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Consumption of lettuce with seaweed extract biostimulant application improved iron homeostasis in a randomized interventional trial of healthy individuals

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Minerals have key roles in the body's metabolism and homeostasis. Biostimulants application to vegetables, such as seaweed extracts derived from *Ecklonia maxima* (SwE), is a useful agronomic approach to improve crop yield and quality by a naturally functionalizing process. We hypothesized that SwE biostimulants would impact the minerals profile of the lettuce and the consumption of lettuce with SwE application would affect blood minerals concentration in the health population. This in turn would impact metabolic pathways essential for human homeostasis. A group 48 healthy adults, of both sexes, was allocated in a double-blinded manner into groups that consumed 100 g a day of control lettuce, lettuce with SwE application or an iron tablet (30 mg) for four weeks. Blood samples were collected at baseline (T0) and at the end of the trial (T2) and compared for differences in serum mineral concentrations, iron, lipid and glucose homeostasis. In lettuce, SwE biostimulant enhanced iron concentration by about 63%. The consumption of lettuce with SwE application increased serum iron by about 38%, transferrin saturation by about 47%, and reduced total cholesterol by about 19% and Low-density lipoprotein by about 22%. Supplementation of iron in tablets has similar effects to lettuce with SwE application but with side effects (diarrhea or constipation). The study offers an innovative perspective by assessing lettuce with SwE application as a natural alternative to iron supplements that are commonly associated with gastrointestinal side effects. The results are of interest in the context of dietary iron deficiency especially among populations that avoid meat-based diets. This research could have broad implications for enhancing the nutritional value of plant-based foods to support dietary health by promoting intersection of sustainable agriculture and human nutrition.

***Clinical trial registration number*: NCT06656871.**

Keywords Functional food, Health, Physiology, Vegan, Vegetarian, Minerals

An adequate intake of minerals through the diet is crucial for many important metabolic functions. In fact, despite the low requirements of these minerals, they are essential nutritional elements for the correct physiological

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functions of the human body and thus ultimately, human health[1]. There are a growing number of people who are at risk of mineral deficiency[2]. They are older individuals[3], persons with chronic illnesses[4], women during pregnancy, children during growth[5–7], athletes and sportive[8,9], people who live on specific diet for example vegetarian or vegan diets or individuals who live at reduced diet to achieve a specific weight[10].

Sustainable agronomic practices are useful to improve yield and quality of vegetable crops[11–15]. Biostimulants represent a suitable way to enhance plant performances[16–20] and the application of seaweed extracts (SwE) derived from *Ecklonia maxima*, is one of the imperative agronomic strategies[21,22]. The SwE is used to boost mineral uptake and accumulation to promote plant growth[23]. In this way, the farmers can reduce the use of chemical fertilizers or other synthetic products[24–26] extremely toxic even at minimal or trace levels[25]. Thus, the SwE is a natural way of stimulating plant growth[22]. So far, the impact of biostimulated plants consumption via SwE in human health is unknown. Here, we hypothesized that SwE biostimulant would impact minerals profile of the lettuce and the consumption of lettuce with SwE application would affect blood minerals concentration in health population. This in turn would impact metabolic pathways essential for human homeostasis. Therefore, we analyzed the minerals profile in the lettuce with SwE application and allocated a group of volunteer healthy adults, of both sexes, in a double-blinded manner into groups that consumed 100 g a day of control lettuce, lettuce with SwE application or an iron tablet for four weeks. It was chosen lettuce because is consumed worldwide in all seasons and from most of the population[27]. The blood samples were collected at baseline (T0) and at the end of the trial (T2) and compared among the groups for differences in the serum mineral concentrations. The main physiological pathways such as iron, glucose and lipid and lettuce minerals profile were analyzed.

Results

Lettuce minerals profile

We analyzed the impact of SwE biostimulant in lettuce mineral profile. The analysis of mineral profile showed that SwE biostimulant impact in iron plant absorption. In fact, iron was significantly increased in lettuce with SwE application compared to control lettuce (Table 1). As shown in Table 1 we performed analysis of other minerals but none differences were observed among the two crops.

Characteristic of the participants

The clinical trial involved a total of 48 volunteers, 15 males and 33 females.All participants were in good health. The first evaluation carried out was the analysis of the anthropometric characteristics of the groups. No significant differences were found in the participant anthropometric characteristics (weight, body mass index, percent of fat mass and visceral adipose tissue) or between the control group, lettuce SwE group and Iron Tablet group (Table 2).

Supplementation of biostimulated SwE lettuce and serum minerals concentration

It was analyzed the serum mineral concentration of calcium, potassium, magnesium phosphorus and iron because they are physiological markers used in the clinical laboratory routine test to verify the health status of patients[28,29]. In the control group, the daily intake of 100 g of control lettuce for 4 weeks did not change the concentration of blood minerals calcium, potassium, magnesium, phosphorus and iron compared to baseline (Table 3). The consumption of lettuce with SwE application (100 g) did not affect the serum concentration of potassium, calcium, magnesium, and phosphorus but significantly increased iron levels (Table 3). In the iron

	Control lettuce (*n* = 3)			Lettuce with SwE application (*n* = 3)			
Lettuce minerals profile (mg/Kg)	Mean	SEM	SD	MEAN	SEM	SD	*p*-value
Fe	**11.752**	2.0	3.582	**19.134**	1.0	1.885	**< 0.0001**
Se	0.034	0.01	0.027	0.037	0.001	0.029	> 0.9999
Mo	0.046	0.001	0.003	0.043	0.009	0.017	> 0.9999
I	1.102	0.03	0.066	1.072	0.03	0.058	> 0.9999
Al	12.028	2.4	4.177	9.854	1.4	2.452	0.3834
V	0.034	0.009	0.019	0.028	0.005	0.010	> 0.9999
Cr	0.092	0.009	0.017	0.115	0.01	0.030	> 0.9999
Co	0.008	0.002	0.004	0.007	0.001	0.003	> 0.9999
Ni	0.056	0.007	0.013	0.067	0.005	0.009	> 0.9999
Cu	0.361	0.03	0.054	0.310	0.06	0.110	> 0.9999
Zn	4.961	0.98	1.710	4.409	1.27	2.216	> 0.9999
As	0.850	0.03	0.052	0.742	0.002	0.050	> 0.9999
Sr	0.854	0.10	0.176	1.288	0.19	0.332	> 0.9999
Cd	0.012	0.002	0.004	0.010	0.004	0.007	> 0.9999
Sn	0.023	0.003	0.006	0.040	0.015	0.027	> 0.9999
Sb	0.002	0.0005	0.001	0.007	0.001	0.002	> 0.9999
Pb	0.111	0.004	0.008	0.102	0.006	0.011	> 0.9999

Table 1. Minerals profile of control and lettuce with SwE application. Significant values are in [bold].

