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Effect of a Phytochemical-Rich Olive Derived Extract on Anthropometric, Hematological And Metabolic Parameters

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Abstract: Background: Extra virgin olive oil is a fundamental component of the Mediterranean diet. It contains several molecules that sustain human well-being by modulating cellular metabolism and exerting antioxidant, anti-inflammatory, anti-ageing effects to protect normal tissues and can exert anti-angiogenic, and pro-apoptotic effects on cancer cells. Metabolites found in different parts of the olive tree, including leaves, also possess properties that might help in cancer prevention and promote wellness in aging. Olive mill wastewater (OMWW), a liquid residue produced during olive oil extraction, represents an environmental issue. However, it is enriched in phytochemicals with potential beneficial properties. Dietary supplements based on OMWW can be produced for nutritional supplementation with advantages to the ecology. **Purpose:** This work aims to measure hematochemical, anthropometric and metabolomic parameters in volunteers assuming an OMWW dietary supplement, Oliphenolia® (OMWW-OL). **Methods:** The supplementation of OMWW-OL 25 mL twice daily for 30 days was tested on a pilot cohort of volunteers with characteristics close to metabolic syndrome. Hematochemical, anthropometric, and metabolomic parameters were analyzed before the intervention, at 30 days and 30 days after stopping consumption. **Results:** 29 volunteers were enrolled and 23 completed the study. The participants' parameters at baseline were measured, and then twice daily at 30 days of treatment and 30 days after assumption discontinuation. Although treatment was with an olive derivative, weight and body mass index instead of increasing slightly decreased, particularly in women. Also, hydration increased, especially in women, while blood pressure, glycemia and insulin decreased. Cholesterol, low-density and high-density lipoproteins and triglycerides were stable while vitamin D levels, alongside calcium, sensibly increased. Albumin also increased and parameters related to sarcopenia displayed promising, although not statistically significant, trends toward positive effects. All the values were in support of an equilibrium with no damaging effects. By mass spectrometry analysis, we also found favorable changes in the vitamin D/histamine and homocysteine/methionine ratios, an increase in a new metabolite of unknown formula, and vitamin D/unknown metabolite ratio. **Conclusions:** Supplementation of OMWW-OL has no detrimental effects and, although with limited potency of a pilot study, might imply the beneficial modulation of several biological parameters, being potentially valuable for people at risk of metabolic syndrome. Some of these parameters could also be relevant as anti-ageing and in cancer prevention.

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1. Introduction

An integral part of the Mediterranean diet, extra virgin olive oil (EVOO) is high in monounsaturated fatty acids, which are regarded as advantageous for protection against diseases [1]. It is also a good source of antioxidants, including polyphenols and vitamin E, which may help protect against oxidative damage in the body, the cause of several chronic degenerative diseases [2,3]. EVOO reduces the risk of cardiovascular pathologies, type 2 diabetes, neurodegenerative diseases, and some cancers by displaying anti-inflammatory, anti-oxidant effects [5]. Olive trees can produce useful metabolites in a variety of tissues, including the leaves. Olive mill wastewater (OMWW) is a large-scale liquid waste product released during olive oil extraction. It is an environmental concern on the one hand, but on the other, it is a rich source of phytochemicals that may have beneficial effects [5-7]. Hydroxytyrosol is the most common polyphenol in OMWW extracts however, verbascoside and oleuropein are also abundant [8]. It has been shown that polyphenols control inflammation and oxidative stress [9], regulate gene expression in metabolic pathways, and have the potential to improve insulin sensitivity [10]. In this way, polyphenols could play an important role in the prevention of metabolic syndrome, which is characterized by hypertension, hyperglycemia, hyperinsulinemia, dyslipidemia, and obesity [11,12]. Thanks to pharmacometabolomics [13], the personalization of drug therapy is possible through the comprehensive analysis of metabolites in a patient's biological fluids. Metabolomics can provide important information on the effect of clinical treatments on the metabolism, and therefore, mass spectrometry (MS) has taken a central role in personalized and precision medicine [14].

In this work, we report the application of a dietary strategy to study the effects of an OMWW-derived supplement, Oliphenolia® (OMWW-OL), on different serum parameters at distinct time points. Our aim was to measure hematochemical, anti-inflammatory, antioxidant, and anthropometric parameters and to assess metabolic pathways before initiation (T0), after 30 days of nutritional intervention (T1), and 30 days after the end of the nutritional intervention (T2).

2. Materials and Methods

2.1. Study Design

This was a single-arm longitudinal interventional study to evaluate the effect of OMWW-OL on a cohort of volunteers with characteristics close to metabolic syndrome as a pilot study. Participants were enrolled in western Sicily within the project "Nutraceutical effects of olive products: Role in the achievement of longevity". The dietary intervention consisted in the consumption of 25 mL of OMWW-OL twice a day, 30 minutes before the main meals (lunch and dinner) for 30 days. Before starting the nutritional intervention, a 7-day washout period was carried out, during which the participants were required to abstain from EVOO and food supplements containing polyphenols. The flowchart of the study design is reported in Figure 1.

