarkers in overweight subjects. Adipose
298 tissue is related to higher secretion of pro-inflammatory cytokines as TNF-α and IL-6
299 and elevated levels of these proteins have been described in overweight individuals [16,
300 17]. These proteins are linked to several disease states [18] and C-reactive is an
301 important predictive marker of cardiovascular events [19]; hence the reduction of their
302 levels with dietary intervention could contribute to a better prognosis on obesity-
303 associated disorders. In our study we observed a noticeable anti-inflammatory effect
304 with the ingestion of broccoli sprouts, with a significant reduction by 38 % and 59 % in
305 IL-6 and C-reactive protein concentrations, respectively.

306 Clinical studies on human participants on the anti-inflammatory properties of *Brassica*
307 products are scarce. Our research group has previously described a significant decrease
308 on markers of inflammatory processes, as the metabolites tetranor-PGEM (from
309 prostaglandins E_1 and E_2) and 11 β-PGF2α (from prostaglandin D2) after consumption
310 of a single portion of broccoli sprouts [20]. Other authors have reported decreases on C-
311 reactive protein levels by 48 % after 10-day broccoli intake (250 g/day) in smokers,
312 confirming our results; however, no changes on IL-6 levels were detected [21].
313 Decreases in IL-6 and C-reactive protein were also observed after 14 days of
314 cruciferous consumption [22], but the amounts used (7g/kg body weight, 14 g/kg body
315 weight) far exceeded those of our experiment.

316 Differences in population studied, study design, type of *Brassica* or amount of product
317 consumed, could explain the different results observed. Our broccoli sprouts contained

318 significant quantities of aliphatic glucosinolates as glucoraphanin, glucoiberin and
319 glucoerucin, which derive from the aminoacid methionine, as well as indolic
320 glucosinolates as methoxy and hydroxy derivatives of glucobrassicin, that derive from
321 the aminoacid tryptophan. Broccoli sprouts are especially rich in glucoraphanin (up to
322 10-fold above adult organ (inflorescence) levels) that drop with the plant growing, as
323 the plant material increases without concomitant synthesis of glucoraphanin [23].
324 Hence, potential beneficial concentrations are easier to achieve with dietary quantities
325 of sprouts *vs* broccoli heads (inflorescences).

326 Levels of glucosinolates and their metabolites isothiocyanates were measured in 24h-
327 urine by UHPLC-MS/MS in order to ascertain the consumption of broccoli and with the
328 aim to find out if any metabolite is related to the changes in the biochemical parameters
329 observed. We did not observe significant levels of intact glucosinolates in 24 h-urine
330 samples, being explainable as these compounds suffer extensive modifications prior to
331 absorption in the gut. They are present in the intact plant as glucosides and, upon tissue
332 damage, the enzyme myrosinase catalyses their rapid hydrolysis of the glucose moiety
333 [24]. The aglycone of each glucosinolate suffers further hydrolytic metabolism to
334 isothiocyanate in the gastrointestinal tract by gut microbiota; these compounds are then
335 absorbed by enterocytes and distributed systemically [25]. In particular, the
336 isothiocyanate sulphoraphane (1-isothiocyanate-4-methyl-sulfinylbutane) is formed
337 from the glucosinolate glucoraphanin (4-methyl-sulphinylbutyl glucosinolate).
338 Therefore, isothiocyanates are the compounds mainly present in human tissues to which
339 can be attributed the biological activities.

340 In humans, the isothiocyanates are metabolized via the mercapturic acid pathway.
341 Conjugation with glutathione is catalysed by glutathione transferase and GSH-
342 conjugates are metabolized rendering SFN-CYS and SFN-NAC. It has been proven that

polymorphisms of these enzymes have a significant impact on ITC metabolism [26]. From the results described, the metabolites SFN-NAC, SFN-CYS and SFN can be considered as good markers of ingestion, as their presence is related only with the broccoli period, indicating the compliance of the experimental diet.