Anthropometric parameters	Adults (9 females, 4 males) n13 subjects in each group Control group			Adults (10 females, 4 males) *n* 14 subjects in each group Lettuce SwE group			Adults (9 females, 4 male *n* 13 subjects in each group Iron tablet group		
	Baseline Mean ± SD	T2 Mean ± SD	*p*-value	Baseline Mean ± SD	T2 Mean ± SD	*p*-value	Baseline Mean ± SD	T2 Mean ± SD	*p*-value
Ages (years)	55.8 ± 10.4			41.8 ± 14.3		0.064	41.6 ± 13.6		0.065
Weight (kg)	79.700 ± 19.667	78.644 ± 17.142	0.8963	62.662 ± 10.770	64.9 ± 9.747	0.5836	70.25 ± 6.38	67.2 ± 8.546	0.4321
Height (cm)	167.55 ± 9,76	166.44 ± 9.91	0.9812	160.78 ± 10.14	160.79 ± 10.14	> 0.9999	167.04 ± 10.75	167.13 ± 11.52	0.9777
Body Mass Index (BMI) Normal range: 18.5–24.9	28.089 ± 3.203	27.689 ± 3.521	0.8042	25.150 ± 3.317	24.146 ± 3.04	0.4189	25.064 ± 2.954	25.915 ± 3.147	0.4753
Fat Mass % Male normal range: 10–20% Females normal range: 20–30%	27.822 ± 4.248	28.667 ± 4.624	0.6920	31.086 ± 6.550	31.57 ± 6.894	0.8696	33.107 ± 5.924	29.064 ± 4.404	0.0507
Muscle Mass % Males normal range: 33–40% Females normal range: 24–30%	27.822 ± 4.248	28.667 ± 4.624	0.6920	31.315 ± 6.759	31.769 ± 7.103	0.8689	34.369 ± 6.596	29.915 ± 5.669	0.0772
Grade of visceral fat level Normal range: 1–12	11.333 ± 4.213	10.889 ± 4.137	0.8242	6.143 ± 2.381	6.385 ± 2.725	0.8077	7.538 ± 2.145	7.571 ± 2.102	0.9682

Table 2. Characteristics of the subjects in the three groups of study. Values are expressed as mean and standard deviation (means ± SD). A *p*-value > 0.05 indicates that there is no significant change.

tablet group, the daily supplementation for 4 week of 1 tablet of iron (30 mg) significantly increased iron levels compared to baseline (Table 3) and did not affect the concentration of the others minerals (potassium, calcium, magnesium, and phosphorus) (Table 3).

Supplementation of biostimulated SwE lettuce and iron homeostasis
The consumption of lettuce with SwE application increased the percent of transferrin saturation by about 47% compared to the baseline and iron by about 38% (Fig. 1 A and B) but did not influence proteins of iron metabolism such as ferritin and transferrin (Fig. 1C and D). Similarly, in the Iron Tablet group, four-week supplementation increased the percent of transferrin saturation (Fig. 1B) compared to baseline but did not influence ferritin and transferrin (Fig. 1C and D). In the Control Group the consumption of 100 g of lettuce, for four weeks, did not affect the serum iron concentration, the percent of transferrin saturation ferritin and transferrin compared to the baseline (Fig. 1).

Supplementation of biostimulated SwE lettuce and glucose homeostasis
In the control group the nutritional intervention for 4 weeks did not modify glucose metabolism (Table 4). In fact fasting glucose, insulin, insulin resistance and sensitivity were similar within the control group (baseline vs. T2). Daily assumption of lettuce with SwE application did not impact glucose homeostasis. No differences in fasting glucose, insulin, insulin resistance and sensitivity was observed within the SwE group (baseline vs. T2) or between the control and the SwE groups. Similarly, in the iron tablet group the nutritional intervention did not affect glucose homeostasis (Table 4).

Supplementation of biostimulated SwE lettuce and lipid homeostasis
The consumption of lettuce with SwE application significantly reduced total cholesterol and LDL values compared to baseline but did not affect HDL and triglycerides. Consumption of Iron in Tablets also reduced total cholesterol and LDL levels compared to baseline but did not affect HDL and triglycerides. Supplementation with control lettuce did not significantly affect lipid homeostasis (Table 5).

Discussion
Here it was investigated the effects of a 4 weeks nutritional intervention with lettuce with SwE application in a cohort of healthy individuals. The results of this study suggest that consumption of lettuce with SwE application could be a good strategy for preventing iron deficiency in healthy individuals.

Plant-based diets are very popular at this time. Right now, there is an estimated population of vegans and vegetarians by about 1 and 13% respectively and we are expecting a rapid rise in these numbers[30]. It is essential to pay more attention to the effects of plants with biostimulant application because studies assessing the effects of consumption in human health are lacking. The study in plant biostimulants focuses on the effects in plant health[31,32]. To promote sustainability the scientific approach that combines sustainable productivity of cultivated plants to human health should be promoted. SwE extract is reported to enhance crop growth and quality by increasing plant minerals uptake[33]. The core purpose was to verify if the consumption of lettuce with SwE application is able, after one month of daily intake, to affect minerals homeostasis in a population of healthy adults and if it in turn influences essential metabolic pathways of human health.

