Insight into the Molecular Signature of Skeletal Muscle Characterizing Lifelong Football Players

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Abstract: Background: Aging and sedentary behavior are independent risk factors for non-communicable diseases. An active lifestyle and structured physical activity are positively associated with a healthier quality of life in the elderly. Here, we explored the proteomic/metabolomic muscular signature induced by lifelong football training associated with successful aging. Methods: The study was performed on nine lifelong football players (67.3 ± 2.8 yrs) and nine aged-matched untrained subjects. We performed a proteomic/metabolomic approach on V. lateralis muscle biopsies; the obtained data were analyzed by means of different bioinformatic tools. Results: Our results indicated that lifelong football training is able to enhance the muscles’ oxidative capacity in the elderly by promoting fatty acids as preferential energetic substrates and hence determining a healthier body composition and metabolic profile; furthermore, we showed that the total polyamine content is higher in lifelong football players’ muscle, enforcing the involvement of polyamines in muscle growth and hypertrophy. Conclusions: Lifelong football training, as a structured physical activity, significantly influences the expression of the proteins and metabolites involved in oxidative metabolism and muscle hypertrophy associated with successful aging.

Keywords: lifelong football training; successful aging; mitochondrial oxidative capacity; polyamines

1. Introduction

Aging is a continuous physiological process of the human body that begins in early adulthood with the onset of multiple changes at the cell/tissue level (cellular homeostasis alteration, DNA accumulation and protein damage) [1–3], eventually leading to a functional decline in organs/systems [4–6]. In fact, this structural and functional decay affects the cardiovascular, the musculoskeletal and the nervous system, initiating various age-related non-communicable diseases (NCDs), such as Type 2 diabetes mellitus (T2DM), hypertension, insulin resistance (IR), cognitive decline and Alzheimer’s disease [7,8]. Another independent risk factor for major NCDs is extensive sedentary behavior (SB), which is defined as any waking behavior characterized by an energy expenditure of 1.5 METs.
(metabolic equivalent of task, corresponding to the amount of oxygen consumed at rest) or less while sitting, lying, or leaning [9–11]. Many older adults are less active than recommended [12]. However, SB does not mean “physical inactivity”. In fact, older people can meet the current recommendations for moderate to vigorous physical activity (MVPA) but be sedentary in other daily activities; on the contrary, they could have a low SB while not being engaged in any structured PA [10,13]. NCDs due to aging and SB can be counteracted through an active lifestyle and planned PA, which are positively associated with a better quality of life in the elderly. Several studies have reported that individuals who engage in PA age successfully, characterized by a low risk of age-related disabilities in comparison with age-matched sedentary adults [14,15]. Regular physical activity, in fact, exerts its effects on metabolic regulation and the maintenance of metabolic homeostasis [16], especially when the WHO’s guidelines, which recommend both aerobic and resistance exercise, are met [3].

In this context, football training represents an intermittent exercise that includes millions of active players of different ages, genders and pathophysiological conditions around the world [17]. Football training, in fact, is characterized by high intensity anaerobic actions interspersed with periods of low-intensity recovery [18]. For this reason, it is an effective tool to stimulate physiological and molecular adaptations leading to an improved health status at all ages, from young to middle-aged and older people [19–22]. As demonstrated, long-term recreational football training improves cardiovascular, skeletal muscle and metabolic fitness, and health-related body composition parameters. Moreover, it increases the expression of key proteins involved in oxidative metabolism, mitochondrial biogenesis, DNA repair, autophagy and protein quality control through the regulation of specific miRNAs, such as miR-1303 [23], together with heat shock proteins (HSP), such as HSP 70/90 and the components of the proteasome protein complex [21,24–26]. Although several molecular markers involved in the adaptation of skeletal muscle to PA and in protective mechanisms against age-related NCDs have been unveiled over the past decade, many questions have yet to be resolved. Here, we explored the different muscular signatures from veteran soccer players (VPG) vs. age-matched active untrained healthy subjects (control group, CG) at the molecular level. We reported the results of a proteomic/metabolomic approach performed on V. lateralis muscle biopsies in order to highlight the molecular mechanisms that characterize successful aging through lifelong football training.