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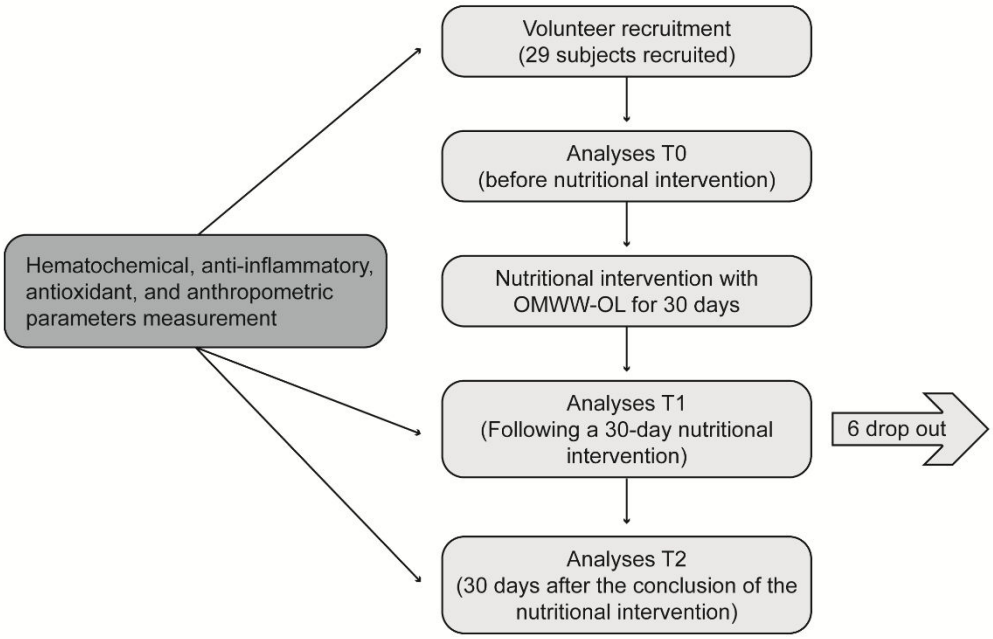

Figure 1. The flowchart of the study design. The study protocol was approved by the Ethics Committee. During a 7-day washout period, the participants were required to abstain from extra virgin olive oil and food supplements containing polyphenols. The nutritional intervention consisted of the consumption of the Oliphenolia® dietary supplement preparation (25 mL) twice a day, 30 minutes before the main meals (lunch and dinner), for 30 days, with no other restriction from the usual diet.

2.2. Study Participants

The subjects enrolled had at least one of the following inclusion criteria: slight dyslipidemia (TC 190–240 mg/dL, triglyceride level ≥150 mg/dL); waist circumference ≥102 cm in men and ≥88 cm in women; altered glucose tolerance (fasting blood glucose ≥100 mg/dL). Exclusion criteria were: any treatment for specific diseases, including drugs to treat metabolic disorders; diagnosis of severe systemic disease; history of treatment with statins or similar drugs or other liposoluble or hypoglycemic drugs; restrictive dietary requirements (e.g., gluten-free diet). The onset of gastrointestinal disorders during the intervention determined the prompt exclusion from the study. No restriction related to gender was considered.

2.3. Ethical Considerations

Before enrollment, each recruited subject was fully informed about the purposes and procedures of the experiment, and their informed consent was obtained. To respect privacy, patients' names were anonymized by assigning each one an encoded alpha-numeric identification code in accordance with the General Data Protection Regulation (GDPR, EU Regulation 2016/679) concerning the protection of individuals with regard to the processing of personal data, as well as the free circulation of such data. The study protocol was conducted in accordance with the 1964 Declaration of Helsinki and its later amendments, and the institutional Ethics Committee (Policlinico Paolo Giaccone, University Hospital) approved the study protocol (Nutrition and Longevity, No. 032017).

2.4. Data Collection

A group of well-trained biologists and physicians from the University of Palermo administered a questionnaire to the participants to collect demographic, clinical, and anamnestic data. Additionally, a weekly food diary was provided to record data on their dietary intake. A database in

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Excel was created to collect and handle all participants' data and information. To obtain informative and reproducible analyses, anthropometric measures were obtained while participants wore light clothes and no shoes. Body weight was measured using an electronic scale calibrated in kilograms. Height was measured with the subject in the supine position with a stadiometer. The bioelectrical impedance analysis (BIA) was also performed in the supine position, wearing light clothes and barefoot. The resistance and the reactance, both in Ohm and the phase angle, were reported; the body compartment measurements (in percentage by weight and kg/m^2) were estimated using regression equations by Bodygram®Plus 1.1.4.4 software (AKERN® Pisa, Italy). Body mass index (BMI, kg/m^2) was calculated using anthropometric measurements, such as height (m) and weight (kg).

2.5. Analysis of Hematochemical Parameters

Overnight fasting blood samples were collected in the morning. The blood was collected in specific tubes without additives in order to separate the serum samples obtained after centrifugation at 2,500 rpm for 15 minutes at 4°C, rapidly frozen, and stored at -80°C for further tests. All hematochemical analyses were measured by standard biochemical assays. The concentration of serum low-density lipoprotein (LDL) cholesterol was calculated by using the Friedewald equation: LDL=total cholesterol-high-density lipoproteins (HDL)-(triglycerides/5). We also collected samples before initiation (T0), after 30 days of nutritional intervention (T1), and 30 days after the end of the nutritional intervention (T2) for spectrometry analysis.

2.6. Evaluation of the Anti-Oxidative Effect

Serum samples from patients were also used for the determination of human oxidized LDL (oxLDL) quantity, using a specific ELISA kit (Cusabio, Houston, TX, USA) following manufacturer instructions. The competitive inhibition enzyme immunoassay method was used in this assay. The microtiter plate was pre-coated with oxLDL. Standards or samples were added to the appropriate microtiter plate wells with horseradish peroxidase-conjugated antibody preparation specific for oxLDL. Pre-coated oxLDL and oxLDL in samples were used to start a competitive inhibition reaction. The absorption was determined with a microplate spectrophotometer (Tecan, Switzerland) at 450 nm. Results were interpreted by constructing a dose/response curve according to the standards provided in the kit.