It was first analyzed the minerals profile of lettuce to verify if SwE application would impact minerals concentration in lettuce and to which extent. It is well known that high mineral value in the soil can lead to plant minerals toxicity and impacting health and growth. Mineral deficiency can bring to chlorosis and necrosis.

	Adults (9 females, 4 males) *n*°13 subjects in each group Age 24–65 years					Adults (10 females, 4 males) *n*°14 subjects in each group Age 24–65 years					Adults (9 females, 4 males) *n*°13 subjects in each group Age 24–65 years				
	Control group					Lettuce SwE group					Iron tablet group				
Serum minerals concentration	**Baseline Mean ± SD**	**SEM**	**T2 Mean ±SD**	**SEM**	***p*-value**	**Baseline Mean ±SD**	**SEM**	**T2 Mean ±SD**	**SEM**	***p*-value**	**Baseline Mean ±SD**	**SEM**	**T2 Mean ± SD**	**SEM**	***p*- value**
Potassium (mmol/L)	4.5 ± 0.4	0.11	4.1 ± 0.4	0.11	0.23	4.2 ± 0.4	0.11	4 ± 0.3	0.08	0.997	4.3 ± 0.3	0.09	4.4 ± 0.3	0.09	0.99
Calcium (mg/dL)	9 ± 0.2	0.07	9.1 ± 0.3	0.09	> 0.99	9.1 ± 0.6	0.16	9 ± 0.4	0.09	> 0.999	9 ± 0.2	0.06	8.9 ± 0.2	0.06	> 0.99
Magnesium (mg/dL)	2.3 ± 0.1	0.02	2.2 ± 0.1	0.03	> 0.99	2.2 ± 0.2	0.04	2.2 ± 0.1	0.03	> 0.999	2 ± 0.2	0.05	2.1 ± 0.2	0.05	> 0.99
Phosphorus (mg/dL)	3.3 ± 1	0.20	3.6 ± 0.6	0.14	0.998	3.5 ± 0.4	0.10	3.6 ± 0.5	0.14	> 0.999	3.7 ± 1	0.15	3.6 ± 0.6	0.15	> 0.99
Iron (µg/dL)	72 ± 22	6.25	70 ± 12	3.36	< 0.0001	73.7 ± 35	9.22	102 ± 32	8.65	0.01	72 ± 23	6.35	99 ± 20	5.50	0.03

Table 3. Serum mineral profile before (baseline) and after (T2) the nutritional intervention. Values are expressed as mean and standard deviation. A *P* value > 0.05 indicates no change. Consumption of lettuce with SwE application and Iron tablets increases blood concentrations within the physiological range.

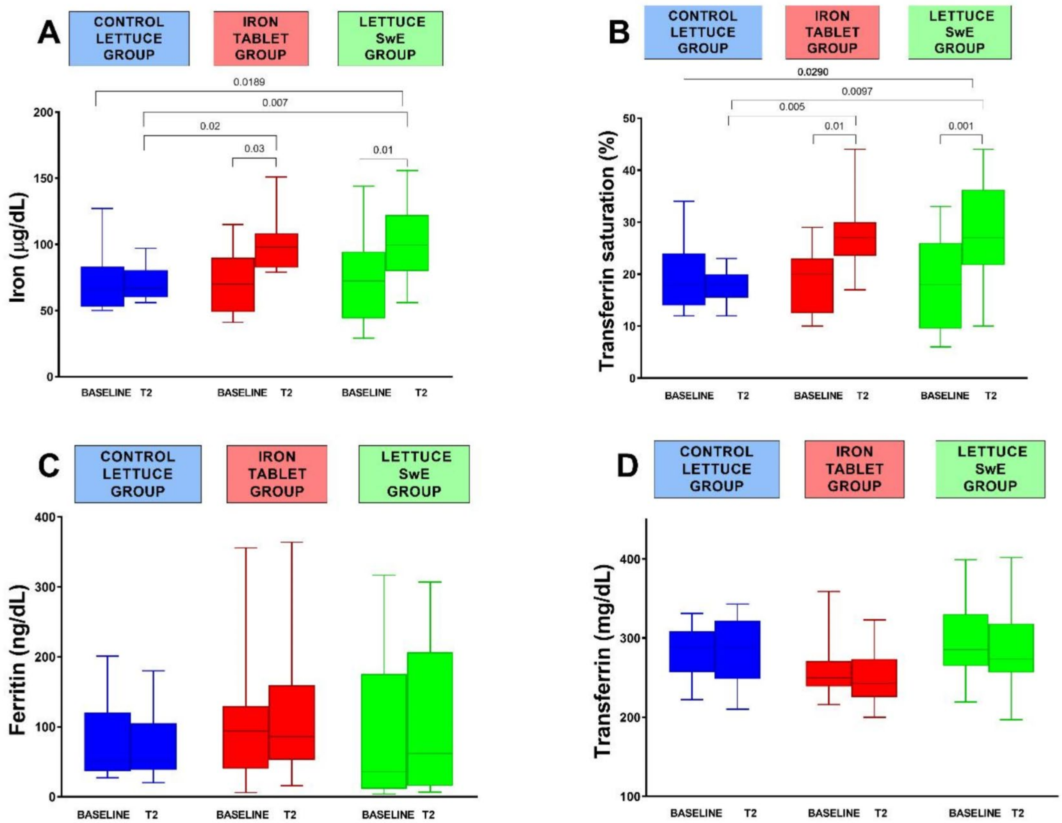

Fig. 1. Markers of iron metabolism measured at baseline and after four weeks of supplementation in the control group, lettuce SwE group and in the iron tablet group. (**A**) Box and whisker of iron. (**B**) Box and whisker plot of transferrin saturation. (**C**) Box and whisker plot of ferritin. (**D**) Box and whisker plot of transferrin.

