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RESEARCH ARTICLE

Cardiovascular benefits from ancient grain bread consumption: findings from a double-blinded randomized crossover intervention trial

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ABSTRACT
Ancient grain varieties have been shown to have some beneficial effects on health. Forty-five clinically healthy subjects were included in a randomized, double-blinded crossover trial aimed at evaluating the effect of a replacement diet with bread derived from ancient grain varieties versus modern grain variety on cardiovascular risk profile. After 8 weeks of intervention, consumption of bread obtained by the ancient varieties showed a significant amelioration of various cardiovascular parameters. Indeed, the ancient varieties were shown to result in a significant reduction of total cholesterol, low-density lipoprotein (LDL)-cholesterol and blood glucose, whereas no significant differences during the phase with the modern variety were reported. Moreover, a significant increase in circulating endothelial progenitor cells were reported after the consumption of products made from the ancient *"Verna"* variety. The present results suggest that a dietary consumption of bread obtained from ancient grain varieties was effective in reducing cardiovascular risk factors.

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Gran; ancient variety; cardiovascular disease; food

Introduction

Cereals can contribute towards a favorable health status, particularly in the prevention of chronic and cardiovascular diseases (Truswell 2002; Mellen et al. 2008). The beneficial effects are mostly attributed to various nutritional components of the grain such as fiber, vitamins (B-group and E), minerals (zinc, magnesium, iron, potassium), phytoestrogens and antioxidant substances (Adom et al. 2003; Gil et al. 2011). Over the past few years, with the aim of magnifying the nutraceutical properties of the grain, researchers have focused their attention both on the selection of particular varieties, as well as in the choice of cultivation methods. In particular, ancient grain varieties are reported as a potential health-promoting food source, due to the higher content of beneficial nutrients such as antioxidant molecules, vitamins and minerals (Dinelli et al. 2007; Vrček et al. 2013). In recent years, our research group has published results derived from three different intervention studies, all in support of this hypothesis. Results were obtained from both healthy subjects and those suffering from irritable bowel syndrome (Sofi et al. 2010, 2013, 2014). Both the presence and quantity of the beneficial components derived from the grain are dependent both on the type of grain as well as from aspects relating to the production, storage and transformation of the grain. One of the most interesting aspects, also from the standpoint of marketing, is the potential beneficial effect of the organic cultivation in improving the nutritional properties of the grain (Vrček et al. 2013). A recent systematic review analyzing all studies, to date, on the supposed beneficial effect of organic agriculture on the nutritional quality of foods produced, reported no significant differences between those foods cultivated in conventional and organic agriculture, respectively (Smith-Spangler et al. 2012). However, to the best of our knowledge, there are no published studies on possible beneficial effects, on cardiovascular risk parameters, in subjects consuming foods grown under different methods of cultivation. We designed the present intervention study with the dual purpose of (1) extending our previous findings, demonstrating the possible beneficial effects of consuming products made with the ancient grain varieties, to a larger population using different novel risk

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parameters and (2) investigating any potential differences existing between varieties derived from either organic or conventional agriculture on the cardio-metabolic risk profile.