2.7. Liquid Chromatography SANIST Mass Spectrometry

Serum metabolites were measured with an untargeted liquid chromatography (LC)-MS SANIST platform. Preliminary MS biomarker identification was obtained based on the accuracy of the derived molecular-mass data (m/z, mass error: 10 ppm), and the ratios of the relative abundance of the different metabolites (i.e., "metabolite ratio") were calculated [16,17]. Thanks to the calculated ratios, metabolites were classified using an unsupervised method [16]. At 300 µL of serum, 600 µL of acetonitrile (CH3CN, Sigma Aldrich, Milan, Italy) was added to precipitate proteins, and centrifuged at 13,000 g for 10 minutes at room temperature. An aliquot of supernatant (600 µL) was collected and spiked with 100 µL of Ultramark polymer solution (10 ng/mL) to check the stability of the LC-MS signal during analysis. A SpeedVac (ThermoFisher, San Jose, CA, USA) was used to dry the remaining 300 µL; 100 µL of doubly distilled water/methanol (1:1, v/v; Sigma Aldrich, Milan, Italy) (1:1, v/v) solution was used to resuspend each sample, and 10 µL was added at internal standard reserpine (10 µL, final concentration 10 ng/mL, Sigma Aldrich, Milan, Italy). The samples were directly injected into the LC-MS apparatus. . A reverse-phase C-18 LC column (50 × 2.1 mm; particle size, 5 µm; pore size, 100 Å; Phenomenex, [Torrance, CA OR San Jose], USA) was employed. The mobile phases consisted of A) water (containing 0.2% formic acid (HCOOH, Sigma Aldrich, Milan, Italy), v/v), and B) CH3CN (100%) and the separation was achieved using an elution gradient. The flow rate was 0.25 mL/min, and the injection volume was 15 µL. Full-scan spectra were acquired in the mass range m/z 40–800. Tandem mass spectrometry (MS/MS) experiments on serum samples were performed with collision-induced dissociation using helium as collision gas. The precursor ions

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were isolated and fragmented (isolation windows, ±0.3 m/z; collision energy, 30% of its maximum value, which was 5 V peak to peak), and then product ions were obtained and analyzed by Orbitrap mass analyzer with a highly accurate m/z ratio (mass resolution m/z 15000; mass error <10 ppm). Compound identification was made based on fragmentation patterns and mass search database (NIST14) library following the European Union database match mode (EU directive 2002/657/EC) [18]. The chromatographic data were elaborated using the Xcalibur Quan Browser (Version 4.1.31.9). The SANIST data elaboration tool was used to predict the metabolomic pathways altered in the serum samples of participating subjects [19].

2.8. Statistical Analysis

Graphics and statistical analyses were performed using GraphPad Prism Software. The Figures show the single values at T0, T1 and T2. Y-axis reports the values of the analysed biomarker. Statistics are performed on comparison between the average of T0/T1 ± SEM, T1/T2 ± SEM T0/T2 ± SEM. Significance was calculated from mean±SD and analyzed through GraphPad Prism Software, using the Student's t-test, and one-way Anova. A value of $p<0.05$ was considered statistically significant. *p <0.05, **p < 0.01, ***p < 0.001, and ****p<0.0001.

3. Results

3.1. Anthropometric, Hematochemical, Inflammatory and Oxidative Parameters

A total of 29 participants were enrolled in this study; 17 were men, and the mean age was 57 years (ranging from 30 to 72 years). Six participants dropped out of the study after the 30-day time point for personal reasons, and 23 completed the study (average age: 59 years). For better visualization the parameters that showed significant differences, as well as those with an interesting trend, upon the intervention are reported in graphics (Figures 2–9; Figure S1-3).

The weight (kg) and BMI of participant did not increase and actually in female subjects decreased slightly but significantly both at 30 days (T1) and 60 days (T2), while no significant changes were observed in men's weight and BMI (Figure 2).

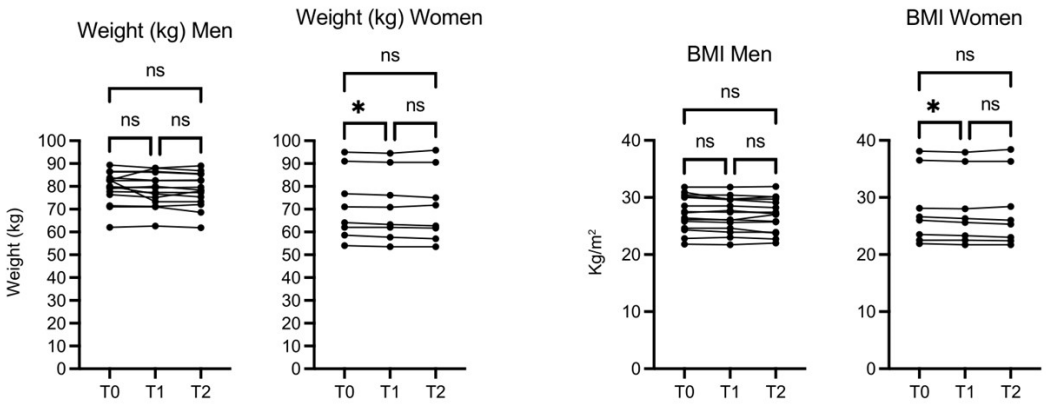

Figure 2. OMWW-OL assumption effects on weight and BMI in volunteers. The images show the single values at T0, T1 and T2. Y-axis reports the values of the analysed biomarker. Statistics are performed on comparison between the average of T0/T1 ± SEM, T1/T2 ± SEM T0/T2 ± SEM.