2. Materials and Methods

2.1. Subjects

The study was performed on 18 healthy male volunteers aged 64–71 years, who enrolled at the University of Copenhagen. Nine were football players who, in the last 10 years, had trained for one session per week (1.5 ± 0.6 h/session) and played in 26 ± 12 football matches (2 × 35 min) per year in local football clubs in Copenhagen; they constituted the veteran football player group (VPG; 67.3 ± 2.8 yrs). The control group (CG; 66.5 ± 1.6 yrs) were nine healthy aged-matched active untrained men. For both groups, the exclusion criteria were a history or symptoms of cardiovascular disease or cancer, Type 2 diabetes, hypertension, nephropathy or musculoskeletal complaints, whereas the inclusion criterion was the ability to perform all the tests required for study participation as previously described [21,25,26]. The anthropometric, biochemical and clinical parameters of the participants are shown in Table 1 and were evaluated as previously reported [21,25,26]. Moreover, the body composition, maximal oxygen uptake (VO$_2$max) and resting heart rate (RHR; bpm) were measured for all subjects as described by Mancini and colleagues [21,25,26]. All subjects were informed about any potential discomforts or risks related to the experimental protocol and gave their informed written consent to participate in the study. This was conducted according to the Declaration of Helsinki and was approved by the local ethics committee of the University of Copenhagen (H-1-2011-013, ClinicalTrials.gov Identifier: NCT01530035).
Table 1. Anthropometric, biochemical and clinical parameters of the subjects participating in the study.

| Parameter                        | VPG                | CG                 |
|----------------------------------|--------------------|--------------------|
| Number of subjects               | 9                  | 9                  |
| Age (yrs)                        | 67.3 ± 2.8         | 66.5 ± 1.6         |
| Height (cm)                      | 179.2 ± 2.2        | 175.0 ± 5.2        |
| Body weight (kg)                 | 79.3 ± 6.4         | 92.7 ± 14.6        |
| BMI (kg/m²)                      | 24.7 ± 1.7 *       | 29.6 ± 4.3         |
| Fat mass (%)                     | 22.9 ± 6.5 *       | 33.4 ± 5.0         |
| Lean mass (kg)                   | 57.3 ± 1.9         | 57.9 ± 7.6         |
| VO₂max (mL/min/kg)               | 34.8 ± 1.5 **      | 25.2 ± 3.1         |
| Fasting blood glucose (mmol/L)   | 5.3 ± 0.4          | 5.5 ± 0.5          |
| Total cholesterol (mmol/L)       | 5.4 ± 1.4          | 5.4 ± 0.5          |
| HDL cholesterol (mmol/L)         | 1.6 ± 0.8          | 1.2 ± 0.1          |
| LDL cholesterol (mmol/L)         | 3.1 ± 1.2          | 3.3 ± 0.5          |
| Triglycerides (mmol/L)           | 0.9 ± 0.5          | 1.1 ± 0.4          |
| Systolic BP (mmHg)               | 124.7 ± 16.4       | 131.2 ± 12.9       |
| Diastolic BP (mmHg)              | 70.2 ± 4.2         | 74.7 ± 10.1        |
| Mean arterial pressure (mmHg)    | 88.3 ± 7.7         | 93.5 ± 10.7        |
| Resting heart rate (bpm)         | 49.2 ± 5.9         | 61.0 ± 14.5        |

Values are reported as the mean ± SD. **p < 0.001; *p < 0.05.

2.2. Muscle Sample Collection and Preparation

Before muscle biopsy, all recruited subjects observed an overnight fast. Muscle biopsies were obtained from the vastus lateralis under local anesthesia by using the Bergstrom technique [21]. The muscle samples were immediately frozen in liquid nitrogen and stored at −80 °C until subsequent analysis. Muscle biopsies were homogenized in a buffer containing 150 mM NaCl, 1% Triton, 5 mM EDTA, 50 mM Tris-HCl and the Complete Mini protease inhibitory cocktail. After 30 min of incubation on ice, the samples were clarified by centrifugation at 16,000× g for 30 min at 4 °C. The protein concentration was determined using Bradford’s reagent (Bio-Rad, Hercules, CA, USA).