	Adults (9 females, 4 males) *n*°13 subjects in each group Age 24–65 years			Adults (10 females, 4 males) *n*°14 subjects in each group Age 24–65 years			Adults (9 females, 4 males) *n*°13 subjects in each group Age 24–65 years		
	Control group			Lettuce SwE group			Iron tablet group		
Glucose profiles	**Baseline Mean ± SD**	**T2 Mean ± SD**	***p*-value**	**Baseline Mean ± SD**	**T2 Mean ± SD**	***p*-value**	**Baseline Mean ± SD**	**T2 Mean ± SD**	***p*-value**
Glucose (mg/dL)	86.1 ± 6.3	83.1 ± 8.0	0.997	81.2 ± 9.3	82.7 ± 9.2	> 0.999	86.8 ± 8.8	87.2 ± 4.3	> 0.999
Insulin (mUI/L)	9.6 ± 4.3	10.2 ± 5.3	> 0.999	8.6 ± 5.1	7.8 ± 4.9	> 0.999	9.3 ± 3.6	5.6 ± 2.0	0.502
HOMA–IR	1.9 ± 1.5	2.3 ± 2.6	> 0.999	1.1 ± 0.7	0.9 ± 0.6	> 0.999	1.1 ± 0.5	1.0 ± 0.3	0.519
HOMA %β	118 ± 35	133 ± 50	0.891	121 ± 22	109 ± 28	0.945	113 ± 41	82 ± 25	0.249
HOMA %S	72 ± 37	85 ± 48	> 0.999	111 ± 40	142 ± 70	0.699	111 ± 40	99.57 ± 24	> 0.999

Table 4. Markers of glucose metabolism measured at baseline and after 4 weeks of intervention in the control group, lettuce SwE group and iron tablet group. Values are expressed as mean and standard deviation. A *P* value > 0.05 indicates no change. Consumption of control lettuce, lettuce with SwE application and Iron tablets did not modify glucose homeostasis.

Similar to human, where minerals have different important function (immune function, regulates fluid balance, enzyme function, energy production)[34] also in the plant minerals have numerous essential role such as providing osmotic for turgor and growth structural components, enzyme activation[35]. The analysis showed a significantly increase in iron concentration of lettuce with SwE application while none differences was observed for the other minerals analyzed among the control and biostimulant lettuce.

For the nutritional intervention the approach previously described[36] in order to avoid bias of dietary intake and physical activity of participants was used. The participants were instructed to record in the food diary quantities and type of food and beverages eaten 1 week before starting the protocol and until the end of it. It

Lipid Profiles	Adults (9 females, 4 males) *n*°13 subjects in each group Age 24–65 years			Adults (10 females, 4 males) *n*°14 subjects in each group Age 24–65 years			Adults (9 females, 4 males) *n*°13 subjects in each group Age 24–65 years		
	Control group			Lettuce SwE group			Iron tablet group		
	Baseline Mean ± SD	T2 Mean ± SD	*p*-value	Baseline Mean ± SD	T2 Mean ± SD	*p*-value	Baseline Mean ± SD	T2 Mean ± SD	*p*-value
Total Chol (mg/dL)	203 ± 28	196 ± 27	> 0.999	197 ± 38	159 ± 33	0.002	202 ± 47	155 ± 33	0.004
Chol LDL (mg/dL)	125 ± 27	127 ± 25	> 0.999	124 ± 30	96 ± 21	0.028	126 ± 30	95 ± 23	0.015
Chol HDL (mg/dL)	57 ± 15	57 ± 13	> 0.999	59 ± 11	57 ± 8	> 0.999	58 ± 11	55 ± 14	> 0.999
Total Chol/HDL (mg/dL)	3.8 ± 1	3.6 ± 0.7	> 0.999	3.3 ± 0.9	2.9 ± 0.7	0.980	3.1 ± 0.9	3.2 ± 0.8	> 0.999
Triglycerides (mg/dL)	104 ± 45	99 ± 41	> 0.999	87 ± 36	94 ± 37	> 0.999	81 ± 38	83 ± 25	> 0.999

Table 5. Markers of lipid metabolism measured at baseline and after 4 weeks of intervention in the control group, lettuce SwE group and iron tablet group. Values are expressed as mean and standard deviation. A *P* value > 0.05 indicates no change. Consumption of biostimulant SwE lettuce significantly reduces total cholesterol and LDL.

was asked not to change lifestyle and nutritional habits and to avoid supplementation or integration at least 20 days before the baseline and for the entire period. The groups of study were homogenous and there were no differences in life-style factors and dietary between the groups of study.

Consumption of lettuce with SwE application ameliorates iron homeostasis in healthy individuals with statistically significant differences. Specifically, through the lettuce (vegetable matrix) the SwE group received about 37% more iron compared to the control group. In fact, iron assumption through the vegetable matrix was by about 0.19 mg/100 g fresh weight for the SwE group and by about 0.12 mg/100 g fresh weight for the control group. This daily difference raised up serum iron by about 38% and transferrin saturation by about 47% in the SwE group at the end of the trial (4 weeks). The results were confirmed by the lack of changes in iron, transferrin saturation, ferritin and transferrin in the control group which got the same quantities of control lettuce for the same period. Of interest, the nutritional intervention with lettuce with SwE application was well tolerated by participants and maintained serum iron and transferrin saturation within physiological levels.

We then investigated if the nutritional intervention with lettuce with SwE application could affect glucose homeostasis. In fact, keeping normal iron metabolism is essential for maintaining blood glucose stability[37]. This is very important because iron can affect glucose homeostasis in cells such as pancreas β cells, and adipose tissue and be dangerous in case of iron overload[38]. We did not observe any differences in glucose, insulin, insulin resistance or sensitivity among the groups. Sugars content in the lattuce was not detected in our trial but there are reports informing that seaweed extract can enhance sugars in lettuce[39]. The higher yield, observed in seaweed extract treated plants, could be related to the seaweed extract polysaccharide content, since sugars are recognized to increase plant productivity by eliciting endogenous hormone homeostasis[40].