The circumference of the chest, wrist, arm, hip, calf, and abdomen did not change significantly after the intervention (data not shown). Women hydration was increased at T2 *vs* T0 ($p\leq0.01$), while hydration in men was increased slightly but not in a statistically significant manner (Figure 3).

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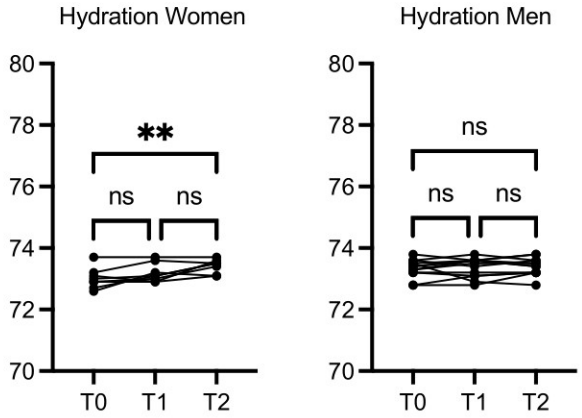

Figure 3. OMWW-OL assumption effects on hydration in volunteers. The images show the single values at T0, T1 and T2. Y-axis reports the values of the analysed biomarker. Statistics are performed on comparison between the average of T0/T1 ± SEM, T1/T2 ± SEM T0/T2 ± SEM.

Blood pressure decreased in a statistically significant manner, both for minimum and maximum values, at T2 *vs* T1, while T1 *vs* T0 and T2 *vs* T0 differences were not significant (Figure 4).

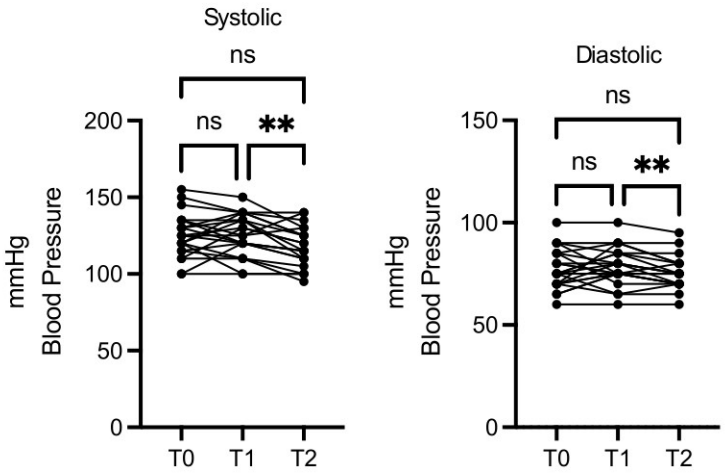

Figure 4. OMWW-OL assumption effects on blood pressure in volunteers. The images show the single values at T0, T1 and T2. Y-axis reports the values of the analysed biomarker. Statistics are performed on comparison between the average of T0/T1 ± SEM, T1/T2 ± SEM T0/T2 ± SEM.

There was also an interesting, although not significant, change in levels of LDL, HDL, and total cholesterol (Figure 5).

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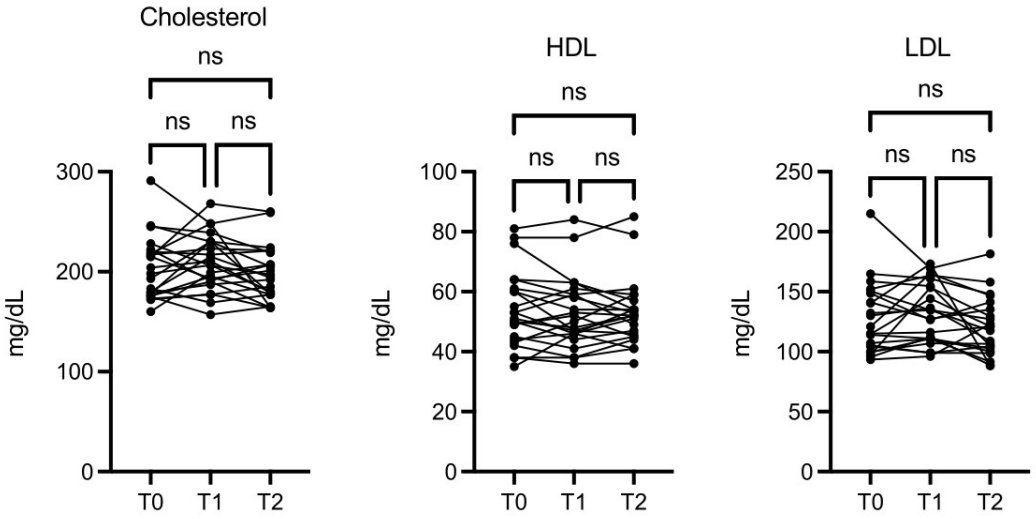

Figure 5. OMWW-OL assumption effects on cholesterol in volunteers. The images show the single values at T0, T1 and T2. Y-axis reports the values of the analysed biomarker. Statistics are performed on comparison between the average of T0/T1 ± SEM, T1/T2 ± SEM T0/T2 ± SEM.

Glycemia values were lower at T2 *vs* T0 ($p \leq 0.05$), and the decrease was more significant at T2 *vs* T1 ($p \leq 0.01$) (Figure 6). Insulin was also decreased at T2 *vs* T0 ($p = 0.05$) (Figure 6).