Materials and methods

Study population

The study population comprised 45 clinically healthy subjects (32 men; 13 women). The median age was 50.1 (range: 25–75 years), with a mean body mass index (BMI = kg/m^2) of 25.5 ± 4.4. All subjects were recruited following consultation from the list of workers and members of the food industry "UNICOOP Firenze" at the worksites at Ponte a Greve, Florence and Castelfiorentino, Tuscany, Italy, between January 2013 and July 2013. Participants were interviewed and examined using standardized methods. Information regarding personal medical history, demographics, family history of coronary or other atherosclerotic diseases, medication used and lifestyle habits in relation to smoking habit, diet, physical exercise, weight, blood pressure, lipids and diabetes were obtained at the time of the interview. Physicians, using standardized protocols, conducted a physical examination, blood pressure measurements, and laboratory tests. In particular, BMI was calculated as weight (kg)/height (m^2). Physicians measured blood pressure on the patient's right upper arm in a sitting position. Raised blood pressure was defined as systolic blood pressure of 140 mmHg or more and diastolic blood pressure of 90 mmHg or more for primary prevention. For secondary prevention, raised blood pressure was defined as systolic blood pressure of 130 mmHg or more and diastolic blood pressure of 80 mmHg or more, as reported by the guidelines of the European Society of Cardiology. Smokers were defined as those who smoked at the time of the physical examination. Diabetic subjects were classified according to the American Diabetes Association on the basis of self-reported data (if confirmed by medication or chart review). Dyslipidemia was defined, either according to the Third report of the National Cholesterol Education Program (NCEP-III), or if the participant reported taking antidyslipidemic drugs, as verified by the physician. Physical activity was assessed as either absent (sedentary lifestyle), mild or moderate, based on the duration and intensity of physical activity over the preceding 6 months. Volunteers were assessed before the onset of the study for good health by a standard medical questionnaire, and were then selected according to certain inclusion criteria. Inclusion criteria for subject participation in the study necessitated that participants: provide a signed consent form, be of an age ranging between 18 and 75 years, be in good general health, be neither pregnant nor lactating, and have neither a gluten allergy nor gastrointestinal disorders (e.g. chronic constipation, diarrhea, inflammatory bowel disease, irritable bowel syndrome, or other chronic gastrointestinal complaints) nor gall bladder problems.

Study design

The study was a randomized, double-blinded, cross-over dietary trial study with 3 different interventions, each lasting for 8 weeks. Starting from January 2013 (1st intervention), participants were randomly assigned to receive bread obtained from the ancient variety "*Verna*", obtained from either organic ($n = 22$) or conventional ($n = 23$) cultivation, respectively. The 2nd intervention started from May 2013 during which all participants were permitted to eat the modern variety "*Blasco*". Thereafter, from June 2013 (3rd intervention phase) participants in both groups were assigned to consume the two remaining ancient varieties "*Gentil Rosso*" and "*Autonomia B*", both cultivated under conventional agriculture. During the whole study period, all participants were instructed to maintain their usual diet and lifestyle habits, and were not permitted to eat other bread. At baseline and at the end of each intervention phase, blood samples were taken from all subjects between 07:00 and 09:30 after a 12-h fasting period. Furthermore, subjects were requested not to engage in strenuous physical activity during the day before the examination.

Blood measurements

Venous blood samples were taken in the morning and collected into evacuated plastic tubes (Vacutainer). Samples were obtained by centrifuging at 3000*g* for 15 min at 4 °C and supernatants were stored in aliquots at −80 °C until analysis. Lipid variables, blood glucose, minerals and serum electrolytes were assessed by conventional procedures. Endothelial Progenitor Cells (EPCs) and Circulating Progenitor Cells (CPCs) were assessed using flow cytometry. EPCs were identified through the expression of CD34, KDR and CD133, and were considered as CD34+/KDR+, CD133+/KDR + and CD34+/CD133+/KDR+. CPCs were defined as follows: cells forming a cluster with a low side scatter, low-to-intermediate CD45 staining, and positive for CD34+, CD133 + and CD34/CD133.

Statistical analysis

Statistical analysis was performed using the statistical package PASW 18.0 for Macintosh (SPSS Inc, Chicago, IL). All variables were checked for normal distribution before data analysis. Data were expressed as arithmetic means ± SD for normally distributed variables, and as median and range or non-normally distributed data. The Mann–Whitney test was used for testing differences between the groups. A general linear model for repeated measurements was performed to compare the effect of the treatments at their different phases of intervention. A multivariable model with adjustments for age, gender, BMI change, smoking habit and hypertension was performed to evaluate the modifications of cardiovascular parameters that were likely determined by the tested intervention (i.e. making a comparison between parameters at the beginning and at the end of each intervention phase). Data for the general linear model were reported as geometric mean and standard error. A p-value <0.05 was considered to indicate statistical significance.