Concerning indole glucosinolates, indole-3-carbinol (I3C) is released by hydrolysis of glucobrassicin (3-indolyl-methylglucosinolate) by myrosinase action. This type of indole glucosinolates are present in seeds, mature plant and some sprouts cultivars, but are not commonly present in all *Brassica* varieties [27, 28]. After ingestion, I3C is modified by the acidic pH in the stomach and dimerizes to 3,3'-diindolylmethane (3,3-DIM) [29]. Hence, the presence of 3,3-DIM in the 24 h-urine samples is related to the metabolism of glucobrassicin derivatives present in our broccoli sprouts.

The increases on broccoli metabolites were significantly related to the decreases in IL-6 and C-reactive protein levels, what suggests their implication in the modulation of these pro-inflammatory proteins. Studies on cellular models have shown that the mechanism of interaction is common in isothiocyanates and similar to that of endogenous hormones as steroids or vitamins A and D. This type of compounds possesses electrophile groups that interact with nucleophilic moieties of transcription factors, down- or up-regulating their activity [30]; it has been shown that sulphoraphane interacts with the redox-sensitive transcription factor Nrf2, to permit its translocation into the nucleus, where it binds to the antioxidant response element (ARE) and activates the synthesis of proteins related to the response to stress, as phase II detoxification enzymes and quinone reductases [31-34]. Besides, SFN inhibits activation of NF-κβ, a central transcription factor in inflammation process and the gene expression of proinflammatory mediators [34, 35]. This signalling pathway is redox sensitive as depends on the balance between ROS intracellular concentration and GSH levels. Changes on GSH levels by SFN may

368 influence in this anti-inflammatory action. Other authors observed an anti-inflammatory
369 effect by induction of Nrf2-pathway of broccoli sprout extract in human skin [36] and
370 nasal lavage cells [32] in healthy subjects as well as in patients with chronic obstructive
371 pulmonary disease (COPD) [37].
372 3,3'-DIM has shown to reduce transcriptional activity of NF- κβ, what results in lower
373 levels of inflammatory mediators as IL-6, in activated macrophages [38] as well in
374 different models of inflammation in mice [39, 40]. It has been pointed out the possible
375 synergistic interaction of both SFN and 3,3'-DIM [7] and the isothiocyanates erucin and
376 sulphoraphane are interconvertible [41], so that the anti-inflammatory effects observed
377 with broccoli sprouts intake are likely due to the combined effects of all the hydrolysis
378 products of glucosinolates.
379 Concerning anthropometric parameters, after 10 week of the daily consumption of
380 broccoli sprouts, weight and body mass index were not altered; however, body fat mass
381 significantly decreased with broccoli intervention. It has been described that the
382 metabolite I3C decreases adipogenesis by supressing pathways of lipid accumulation
383 mediated by PPARγ [42]; however, we did not detect I3C in 24 h-urine as it is mainly
384 excreted in its metabolite 3,3'-DIM. We did not observe a significant correlation
385 between the increase in 3,3'-DIM and the decrease in fat mass; no further experiments
386 were performed to corroborate an additional hypothesis about the effects on
387 adipogenesis.
388 A limitation of this study is the lack of a parallel randomised control group which would
389 be ideal to stablish causality links between broccoli intakes and the change in biomarker
390 levels. The post-intervention follow up is not an ideal control period as several other
391 factors might have changes in the individuals or the environment. However, the strong
392 changes observed in the inflammatory markers at the end of the intervention and their

recuperation afterwards could be an indication of the beneficial effect of the broccoli that will have to be tested in a proper trial. On the other hand, overweight is frequently associated with other pathologies as hypertension, cardiovascular events, insulin resistance or type 2 diabetes and, due to the complex interactions among them, we limited the study to people with overweight status according to WHO criteria, but without any pathology or clinical disorder. Hence, our result can only be extrapolated to these type of population and not to the overall that could include people with some concomitant pathologies.

CONCLUSIONS

The consumption of broccoli sprouts in a real dietary serving is able to affect IL-6 and C-reactive protein levels in overweight subjects, hence attenuating chronic inflammation. Further research with broccoli sprouts including other biomarkers and mechanistic studies are necessary to elucidate the role of this healthy rich and nutritious food product, but these promising results support the current evidence on the properties of this *Brassica* specie for disease prevention.