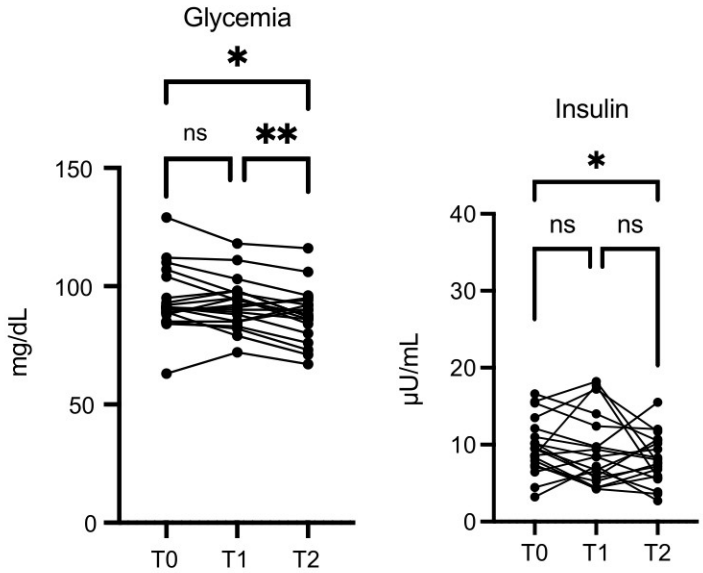

Figure 6. OMWW-OL assumption effects on glycemia and insulin in volunteers. The images show the single values at T0, T1 and T2. Y-axis reports the values of the analysed biomarker. Statistics are performed on comparison between the average of T0/T1 ± SEM, T1/T2 ± SEM T0/T2 ± SEM.

Vitamin D level was significantly increased at T1 *vs* T0 ($p \leq 0.05$), T2 *vs* T0 ($p \leq 0.001$), and T2 *vs* T1 ($p \leq 0.01$) (Figure 7). This was not due to seasonal variation. Calcium was also increased at T1 *vs* T0 ($p \leq 0.05$, Figure 7).

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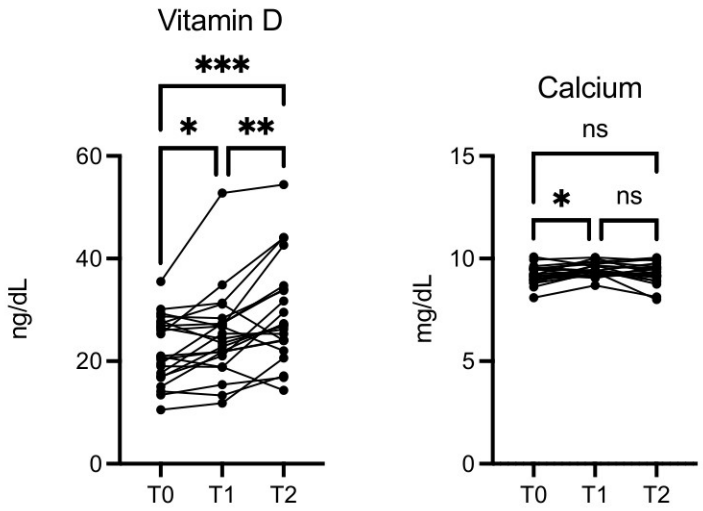

Figure 7. OMWW-OL assumption effects on vitamin D and calcium in volunteers. The images show the single values at T0, T1 and T2. Y-axis reports the values of the analysed biomarker. Statistics are performed on comparison between the average of T0/T1 ± SEM, T1/T2 ± SEM T0/T2 ± SEM.

The free triiodothyronine (FT3) level was significantly higher at T1 *vs* T0 and at T2 *vs* T0, while free thyroxine (FT4) was significantly higher only at T1 vs T0 (Figure 8). Thyroid-stimulating hormone (TSH) levels did not change significantly (data not shown).

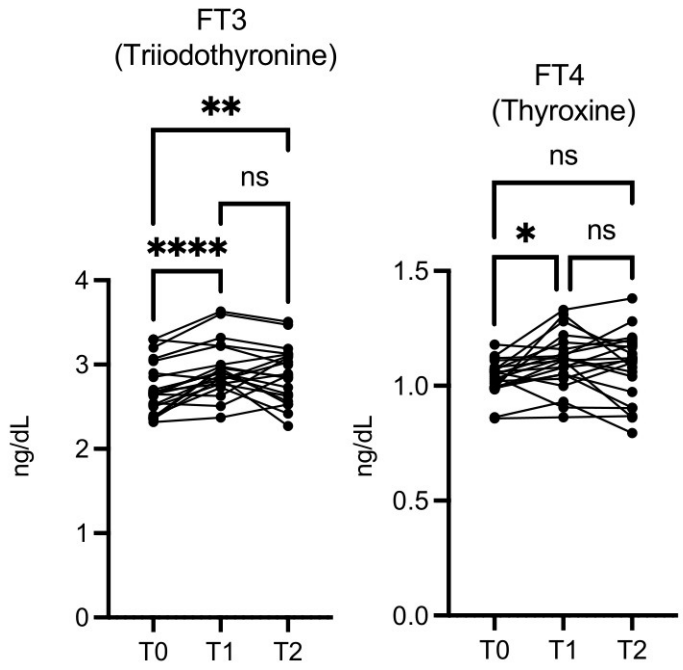

Figure 8. OMWW-OL assumption effects on thyroid hormones (T3 and T4) in volunteers. The images show the single values at T0, T1 and T2. Y-axis reports the values of the analysed biomarker. Statistics are performed on comparison between the average of T0/T1 ± SEM, T1/T2 ± SEM T0/T2 ± SEM.