Results

Clinical characteristics

Ten participants were smokers (22.2%) at the time of the study. Twelve participants were hypertensive (26.7%), one was diabetic and one dyslipidemic, all under good control due to the respective treatments taken.

1st intervention phase

During the 1st intervention phase with the ancient variety "*Verna*", a significant reduction in blood glucose, total cholesterol and low-density lipoprotein (LDL)-cholesterol were reported. Blood glucose decreased by 5.6%, whereas total cholesterol and LDL-cholesterol levels decreased by 3.1% and 3.9%, respectively (Figure 1). In addition, the intervention phase with "*Verna*" resulted in a significant increase of some minerals such as potassium and sodium (Table 1). With regard to the different methods of cultivation, no significant differences in the biochemical parameters were observed between subjects consuming the

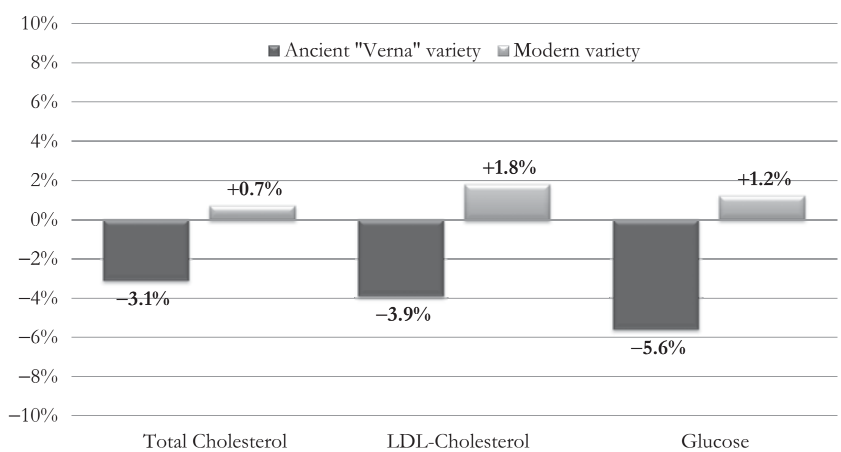

Figure 1. Percentage of modification for selected biochemical parameters during the phase of intervention with the ancient "*Verna*" variety and during the period with the modern variety.

Table 1. 1st phase of intervention with the ancient "*Verna*" variety.

Variable	Pre	Post	Change	*p* Value
White blood cells, *n*	6.68 (6.04–7.32)	6.65 (6.05–7.26)	−0.03 (−0.46; 0.41)	0.2
Glucose (g/L)	0.89 (0.85–0.93)	0.84 (0.81–0.87)	−0.05 (−0.09; −0.02)	0.003
Total cholesterol (mg/dL)	214.54 (201.01–228.08)	208.03 (195.52–220.54)	−6.51 (−12.21; −0.82)	0.03
LDL-cholesterol (mg/dL)	132.94 (121.17–144.71)	127.76 (116.52–138.99)	−5.18 (−9.86; −0.50)	0.03
Triglycerides (mg/dL)	103.57 (86.29–120.84)	114.11 (92.74–135.48)	10.54 (−8.08; 29.17)	0.3
HDL-cholesterol (mg/dL)	64.94 (59.63–70.26)	59.77 (54.89–64.66)	−5.17 (−7.18; −3.17)	0.0001
Sodium (mEq/L)	141.17 (140.79–141.56)	141.60 (141.18–142.03)	0.43 (0.04; 0.82)	0.03
Potassium (mEq/L)	4.46 (4.33–4.60)	4.54 (4.39–4.69)	0.08 (0.004; 0.15)	0.04
Calcium (mg/dL)	9.40 (9.29–9.52)	9.50 (9.40–9.60)	0.10 (−0.02; 0.21)	0.09
Magnesium (mg/dL)	2.12 (2.08–2.17)	2.11 (2.06–2.17)	0.01 (−0.06; 0.05)	0.8
Iron, microg/dL	78.26 (69.42–87.09)	82.51 (71.71–93.32)	4.26 (−5.10; 13.62)	0.4

Data are reported as geometric mean and (range). General linear model for repeated measurements adjusted for age, gender, BMI, smoking habit and hypertension.