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STATEMENT OF AUTHORSHIP

López-Chillón MT carried out data analyses and contributed to the interpretation of the findings; Carazo-Díaz MC and Prieto-Merino D performed the statistical analysis; Zafrilla P contributed to data analysis and discussion of the manuscript; Moreno D.A. Principal Investigator and general management of the AGL-2013-46247-P project, contributed with the funding the study, design of experiment, discussion and writing of manuscript. Villaño D contributed to the interpretation of analyses and discussion of the manuscript, statistical management of data and writing of manuscript.

CONFLICT OF INTEREST STATEMENT AND FUNDING SOURCES

All Co-authors declare no conflicts of interest.

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562

563 **FIGURE LEGENDS**

564 Figure 1. Evolution of ratios of continuous variables in each visit according to model
565 (1): a) changes in weight, BMI and body fat mass; b) changes in IL-6 and C-reactive
566 protein levels

567 Figure 2. Example of how changes in IL-6 during broccoli intake depends on baseline
568 values of IL-6 (variables log-transformed).

569 Figure 3. Proportion of individuals and 95 % IC of metabolites detected at each visit

570 Figure 4. Changes in binary variables over periods

571

Table 1. Baseline characteristics of volunteer (n=40; 21 men, 19 women)

Variable	Mean ± standard deviation
Age (years)	46 ± 6
Height (m)	1.72 ± 0.08
Weight (kg)	85.8 ± 16.7
BMI (kg/m^2)	28.9 ± 4.0
Body fat mass (%)	30.34 ± 7.54

Table 2. Glucosinolate contents in broccoli sprouts daily portions (mg/30 g F.W)

	Mean ± Standard deviation (n=3)
Glucoiberin (GIB)	19.28 ± 0.98
Glucoraphanin (GRA)	51.08 ± 1.06
4-Hydroxiglucobrassicin (HGB)	3.67 ± 0.41
Glucoerucin (GER)	10.14 ± 1.20
Glucobrassicin (GBS)	9.69 ± 0.95
4-Methoxyglucobrassicin (MBG)	7.14 ± 0.61
Neoglucobrassicin (NBG)	20.11 ± 1.66
Aliphatic Glucosinolates (Σ)	80.50 ± 2.18
Indolic Glucosinolates (Σ)	40.62 ± 2.07
Total (Σ)	121.11 ± 4.00

Table 3. Changes observed with broccoli treatment as well as during follow-up period. Values are expressed as mean (confidence interval 95 %)

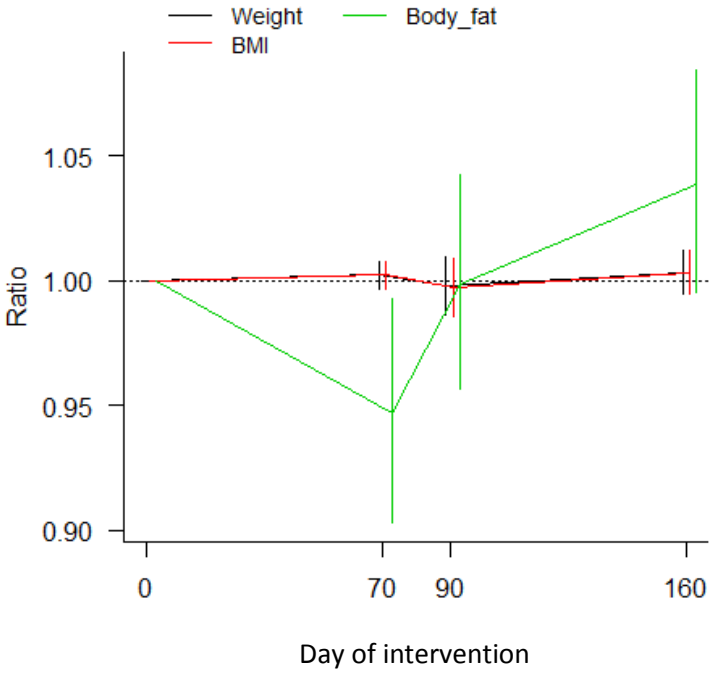
Variable	Day 0	Day 70	Day 90	Day 160
Weight (kg)	85.79 (80.38–91.20)	85.69 (80.42–90.95)	83.83 (79.47-88.18)	84.04 (79.66-88.41)
BMI (kg/m^2)	28.88 (27.56-30.20)	28.93 (27.63-30.23)	28.49 (27.40-29.57)	28.60 (27.52-29.68)
Body fat mass (%)	30.34 (27.29-33.39)	29.32 (26.93-31.71)	30.29 (27.87-32.72)	32.09 (29.69-34.49)
IL-6 (pg/mL)	4.76 (4.21-5.31)	2.11 (1.61-2.61)	1.20 (0.88-1.52)	2.66 (1.89-3.44)
C-reactive protein (µg/mL)	2.42 (1.45-3.40)	1.52 (0.70-2.34)	1.92 (1.02-2.82)	2.32 (1.07-3.56)
SFN-NAC (µM)	0.193 (0.00-0.41)	2.301 (1.85-2.75)	0.023 (0.01-0.04)	0.094 (0.00-0.19)
SFN-CYS (µM)	0.116 (0.00-0.26)	0.800 (0.57-1.03)	0.078 (0.00-0.22)	0.081 (0.00-0.19)
3,3-DIM (µM)	0.484 (0.38-0.59)	0.707 (0.61-0.80)	0.449 (0.33-0.57)	0.461 (0.36-0.56)
SFN (µM)	0.098 (0.00-0.23)	0.543 (0.40-0.69)	0.038 (0.00-0.13)	0.022 (0.01-0.03)