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The albumin level was significantly increased at T1 *vs* T0 ($p \leq 0.001$) and T2 *vs* T0 ($p \leq 0.01$) (Figure 9).

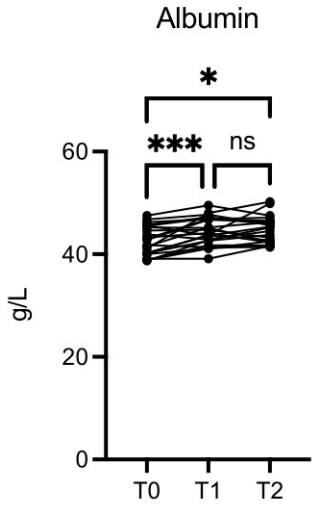

Figure 9. OMWW-OL assumption effects on albumin in volunteers. The images show the single values at T0, T1 and T2. Y-axis reports the values of the analysed biomarker. Statistics are performed on comparison between the average of T0/T1 ± SEM, T1/T2 ± SEM T0/T2 ± SEM.

Sarcopenia associated markers such as, skeletal muscle index (SMI), skeletal muscle mass (SMM), fat mass index (FMI), fat-free mass index (FFMI), apendicular skeletal muscle mss (ASMM) and basal metabolism (Mbasal), although not in a significant manner, showed a trend toward improvements (Figure S1). In our investigation transferrin, ferritin, and sideremia showed no significant differences (Figure S2). At the end of the intervention, liver enzymes, including alkaline phosphatase, and markers of kidney function (creatinine, urea, uric acid, osteocalcin) (data not shown), and magnesium (Figure S3) did not show variations. Potassium significantly decreased at T2 *vs* T1 (Figure S3).

The values of oxidative stress parameters (TEAC, MDA and PSH) were not significantly different between the time points considered (data not shown).

In general the data show that the vital parameters remain in the standard range, there is no detrimental effect with some positive encouraging trends to health benefit.

3.2. Mass Spectrometry Serum Characterization

SANIST profiling revealed three chromatographic peak-area ratios, correlating them with enzyme functionality alteration. The detected potential biomarkers were identified using the approach shown in Figure 10.

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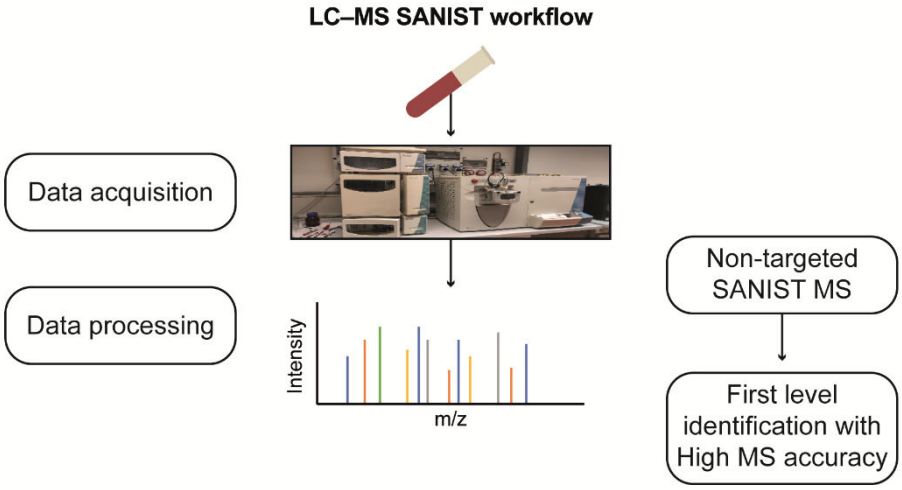

Figure 10. Workflow for non-targeted LC-MS SANIST metabolites profiling.

Mass spectra comparisons for LC-MS data obtained with the NIST library are shown in Figure 11. SANIST technology [16] was used to obtain and compare metabolomics profiles. An Ultimate 3000 UPLC (ThermoFisher) LC system fitted with an Orbitrap mass spectrometer (ThermoFisher) coupled to a surface-activated chemical ionization (SACI)/ESI source was used to analyze metabolic profiling in both ESI-positive and ESI-negative ion modes.

The ion at m/z 150 → 133 (loss of a –OH group) (red) was identified by NIST as methionine (C5H11NO2S) present in the library spectrum (blue), with a 92% identification score (direct match factor: 885; reverse match factor: 935) (Figure 11A). Methionine was confirmed by comparison to LC and MS/MS data acquired for the certified analytical standard, following the EU directive (EU directive 2002/657/EC). The ions m/z 4.0 → 381 and 112 → 95 were identified using the same approach and corresponding to calcitriol and histamine, respectively (Figure 11B and C). We also identified homocysteine with ion at m/z 136 → 90 (Figure 11D).