Table 2. Comparison between conventional and organic cultivations at the 1st phase of intervention with the ancient *"Verna"* variety.

Variable	Conventional pre	Conventional post	Change	*p* Value	Organic pre	Organic post	Change	*p* Value
White blood cells, *n*	6.63 (5.43–7.83)	6.67 (5.56–7.77)	0.04 (−0.75; 0.68)	0.9	6.73 (6.04–7.41)	6.64 (5.90–7.38)	−0.09 (−0.57; 0.75)	0.8
Glucose (g/L)	0.90 (0.85–0.96)	0.83 (0.77–0.89)	−0.07 (−0.14; −0.01)	0.02	0.88 (0.82–0.94)	0.85 (0.81–0.89)	−0.03 (−0.06; −0.005)	0.01
Total cholesterol (mg/dL)	213.44 (190.29–236.60)	205.78 (189.22–222.33)	−7.67 (−17.31; 1.97)	0.1	215.71 (196.88–234.53)	210.41 (190.97–228.86)	−5.29 (−12.19; 9.60)	0.1
LDL-cholesterol (mg/dL)	133.59 (112.92–154.26)	127.71 (110.64–144.78)	−5.88 (−13.27; 1.50)	0.1	132.25 (115.62–148.88)	127.81 (111.62–144.01)	−4.44 (−9.66; 0.79)	0.09
Triglycerides (mg/dL)	111.33 (82.56–140.11)	114.44 (83.68–145.21)	3.11 (−24.51; 30.73)	0.8	95.35 (75.01–115.69)	113.77 (78.59–148.94)	18.41 (−40.81; 3.99)	0.09
HDL-cholesterol (mg/dL)	62.17 (54.34–69.99)	57.78 (51.11–64.45)	−4.39 (−6.97; −1.81)	0.003	67.88 (58.69–77.07)	61.88 (54.14–68.62)	−6.00 (−8.95; −3.05)	0.001
Sodium (mEq/L)	140.83 (140.29–141.37)	141.67 (141.16–142.17)	0.83 (−0.23; 1.43)	0.08	141.53 (140.98–142.08)	141.53 (140.86–142.30)	0 (−0.57; 0.57)	0.1
Potassium (mEq/L)	4.53 (4.36–4.71)	4.61 (4.43–4.78)	0.08 (−0.02; 0.16)	0.2	4.39 (4.16–4.62)	4.47 (4.21–4.71)	0.08 (−0.06; 0.22)	0.09
Calcium (mg/dL)	9.46 (9.30–9.61)	9.57 (9.42–9.71)	0.11 (−0.09; 0.32)	0.09	9.35 (9.19–9.50)	9.43 (9.28–9.58)	0.08 (−0.08; 0.25)	0.07
Magnesium (mg/dL)	2.12 (2.06–2.19)	2.08 (1.98–2.19)	−0.04 (−0.15; 0.07)	0.4	2.12 (2.05–2.20)	2.15 (2.08–2.22)	0.03 (−0.03; 0.09)	0.3
Iron (microg/dL)	80.06 (64.79–95.32)	79.33 (63.84–94.83)	−0.72 (−11.58; 10.13)	0.9	76.35 (66.48–86.23)	85.88 (75.46–96.31)	9.53 (−1.02; 20.08)	0.07

Data are reported as geometric mean and (range). General linear model for repeated measurements adjusted for age, gender, BMI, smoking habit and hypertension.