Table 4. Evolution of mean values (ratios) on the time points *

Variable	Mean	Ratio	95% CI	P-value
Weight (kg)	86.141			
From D0 to D70	86.346	1.002	(0.997 to 1.008)	0.38388
From D0 to D90	85.983	0.998	(0.987 to 1.010)	0.74963
From D0 to D160	86.424	1.003	(0.994 to 1.012)	0.45589
Body mass index (kg/m^2)	28.877			
From D0 to D70	28.945	1.002	(0.997 to 1.008)	0.39007
From D0 to D90	28.797	0.997	(0.986 to 1.009)	0.62441
From D0 to D160	28.971	1.003	(0.994 to 1.012)	0.46125
Body fat mass (%)	28.834			
From D0 to D70	27.298	0.947	(0.903 to 0.993)	**0.02586**
From D0 to D90	28.795	0.999	(0.956 to 1.043)	0.94899
From D0 to D160	29.955	1.039	(0.996 to 1.084)	0.07644
IL_6 (pg/mL)	4.594			
From D0 to D70	1.748	0.381	(0.298 to 0.486)	<**0.00001**
From D0 to D90	0.896	0.195	(0.149 to 0.255)	<**0.00001**
From D0 to D160	2.170	0.472	(0.366 to 0.609)	<**0.00001**
C-reactive protein (µg/mL)	1.431			
From D0 to D70	0.847	0.592	(0.405 to 0.865)	**0.00915**
From D0 to D90	1.459	1.020	(0.677 to 1.536)	0.92162
From D0 to D160	1.553	1.085	(0.665 to 1.771)	0.72756
DIM_3_3 (µM)	0.334			
From D0 to D70	0.650	1.947	(1.705 to 2.223)	<**0.00001**
From D0 to D90	0.335	0.757	(0.539 to 1.063)	0.10484
From D0 to D160	0.376	0.850	(0.689 to 1.048)	0.12312

*: data are adjusted by baseline levels

a)


Weight
Body_fat
BMI
1.05
1.00
0.95
0.90
Ratio
0
70
90
160
Day of intervention

b)