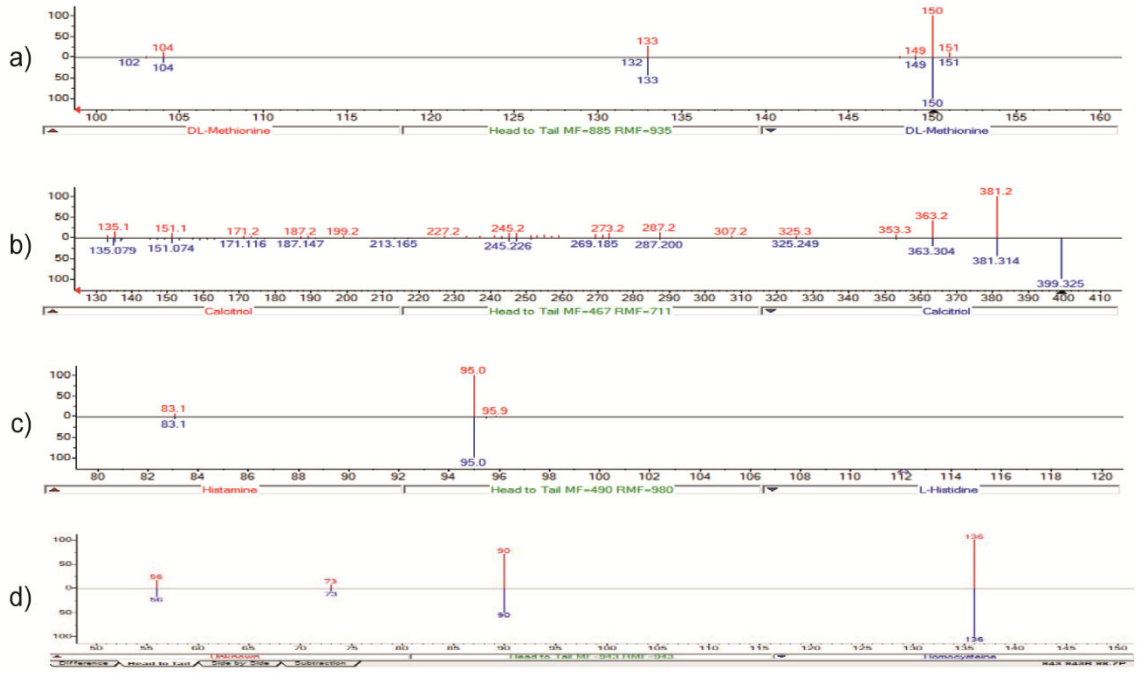

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Figure 11. Biomarkers detected by NIST MS Search Program.

Furthermore, we observed a peak at m/z 371 (data not shown), but the corresponding metabolite was not identified using both an MS and an MS/MS database search. However, this unknown metabolite is very interesting because the similarity approach in MS/MS search appears to be highly similar to a peptide molecule. Due to the absence of a commercial internal standard, verifying the data using EU Directive 2002/657/EC was not possible. In order to determine whether the metabolite at m/z 371 was present in the dietary supplement, OMWW-OL was analyzed using the LC-SANIST-MS/MS method. Nonetheless, it was evidently absent from the analysis of 10 OMWW-OL samples.

We observed a 10-fold increase in the homocysteine/methionine ratio (m/z 134/150) ($p=4 \times 10–5$), the vitamin 25 (OH) D3/histamine ratio (m/z 401/112) ($p=5 \times 10–5$) and the vitamin 25 (OH) D3/not identified metabolite ratio (m/z 401/371) ($p=6 \times 10–5$) with respect to T0.

The mass spectrometry data show very promising beneficial effects on metabolites with the discovery of a new molecule induced during treatment.

4. Discussion

EVOO is an essential Mediterranean diet component rich in bioactive compounds [20]. Several studies suggest the role of its consumption in reducing the incidence of different diseases, such as cancer, cardiovascular pathologies, type 2 diabetes, and neurodegenerative disorders [21–25]. Every year, olive tree cultivation and oil extraction generate a large amount of byproducts, primarily OMWW, which can have a negative environmental impact. However, OMWW is rich in phenolic compounds displaying potent antioxidant, anti-inflammatory, vascular protective, and anti-aging properties [26–29] and could potentially be useful in the pharmaceutical and food industries [20,28,30–35].

Here, we analyzed the effects of OMWW in the dietary supplement preparation [Oliphenolia®, OMWW-OL) on a cohort of volunteers with characteristics close to metabolic syndrome. Our data showed that OMWW-OL intake for 30 days had no negative effects on liver and kidney function and did not affect magnesium levels and iron absorption, transport, and storage. Several studies observed that diets, including daily EVOO, are effective for weight loss [36–38]; however, data on women were still lacking. Interestingly, our work found that OMWW-OL was associated with a small decrease in female participants' weight and BMI even in a short time. Furthermore, the finding that hydration was significantly increased in females is a very promising result, as dehydration can significantly impair cognitive and physical abilities and has been associated with obesity, chronic diseases, and decreased longevity [39,40]. OMWW-OL might have the potential to ameliorate the state of hydration and, consequently, improve the metabolism, weight, BMI, and aging process, especially in females. Interestingly, hydration, weight, and BMI are more regulated in women than men, and in accordance with recent literature, including from our group, gender differences in aging parameters were revealed [41].

In agreement with several researches, we found that OMWW-OL reduces blood pressure. This effect is probably related to its high polyphenol content [42,43], which would also confer potential cardioprotective properties, as observed for EVOO [44]. We also observed a glycemia decrease after OMWW-OL supplementation in our population. This is consistent with the results obtained by Violi et al. [45], who showed that supplementation of EVOO at lunch is linked to lower post-prandial glycemic levels. Although at a non-significant level, cholesterol and LDL showed a decreasing trend while insulin decreased significantly. Besides its role in diabetes, insulin increases cancer risk by promoting the proliferation of normal, preneoplastic, and neoplastic cells [46]. By decreasing glycemia and insulin levels, OMWW-OL could play a role in preventing metabolic syndrome and cancer.