"*Verna*" variety cultivated either under organic or conventional agriculture, respectively (Table 2).

2nd intervention phase

No significant changes in the biochemical parameters were observed during the phase of intervention with bread obtained from the modern variety of wheat. Glucose was reported to change from 0.84 to 0.85 g/L ($p = 0.5$), total cholesterol from 210.5 to 211.9 mg/dL ($p = 0.6$), LDL-cholesterol from 128.8 to 126.5 mg/dL ($p = 0.4$), and triglycerides from 109.1 to 108.5 mg/dL ($p = 0.9$), respectively. On the other hand, a significant decrease in the mineral content of the study population was shown. In particular, sodium levels decreased from 141.7 to 141.03 mEq/L ($p = 0.03$), potassium from 4.58 to 4.39 mEq/L ($p = 0.001$), calcium from 9.52 to 9.39 mg/dL ($p = 0.04$) and iron from 82.7 to 65.3 microg/dL ($p = 0.005$), respectively.

3rd intervention phase

The third intervention phase, that included the consumption of the remaining 2 ancient varieties, namely "*Gentil Rosso*" and "*Autonomia B*", showed a significant reduction in total cholesterol by 7.8%, and LDL-cholesterol by 6.8% (Table 3). "*Gentil Rosso*" showed a greater and significant reduction in total cholesterol and LDL-cholesterol with respect to "*Autonomia B*". In fact, after the consumption of "*Gentil Rosso*", total cholesterol was decreased from 221.2 (201.3–241.1) mg/dL to 200.6 (176.9–224.4) mg/dL, ($p = 0.001$), and LDL-cholesterol from 130.4 (112.2–148.5) mg/dL to 121.2 (103.2–139.2) mg/dL, ($p = 0.003$). The consumption of "*Autonomia B*" resulted in no significant changes.

Modifications in CPCs and EPCs

In order to fully elucidate the possible beneficial role of such ancient grains on the cardiovascular risk profile of healthy subjects, we analyzed the number of EPCs and CPCs, novel parameters of endothelial dysfunction. The 1st intervention phase with "*Verna*" resulted in a significant increase in the number of CPCs (Figure 2) (CD 34+: +9.6%; $p = 0.04$, and CD 133+: +18.1%; $p = 0.02$), whereas in the intervention phase with the modern variety, a significant decrease was observed for both parameters. No significant changes for EPCs were reported in all the three intervention phases.

Discussion

The aim of the present randomized, double-blinded, crossover dietary intervention study was to evaluate

Table 3. 3rd phase of intervention with the ancient "*Gentil Rosso*" and "*Autonomia B*" varieties.

Variable	Pre	Post	Change	*p* Value
White blood cells, *n*	6.68 (6.09–7.28)	6.44 (5.81–7.07)	−0.24 (−0.6;0.09)	0.1
Glucose (g/L)	0.85 (0.79–0.90)	0.82 (0.78–0.85)	−0.03 (−0.07;0.09)	0.1
Total cholesterol (mg/dL)	218.62 (205.79–231.44)	201.62 (187.06–216.17)	−17.00 (−25.65; −8.35)	0.001
LDL-cholesterol (mg/dL)	130.14 (117.89–142.40)	121.19 (109.43–132.95)	−8.95 (−17.29; −0.61)	0.04
Triglycerides (mg/dL)	101.09 (84.07–118.12)	93.81 (76.88–110.74)	7.29 (−23.08; 8.51)	0.3
HDL-cholesterol (mg/dL)	61.52 (56.86–66.19)	60.76 (56.21–65.31)	−0.76 (−3.74; 2.21)	0.6
Sodium (mEq/L)	141.52 (140.74–142.31)	141.14 (140.57–141.71)	−0.38 (−1.29; 0.53)	0.4
Potassium (mEq/L)	4.36 (4.22–4.54)	4.30 (4.18–4.53)	−0.06 (−0.15; 0.07)	0.4
Calcium (mg/dL)	9.30 (9.14–9.48)	9.30 (9.11–9.51)	0 (−0.22; 0.22)	1
Magnesium (mg/dL)	2.16 (2.11–2.22)	2.18 (2.12–2.24)	0.02 (−0.03; 0.07)	0.5
Iron (microg/dL)	63.47 (51.16–75.88)	75.62 (57.96–93.28)	12.05 (−5.18; 29.28)	0.2

Data are reported as geometric mean and (range). General linear model for repeated measurements adjusted for age, gender, BMI, smoking habit and hypertension.