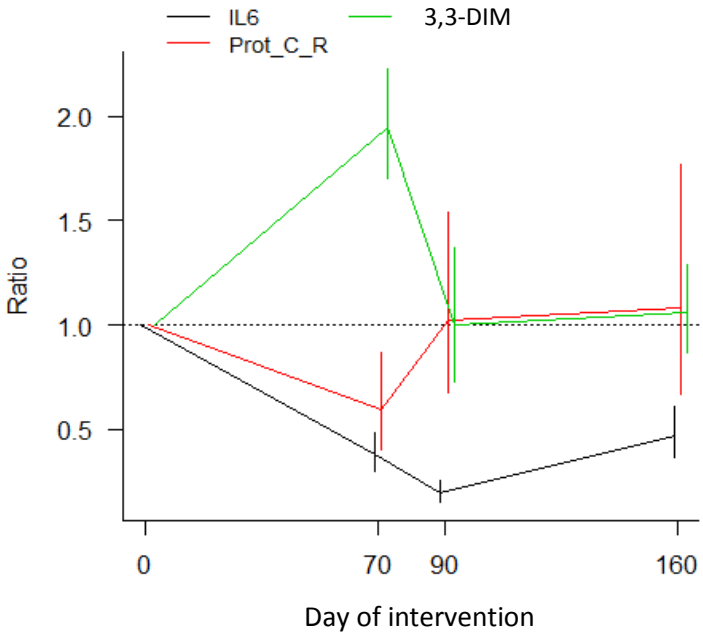

IL6
3,3-DIM
Prot_C_R
2.0
1.5
1.0
0.5
Ratio
0
70
90
160
Day of intervention

Figure 1.

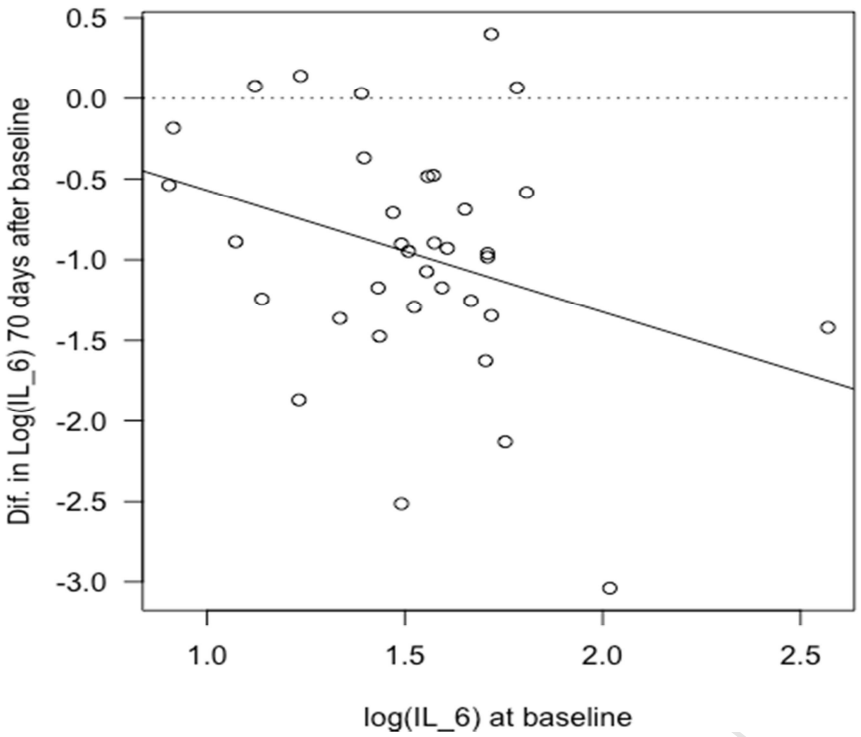


Figure 2.