After nutritional intervention with OMWW-OL, we observed an increase in the FT3 and FT4 serum levels while TSH levels remained stable. Since low FT3 levels are associated with aging [47], the ability of OMWW-OL to increase FT3 and FT4 supports the potential anti-aging effect shown in a previous publication of one of our institutions [48]. In this study, we also observed a significant

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increase in albumin values. Albumin is involved in many bioactive functions, such as extracellular antioxidant defenses [49] and the transport of various ligands [50,51]. Decreases in albumin levels are associated with an inflammatory state [52] and are observed in cancer, infections, malnutrition, liver illness, and kidney disease [53]. Conversely, high albumin concentrations may protect against early glycemic deterioration and type 2 diabetes progression [54]. Therefore, the increased albumin levels observed with OMWW-OL intake may correlate to the anti-inflammatory activity of this olive byproduct and may contribute to metabolic syndrome prevention.

Since we observed a potassium reduction, patients with chronic kidney disease, who are typically recommended to reduce potassium consumption or eliminate it from their blood [55–57], might find this result especially significant. Given the absence of any relevant kidney toxicity in the short time of supplementation, OMWW might contribute to maintaining safe potassium levels in patients with chronic kidney disease.

Several works have observed that vitamin D appears to be effective therapeutic agents to prevent the production of reactive oxygen species and stop cytokine-mediated inflammation [58–61]. However, the literature does not provide much information on the impact of natural compounds on endogenous vitamin D levels. Our finding that a significant increase in vitamin D is achieved by supplementing with OMWW-OL supports a previous observation that *in vitro* digestion using olive oil resulted in higher absorption of the vitamin D precursor, calcifediol, than using other types of edible oils [62]. Since the nutritional intervention also led to increased calcium levels, our results suggest that OMWW-OL may promote calcium balance either by promoting calcium absorption directly or by increasing vitamin D. Calcium is essential for preserving physiological functions and preventing aging by promoting bone mineralization and sustaining heartbeat, blood clotting, and muscle and neuron function [63–66]. Naturally, calcium levels decline as we age, and calcium deficiency has been increasingly linked to aging [64,65].

Sarcopenia, which is an age-related reduction in muscle mass, muscle strength, and/or diminished physical function [67], could also be controlled from the intervention with OMWW-OL. The supplementation with OMWW-OL was able to modulate, although at a non-significant level, a decrease in the sarcopenia-associated marker, FM, FFM and ASMM.

Further insights into the dietary intervention were achieved through MS, and differences were observed in the methionine cycle, which involves the regeneration of methionine from homocysteine [68]. The low homocysteine/methionine ratio found in our metabolomic analysis could be associated with active homocysteine metabolism, which is involved in the effective prevention of both oxidative stress and infectious diseases [69]. Elevated homocysteine levels increase the frequency of cardiovascular disease [70–74] with which metabolic syndrome is associated [75]. Therefore, also according to the decrease cardiovascular risk previously observed for olive oil [76], OMWW-OL could reduce cardiovascular risk by lowering homocysteine levels.

The absence of the metabolite at m/z 371.192 in OMWW-OL suggests that the detection of this metabolite *in vivo* is due to reduced vitamin D25 oxidation. Therefore, the potential antioxidant activity of OMWW-OL may preserve vitamin D25 and its functionality. Interestingly, the vitamin D/histamine ratio is also high, suggesting a decrease in histamine, an inflammatory mediator [77–79]. Since vitamin D regulates the immune system, particularly mast cells that carry histamine, the decrease in histamine observed with OMWW-OL intervention could be a secondary effect of the vitamin D increase [80,81].

5. Conclusions

Here, we have shown that OMWW-OL has no detrimental effect on a variety of parameters, and also that in a preliminary way the study indicates the potential to improve biological parameters in volunteers at risk for metabolic syndrome, according to a trend towards a favorable modulation of several biological parameters. The absence of significant variations in most hematochemical parameters indicates that regular Oliphenolia® ingestion has no adverse effects on lipid levels, liver or kidney function.

Some aging-associated parameters were modulated favorably. In addition to extending the period without needing treatment and slowing the aging process, it could be hypothesized that OMWW-OL could help prevent or postpone the onset of age-related disorders, improving quality of life and reducing healthcare costs. MS data revealed improved vitamin D/histamine, homocysteine/methionine, and vitamin D/unknown metabolite ratios.

One of the study's limitations is the limited sample size; therefore, the information must be considered preliminary, although encouraging, and needs to be strengthened in further research. Our data suggest that OMWW-OL may also have a promising effect in protecting cells from reactive oxygen species-induced injuries due to its high polyphenol content and vitamin D-boosting effect. The possibility of targeting aging instead of treating age-related pathologies is very attractive, as it can improve the quality of life and ensure longevity. This approach would align with positive biology, which researches the biological mechanisms involved in well-being [82]. Some of these parameters could also be relevant in cancer prevention or interception.

Future studies are needed to better elucidate the structure of the potential new metabolite and to investigate biological correlates.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Effects of OMWW-OL assumption on muscle and sarcopenia parameters in volunteers, Figure S2: Effects of OMWW-OL assumption on iron parameters in volunteers, Figure S3: Effects of OMWW-OL assumption on potassium and magnesium values in volunteers.

Author Contributions: A Aiello: clinical studies, LC: mass spectrometry, LC, DMN, PC: manuscript editing and illustration, SN: data analysis and editing, SC: mass spectrometry and metabolites identification, GA, GC, CC, AZ: study design and clinical studies, A Albini: manuscript drafting, and coordination.

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Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

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