2.3. Electrophoretic Separation and In-Gel Digestion

For each group (VPG and CG), the nine protein samples were pooled, incubated for 5 min at 95 °C and separated by 10% SDS-PAGE. Gel bands were stained with Colloid Blue Stain Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s procedure. Gel images were acquired using the scanner GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA). The whole gel lanes were manually cut into 2 mm gel slices and further processed as previously described [27]. In particular, each slice was first washed with acetonitrile (ACN) and then with 50 mM ammonium bicarbonate. Protein bands were reduced by incubation with 10 mM DTT for 45 min at 56 °C and alkylated in 55 mM iodoacetamide for 30 min in the dark at room temperature. Enzymatic digestion was carried out with 10 ng/µL of modified trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate for 45 min at 4 °C. Peptide mixtures were extracted as previously reported and resuspended in 0.2% (v/v) formic acid for mass spectrometry (MS) analysis.

2.4. LC-MS/MS Analysis, and Protein Identification and Quantitation

Mass spectrometry analysis was performed by using an LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany) equipped with the nanoEASY II Nanoseparations chromatographic system (75 µm – L, 20 cm column, Thermo Scientific, Bremen, Germany) as previously described [28]. The peptide analysis was performed at a resolution of 30,000 and used the data-dependent acquisition of an MS scan (400–1800 m/z) followed by MS/MS scans of the five most abundant ions. Raw MS/MS data in Mascot format text (mgf) were processed by the Proteome Discoverer platform (version 1.4, Thermo Fisher Scientific,
Waltham, MA, USA) interfaced with an in-house Mascot server (version 2.3, Matrix Science, London, UK). Proteins were identified with the following search parameters: UniProt as the database, limited to the Homo sapiens taxonomy; trypsin as a specific proteolytic enzyme; 1 missed cleavage allowed; 10 ppm precursor tolerance and a 0.6 Da fragment ion tolerance; carbamidomethylation of cysteine as a fixed modification; conversion of N-terminal glutamine to pyro-glutamic acid and oxidation of methionine as variable modifications. Only proteins with at least 2 assigned peptides with an individual Mascot score $> 19$ were considered to be significant. For the label-free quantitative analysis, spectral counts (SpC) values were used to estimate the proteins’ abundance, as previously reported [29].

In order to perform a semi-quantitative comparative analysis, the protein abundances in each of the proteomes considered were expressed as Rsc, calculated according to the following formula:

$$
\text{Rsc} = \log_2 \left[ \frac{(n2 + f)}{(n1 + f)} \right] + \log_2 \left[ \frac{(t1 - n1 + f)}{(t2 - n2 + f)} \right]
$$

Specifically, Rsc is the log ratio of abundance between Samples 1 (VPG) and 2 (CG); $n1$ and $n2$ are the SpCs for the given protein in Groups 1 and 2, respectively; $t1$ and $t2$ are the total number of spectra of all proteins in the two sample groups; and $f$ is a correction factor set to 0.5 that was used to eliminate discontinuity due to SpC = 0 [30].

In this study, proteins with Rsc $\geq 1.40$ or $\leq -1.40$ were considered to be differentially represented in VPG versus CG groups. The mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium (available at http://www.proteomexchange.org, accessed on 28 October 2022) via the PRIDE [31] partner repository with the dataset identifier PXD037792.