Because iron is an essential regulator also of lipid metabolism[41], it was investigated if supplementation with lettuce with SwE application could impact lipid homeostasis. It was observed significant reduction in total cholesterol and LDL in the SwE group compared with control lettuce suggesting that iron delivered by the vegetal matrix was able to ameliorate lipid homeostasis, confirming that improving iron homeostasis ameliorates also lipid metabolism. We don't know the mechanism of action by which iron enhances lipid homeostasis. It may act by influencing adipokines such as adiponectin or leptin[42,43]. In fact, adiponectin and leptin treatment improved lipid profile following iron overload[44]. Iron also influences macrophages of the adipose tissue. In the adipose tissue there is a subpopulation of anti-inflammatory M2 macrophages that are iron-modulated. They respond by caching up the excess of iron and release it in case of deficiency[45]. Modulation of iron homeostasis in the adipose tissue may positively impact fat storage.

To confirm that the effects following SwE supplementation were due to the improvement of iron homeostasis, we analyzed and compared control and lettuce with SwE application group with the iron tablet group. This last study group was supplemented daily with 30 mg of iron supplemented in tablets. We observed that the treatment increased serum iron, transferrin saturation, total cholesterol and LDL similarly to the SwE group, supporting the hypothesis that the effects were due to improvement of iron homeostasis. However, there was about 40% of drop out due to collateral effects such as diarrhea or constipation. The Strengths, Weaknesses, Opportunities and Threats (SWOT) of the study is reported in Fig. 4.

We are conscious about the limitations of the study and specifically the relatively short intervention period that lasted after 4 weeks, and the small sample size that was cohort of 48 subjects that may affect the generalizability of the findings. Future research with a bigger population and longer treatment time are required. Moreover, further study should explore not only the long-term effects of SwE-enriched crops but also expand the study to different populations by including subjects for example with iron deficiencies. In conclusion, this study shows that biostimulation of SwE lettuce may be used to improve iron homeostasis and be a suitable tool to prevent deficiency and anemia that are major threats to public health worldwide. This research could have broad implications for enhancing the nutritional value of plant-based foods to support dietary health by promoting intersection of sustainable agriculture and human nutrition.

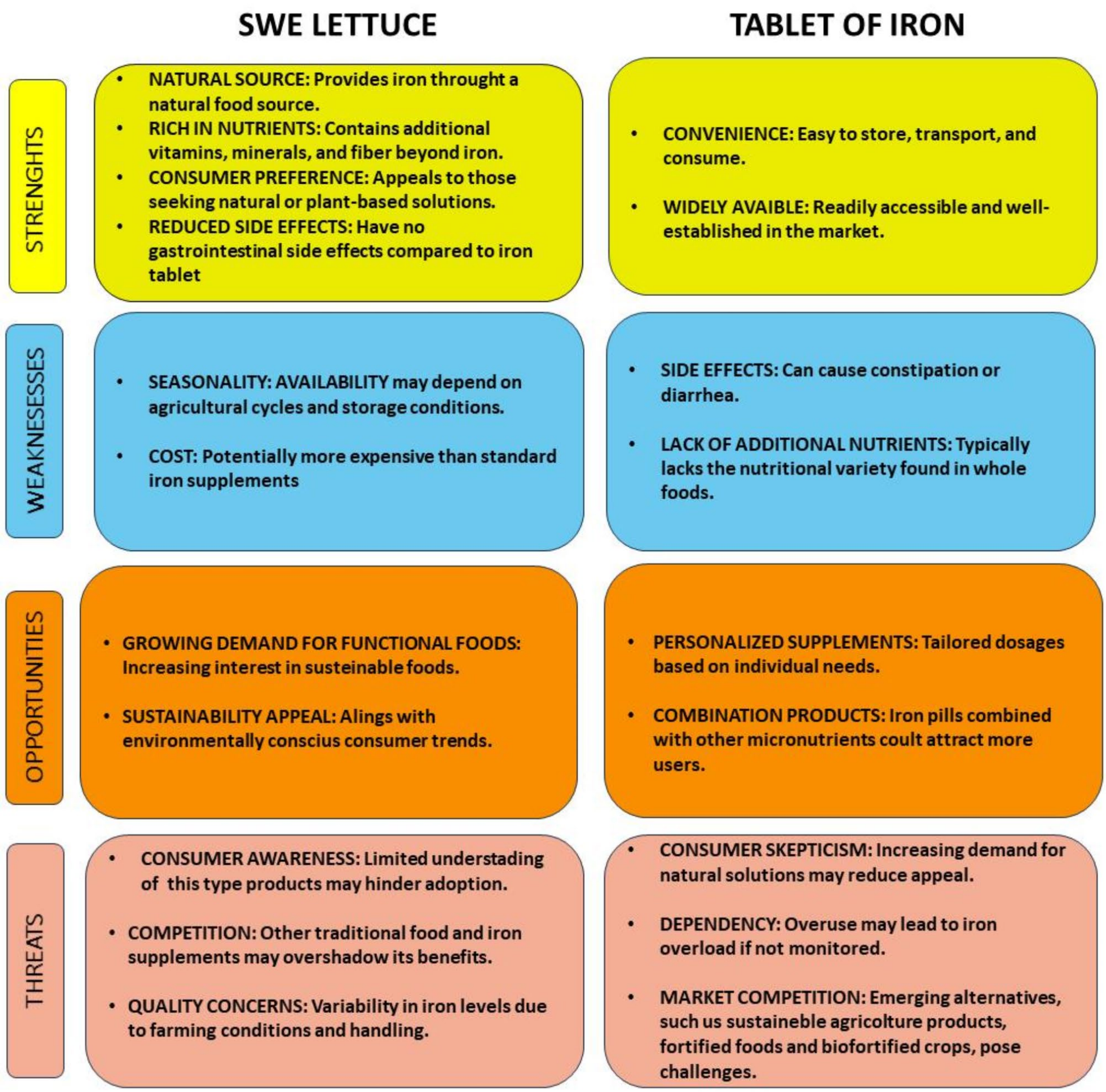

Fig. 2. The Strengths, Weaknesses, Opportunities and Threats (SWOT) of the study for the lettuce group that received lettuce biostimulated with *Ecklonia Maxima* seaweed (Lettuce with SwE application, 100 g/day) versus the iron tablet group that received iron supplementation (30 mg, 1 tablet/day of iron).