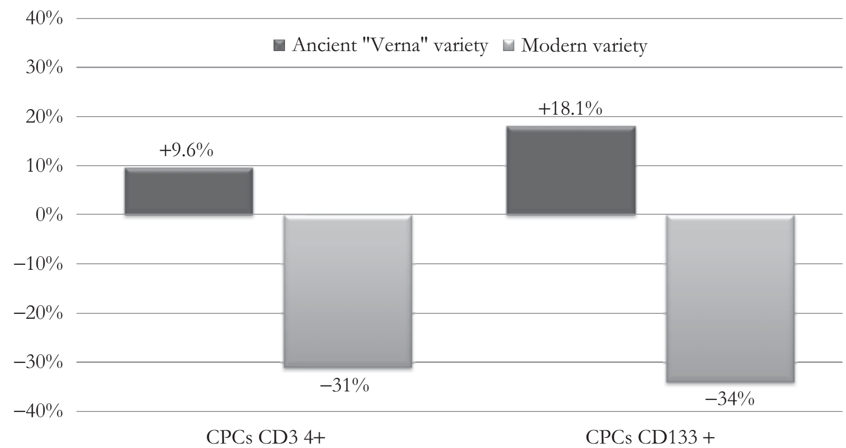

Figure 2. Percentage of modification for CPCs during the phase of intervention with the ancient "*Verna*" variety and during the period with the modern variety.

the possible beneficial effects from the consumption of bread obtained from three different ancient grain varieties on cardiovascular risk biomarkers of clinically healthy subjects. The principle findings of the investigation demonstrated that the consumption of bread obtained by ancient varieties of grain (*Verna, Autonomia B, Gentil Rosso*) effected a significant reduction in some biochemical parameters, such as total and LDL-cholesterol, glucose and a novel biomarker, namely CPCs. These modifications were not observed in the intervention period with the modern variety. Actually, a significant worsening in the mineral profile of the study population was noted.