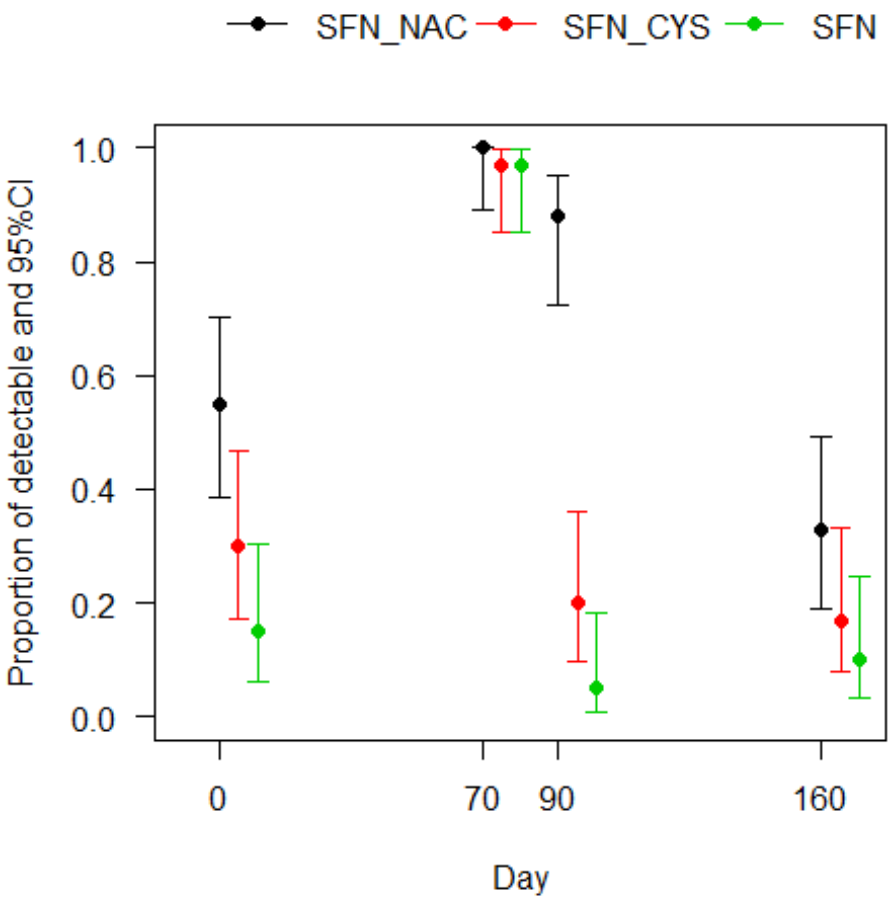


Figure 3.

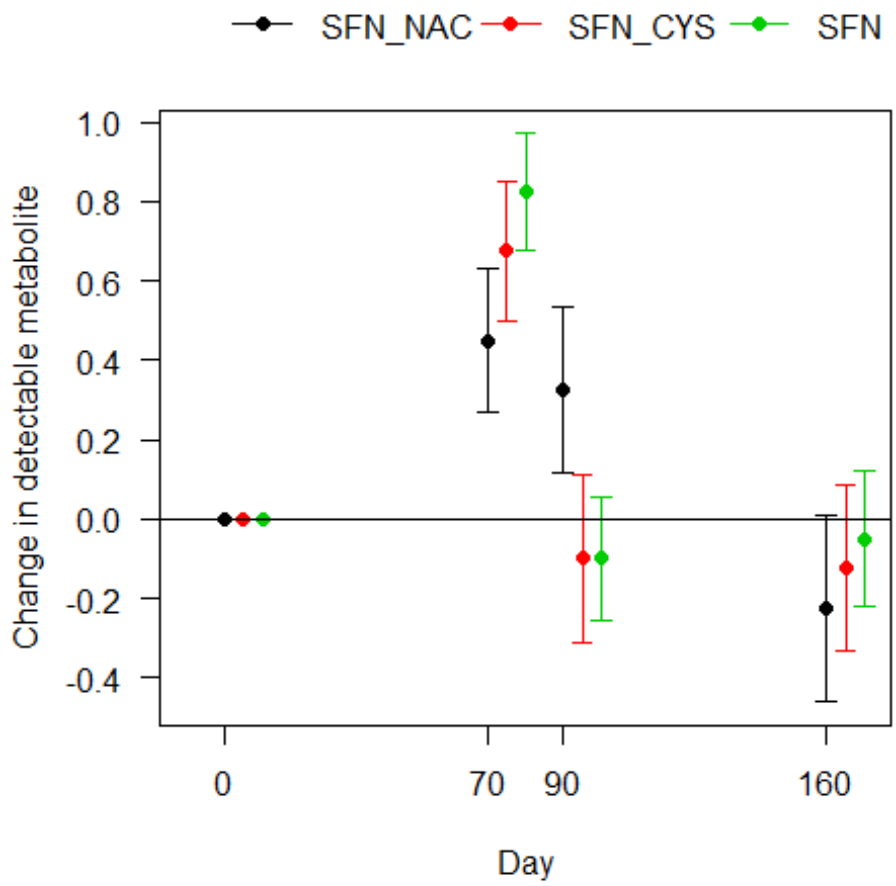


Figure 4.