Methods

Agronomic trial

The agronomic trial was conducted in an experimental polyethylene-covered tunnel located in Palermo at the Agricultural, Food and Forestry Science Department of Palermo University. On 10 March 2023 lettuce (*Lactuca sativa L.* var. *canasta*) (Syngenta Seed, Basel, Switzerland) plants were transplanted at the stage of 4–5 true leaves in a 3.5 L plastic pot (15 × 15 × 20) containing peat moss/coconut fiber (40/60, v/v) substrate mix (base cultivation substrate coconut mix, Bioflor srl, Italy) characterized by pH 6.2, EC 0.5 dS/m, bulk density of 150 kg/m^3 and total porosity of 90 (v/v). 2.0 kg of substrate was used for each pot. Irrigation was carried out by providing a quantity of water determined on the basis of the solar radiation of the previous day as previously reported[46]. All cultivation practices required for lettuce cultivation in Mediterranean environmental conditions were performed[47]. From the 10th day after transplanting, plants were exposed to biostimulant treatments. *Ecklonia maxima*-based biostimulant (Kelpstar, Mugavero, Italy), containing 11 mg L^{-1} of auxin and 0,03 mg L^{-1} of cytokinin, was supplied via foliar spray at 3 ml L^{-1} (recommended dose) every 7 days using 0.5 L m^{-2} of solution. Control plants were sprayed only with water. Treatments were organized in a randomized block design with 3 replicates per treatment. Each block contained 30 plants with a total of 90 lettuce plants. All plants were harvested 70 days after transplant. The weight of control plants were about 600 g and the height were about 24 cm while the weight of the plants exposed to biostimulant treatments were 660 g and the height were 25 cm. Climatic data of the tunnel were recorded by a data logger (see supplementary materials).

Analysis of lettuce minerals profile

For determination of minerals in lettuces was performed an analysis by inductively coupled plasma mass spectrometry (7700x series ICP-MS, Agilent Technologies, Santa Monica CA, USA). The extraction procedure and the ICP-MS analysis were carried out as previously reported[48]. Briefly, 0.5 g of the samples were digested using a Ultrawave digestion system (Milestone, Sorisole, Italy) with 3 mL of 67% (V/V) ultrapure nitric acid in borosilicate vessels. Digestion was conducted in a Ultrawave digestor (Milestone, Sorisole, Italy) The samples digested were diluted up to 50 ml with ultrapure water (milliQ) deionized water until the ICP-MS analysis. The instrument parameters were. nebulizer carrier gas flow, 1.2 L/min; plasma gas flow (15 L/min) reflected powered <5; RF power, 1550 W. For calibration certified reference standards from VWR International LTD (Randon, Pennsylvania, USA) were used. The analysis was carried out on the basis of calibration curves, constructed by the linear interpolation of at least 7 points corresponding to the readings of 7 standard solutions and white calibration, admitting a maximum error of 5% on the reading of the single standards and a correlation coefficient r2 > 0.999.

Nutritional intervention

The study protocol received ethical approval from the University Hospital of Palermo's Ethics Committee Number 02/2023. Full data of first trial registration 24/10/2024, clinical trial registration number NCT06656871. The study was conducted in accordance with the established ethical principles outlined in the Declaration of Helsinki, an internationally recognized framework guiding ethical research involving human subjects.

Fifty-five healthy individuals were recruited for the study (Fig. 2). These participants were volunteers residing in the metropolitan area of Palermo, Italy (Sicily). The baseline demographic characteristics of the 48 participants were the age range was 24–65, the sex was 15 males and 33 females, and the socio-economic status (SES) was medium SES. It is important to underline that the metabolic, hepatic and hematologic levels did not differ in adulthood. In fact, total and basal expenditure and fat-free mass are all stable from ages 20 to 60, regardless of sex[49]. Some longitudinal studies find small declines in body weight (not in excess of 0.3% per year) in older men and women after the age of 60 [50].

Seven participants were excluded from the trial because they did not meet the eligibility criteria. The study then divided into randomly assigned cohorts. The random allocation sequence was obtained by the use of a computer to generate a random number table. More specifically, it used a random number generator computer program (excel) that generated from the list for each group the random numbers. Thus, the cohorts of participants were randomly assigned to the control or experimental groups and given random numbers by a third party, who encoded the interventions with matching random numbers. During the whole data collection period, the investigators, the medical staff, and the participants of the study were blinded to the process allocation. During the process of data analysis and sample assessment the investigators were also blinded. Thus, the participants in a double-blinded manner were allocated to a control group with 13 subjects including 9 females and 4 males, a lettuce SwE group with 14 subjects including 10 females and 4 males, and an iron tablet group with 21 subjects including 14 females and 7 males by considering the approximate dropout rate of about 40% due to gastrointestinal disturbance[51,52].

The control measures that were adopted in the clinical trial were (1) calling twice a week the participants to verify compliance to the clinical trial and (2) the verification of adherence to their usually diet (not changes in dietary habits) by using food diary. For the calculation of effect size, an a priori power calculation was performed by utilizing statistical significance level a of 5% and b probability of 20% was on the basis of previous studies of hematological parameters which estimated a sample size of eight people[48]. To power the secondary outcomes and avoid type 2 errors at least 13 participants in each group were included. Participants in the control group and lettuce SwE group finished the four-week nutritional intervention without any dropouts. Eight participants in the iron tablet group did not complete the clinical trial because of diarrhea and constipation. Thus, the iron group was of 13 persons, 9 females and 4 males (Fig. 2). To monitoring and auditing the execution processes of the clinical trial the following control measures were adopted: Not allowing enrollment if subjects do not meet al.l the inclusion and exclusion study criteria, not allowing enrollment if signed informed consent was not present, control the application of the protocol by scheduling visit window period, the analysis to be taken in the visit, by calling twice a week the participants to verify adherence to the protocol and any deviation or non-compliance with the protocol, by using the food diary tool to check not changes in dietary habits.