In recent years, consumers have increased awareness regarding the nutraceutical properties of grain for the prevention of chronic and cardiovascular diseases. It has been demonstrated that several beneficial components of cereals are all dependent on the production, storage, processing, and climatic conditions of the source grains. It has been reported that different varieties of grains contain different levels of B-group vitamins and antioxidants (Di Silvestro et al. 2012). In this regard, ancient grain varieties have been found to present a better nutraceutical profile with respect to modern varieties, especially in terms of antioxidant substances, vitamins and minerals (Dinelli et al. 2007; Sofi et al. 2010; Di Silvestro et al. 2012; Sofi et al. 2013, 2014). In the past 5 years, we have conducted three different dietary intervention studies with ancient varieties of grain in different study populations. Consequently, we reported that the consumption of products obtained with ancient grains is effective in reducing biomarkers associated with the occurrence of cardio-metabolic diseases (Sofi et al. 2010, 2013, 2014). In the present study, the most interesting results were those related to the reductions of biomarkers in subjects consuming the ancient "*Verna*" variety. This is in line with previous findings obtained in 2010 by our group using the same ancient grain variety (Sofi et al. 2010). "*Verna*" appears to be able to impose a significant beneficial effect on total cholesterol, LDL-cholesterol, as well as for blood glucose. Such improvements are likely related to the higher mineral content and antioxidant substances present in the flour of this ancient variety, as reported previously (Sofi et al. 2010; Di Silvestro et al. 2012). Of great interest, for the first time, we were also able to find a significant increase in CPCs after consumption of bread obtained by "*Verna*". CPCs are cells derived from the bone marrow that contribute to vascular repair, regeneration and re-endothelialization, thereby providing protection against the initiation of atherosclerosis (Cesari et al. 2013). Decreased levels of CPCs have been found to be associated with ageing, hypertension, smoking habit, hypercholesterolemia and BMI, while increased levels have been reported to be associated with the use of some pharmacological treatments such as statins, erythropoietin, peroxisome proliferator-activated receptor gamma-agonists and angiotensin II receptor antagonists. Lifestyle factors, such as diet, may influence this process (Van Craenenbroeck & Conraads, 2010). However, to the best of our knowledge, no evidence supporting the possible relationship between foods and their respective components, and these cells are currently available. The possibility that the consumption of "healthy" foods may modulate the number of these progenitor cells is somewhat appealing and intriguing, but needs further confirmation. Together with "*Verna*", two additional ancient grain varieties (*Gentil Rosso* and *Autonomia B*), which have never been investigated previously, in terms of possible beneficial effects on circulating biomarkers in humans, were tested. A significant improvement in some of these parameters, such as total and LDL-cholesterol, were demonstrated. Therefore, we hypothesize that the consumption of products made with such ancient varieties could determine a beneficial effect on biological disease markers. An additional aim of this study was to investigate, in a subgroup of the study population, the potential effects of attributable to either conventional or organic cultivation on biochemical parameters. In recent years, organic food products have gained much attention in developed countries, due to increasing interest in more ecologically friendly practices, especially because they are considered among consumers as both healthier and safer than those offered by conventional practices (Smith-Spangler et al. 2012). Scientific evidence is in high demand for the purpose of promoting consumer confidence in organic foods. However, evidence thus far is not yet available to consolidate these claims. There have been contrasting results among individual experiments (Worthington 2001; Magkos et al. 2003; Dangour et al. 2010). Some have showed that organic products are healthier and have higher nutritional value than conventional ones, whereas another more recent investigation reported a similar nutritional value between organic and conventional products (Smith-Spangler et al. 2012). In our study, no significant differences in the biochemical parameters were found in subjects consuming "*Verna*" cultivated under these two different methods. Such a result is in line with a recent systematic review that encompassed all the studies comparing the two different methods of cultivation in terms of nutrient content (10). Based on the substantial similarity of nutritional profiles between products made with different methods of cultivation, these results suggest that ancient varieties seem to maintain their beneficial effects regardless of whether they are cultivated under either conventional or organic methods, respectively. Nevertheless, further and larger studies need to be conducted in order to better address this interesting issue.

Our study has several limitations. Firstly, the number of participants (45 total) though greater than our previous studies, is still considered a restriction. Further and larger studies need to be conducted before drawing any firm conclusion on the effects of such food products on human health. The results of the present study are a promising basis for a more comprehensive evaluation of this aspect of clinical nutrition. Secondly, the assessment of dietary habits and physical activity in our study population was not conducted. The possibility that changes in dietary and/or lifestyle habits may have significantly affected parameters investigated cannot be excluded, although, before enrollment, all subjects were instructed by physicians and by an expert dietician to maintain their usual lifestyle habits.

Conclusions

In conclusion, the present results confirmed our earlier studies on the beneficial effects on cardiovascular biomarkers of ancient grain varieties on subjects consuming bread. In the past decades, breeding strategies have been predominantly aimed at improving the yield production of wheat, at the expense of the nutritional profile. This led to the progressive abandonment of the ancient varieties, which are not suitable for the high-input conventional cultivation system. The present findings highlight that ancient grain varieties could be useful in ameliorating the profile of important biomarkers in consumers, thereby possibly stimulating producers to use and implement these varieties in their current breeding strategies.

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Disclosure statement

The Authors declare that they have no competing interests.

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