Participants received instructions to maintain their standard dietary and lifestyle practices, and to abstain from the use of any products containing dietary supplements[53]. The subject cohort provided responses to questionnaires designed to collect anamnestic data, dietary patterns via food diaries, and lifestyle habits. This information was subsequently analyzed by a team of nutritional specialists and medical professionals. Study participants were selected following specific criteria that are reported in Table 6. Written informed consent was obtained from all participants. To safeguard participant anonymity and privacy, a unique alphanumeric code was assigned to each individual. Participants with a history of blood, heart, digestive or metabolic disorders or recent viral infections were not considered for the study. Furthermore, individuals who were using supplements, medications for more than 6 weeks before the study or those with known sensitivities to lettuce were excluded[9]. Pregnant or breastfeeding women were also excluded from participation. Participants recorded their food consumption for 8 days before the start of the study and continued until its completion[48].

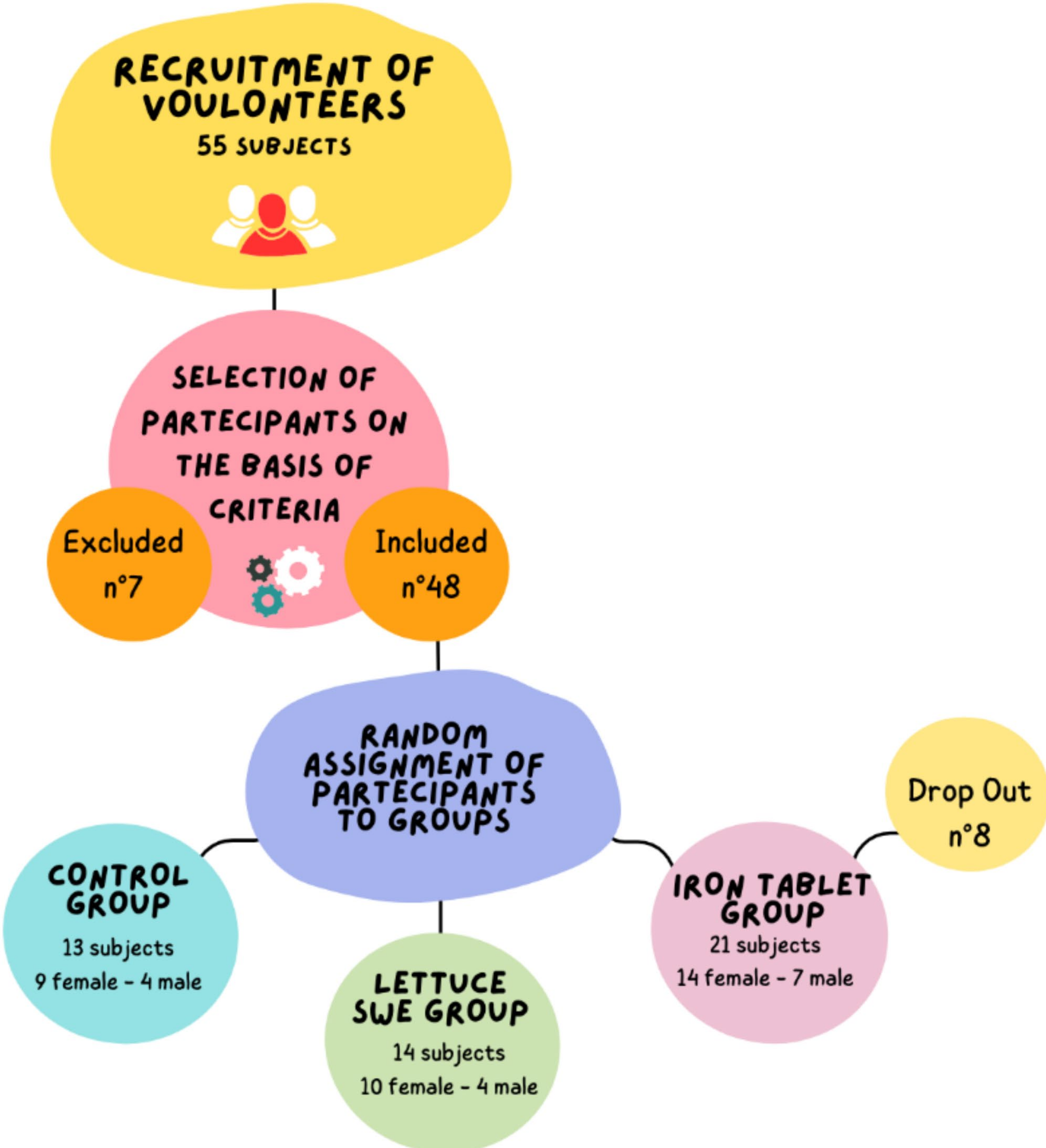

Fig. 3. Flow chart of the study describing a cohort of healthy subjects that was divided into a control group that received lettuce (control lettuce 100 g/day), the lettuce group that received lettuce biostimulated with *Ecklonia Maxima* seaweed (Lettuce with SwE application, 100 g/day) and the iron tablet group that received iron supplementation (30 mg, 1 tablet/day of iron).

Control Lettuces, Lettuce SwE and Iron tablets were provided to the participants for four weeks. The lettuce groups consumed a standardized portion of 100 g of lettuce daily according to our previous study[54] while the iron tablet group got one tablet/day of iron (iron bisglycinate) for the duration of four weeks that according to literature seems to exert less gastrointestinal adverse effects that other iron formulation[55]. Lettuce was provided freshly every three days and was kept in the fridge. Participants in both groups were instructed to maintain a detailed food diary throughout the study period. During the initial study visit (BASELINE), healthy volunteers underwent standardized anthropometric assessments. These assessments included the measurement of weight, height, body mass index (BMI), fat-free mass, fat mass, and visceral fat as previously shown[56–58].

Selection criteria	Inclusion criteria	Exclusion criteria
Absence of metabolic disorders; blood-related and gastrointestinal dysfunction; cardiac anomalies, recent viral infection and food allergies	Age 20–70 years	Chronic disease
Not taking drugs	Italian Ethnicity	Use of drugs
Absence of obesity	Body mass index between 18.5 and 28.5 kg/m^2	Pregnancy, Exogenous hormones, Breastfeeding
Not taking supplements, absence of sensitivity to lettuce	Clinically Healthy	Use of supplements, sensitivity to lettuce

Table 6. Selection, inclusion and exclusion criteria.

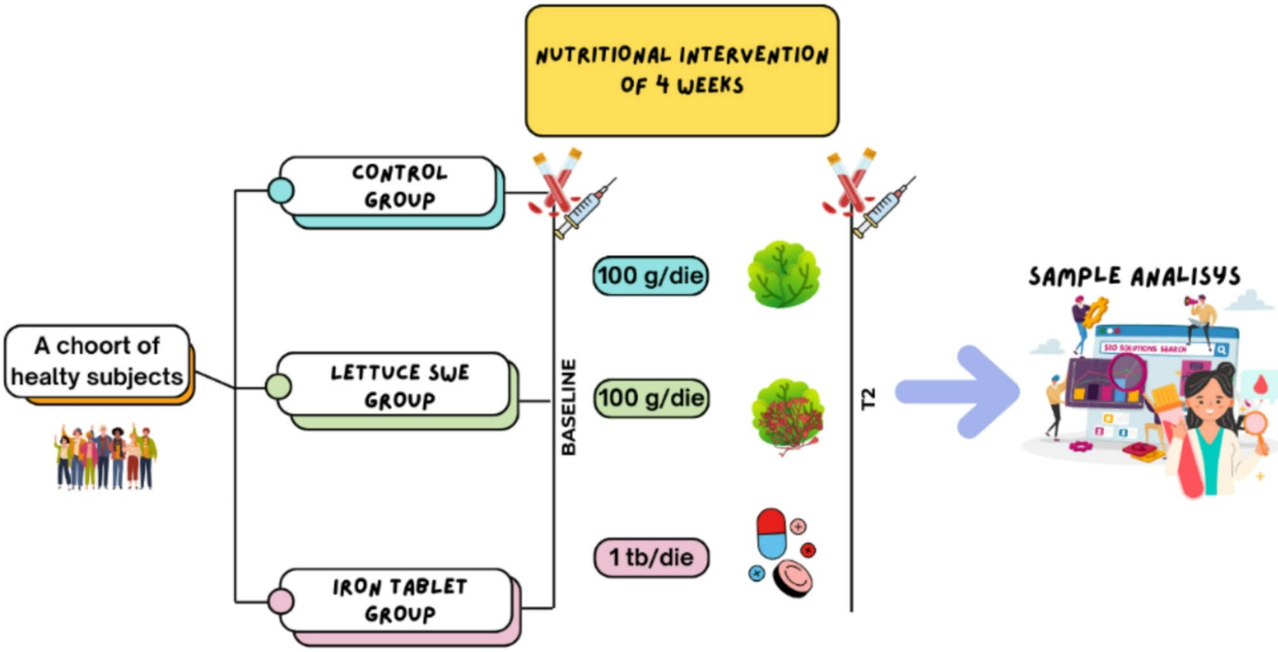

Fig. 4. A cohort of healthy subjects was divided into a control group, receiving control lettuce, lettuce SwE group, receiving biostimulated lettuce and iron tablet group receiving iron supplement in tablet for a total of 4 weeks. Blood samples were collected in specific tubes before (baseline) and after the nutritional intervention (T2).

At baseline (T0 = BASELINE) and after four weeks (T2) blood samples were obtained. Subsequently, the collected blood samples were analyzed for standardized biochemical assays. These analyses, designated as baseline (BASELINE) were performed prior to the commencement of the clinical trial and repeated at the end, after 4 weeks for the follow-up (TFig. ig. 3). Specifically, primary outcome assessing serological levels of minerals (potassium, calcium, iron, phosphate, magnesium) at baseline and after 4 weeks. Secondary outcomes measured iron, glucose and lipid metabolism markers descripted below.

Analysis of the blood samples

Following an overnight fast, participants underwent peripheral venipuncture between 7:00 AM and 8:00 AM and blood samples were collected. As previously reported, the blood sample was dispensed in VACUETTE serum tubes (centrifugation at 1300x g for 15 min)[59,60]. Blood tests were examined and compared between the groups at baseline and after four weeks (T2) to evaluate differences in serum mineral concentrations (calcium, potassium, iron, magnesium, phosphorus), iron homeostasis (ferritin, transferrin, percent of transferrin saturation), lipid profile (total cholesterol, HDL, LDL, triglycerides, CHOL/HDL ratio) fasting glucose and insulin by an automated procedure using the Roche COBAS c503, according to standard commercially available assays supplied by Roche Diagnostics[6,9,36,61–64]. Insulin resistance (HOMA-IR), β-cell function (HOMA-% β) and insulin sensitivity (HOMA-%S) were calculated as previously reported[64].

Statistical analyses

Statistical analyzes were carried out using GraphPad Prism software. The comparative analysis of minerals in lettuce was performed by Student t-tests. To compare the baseline characteristics of the groups student t tests were used. The comparison between the different groups of participants and within the same group between baseline and T2 was carried out using One-Way ANOVA followed by Sidak test. Values were expressed as mean ± standard deviation (mean ± SD). P-value ≤ 0.05 was considered to be statistically significant.

Data availability

The datasets analysed during the current study are available from the corresponding author on reasonable request.

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Declarations

Competing interests
The authors declare no competing interests.

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