2.5. Bioinformatic Analysis

The differentially expressed proteins identified here were classified according to the Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.7, http://david.abcc.ncifcrf.gov/, accessed on 11 November 2020). Based on Fisher’s exact test, the DAVID tool determines the protein enrichment in the Gene Ontology (GO) annotation terms, particularly biological processes. Only annotation categories with a $p$-value of $\leq 0.05$ were considered to be significant. Moreover, the Search Tool for the Retrieval of Interacting Genes (STRING) (version 11, http://string-db.org/, accessed on 11 November 2020) was used for generating the protein–protein interaction networks. The STRING tool imports known and predicted protein–protein associations including physical and indirect interactions. An interaction score of 0.7 (high confidence) was set, considering the following sources: neighborhood, co-occurrence, co-expression, experiments and databases. STRING was also used to analyze the most statistically significant and non-redundant biological processes among the differentially expressed proteins from the proteomic dataset. Only annotation biological processes with a false discovery rate (FDR) $\leq 0.05$ were considered significant. In addition to the identification of functional annotations and biological networks, a pathway analysis was also performed according to the Reactome database (https://www.reactome.org, accessed on 11 November 2020).

2.6. Western Blotting Analysis

Protein extracts (30 µg) from three independent replicates of the VPG and CG were resolved on a 4–20% precast gradient of polyacrylamide gels (Bio-Rad) and then transferred onto nitrocellulose membranes by using the Bio-Rad Trans-Blot Turbo apparatus. The membranes were analyzed by Western blotting as previously described [25] and incubated with the following primary antibodies: rabbit anti-CPT1B (1:1000, Abcam, Cambridge, UK) and mouse anti-ACAA2 (1:500, Santa Cruz, Dallas, TK, USA). A mouse anti-GAPDH (Sigma-Aldrich, St. Louis, MI, USA) antibody was used as a loading control at a dilution of 1:500. Immunoblot detection was carried out using horseradish peroxidase-conjugated anti-mouse (1:5000) or anti-rabbit (1:5000) secondary antibodies (GE Healthcare, Chicago,
IL, USA) and the enhanced chemiluminescence advanced Western blotting detection kit (GE Healthcare). The signals were visualized by X-ray film exposure. Digital images were acquired by GS-800 calibrated densitometer scanning (Bio-Rad). Densitometric measurements were made with Image J2 software; in particular, the raw densitometry signal of each protein band was quantified and normalized against the corresponding GAPDH band. The results were shown as the percentage of the mean of controls and statistically evaluated by Student’s t-test (p-values < 0.05).

2.7. MS-Based Metabolite Profiling

For metabolite extraction, muscle biopsies from the VPG and CG were homogenized in 1 mL of 50:50 cold methanol/0.1 M HCl and then centrifuged at 15,000 × g for 30 min at 4 °C to recover the supernatant containing the metabolites; whereas proteins were extracted from the pellet to estimate the protein concentration as previously described [32]. The metabolite mixture was dried under nitrogen and analyzed by MS/MS to evaluate the amino acid (AA) and acylcarnitine (AC) levels. The metabolite sample was resuspended in methanol containing standard mixtures of labeled AA and AC, incubated for 20 min at room temperature and dried under nitrogen [33]. The metabolites were esterified with 3N HCl/n-butanol at 65 °C for 25 min. The derivatized samples were dried again under nitrogen and resuspended in 300 µL of acetonitrile/water (70:30) containing 0.1% formic acid. For the MS/MS analyses, 100 µL of three independent aliquots of each sample was injected in the API 4000 triple quadrupole mass spectrometer (Applied Biosystems-Sciem, Toronto, ON, Canada) coupled with an 1100 series Agilent high-performance liquid chromatography system (Agilent Technologies, Waldbronn, Germany). MS/MS analyses for AAs and ACs were performed according to parameters that have been previously described [33]. The MS/MS data were quantitatively analyzed by comparing the metabolites and the corresponding internal standard areas using ChemoView v1.2 software. The AA and AC contents were normalized against the total amount of protein from the cellular extract. MetaboAnalyst 4.0 (http://www.metaboanalyst.ca, accessed on 15 January 2021) was used to perform the multivariate statistical analysis. The metabolome dataset, including 50 metabolites’ concentrations, was log10-transformed and scaled according to the Pareto scaling method. Partial least squares-discrimination (PLS-DA) was carried out to evaluate the dataset’s homogeneity and estimate the variable importance in projection (VIP). VIP is the weighted sum of squares of the PLS loadings, taking the amount of variation explained in each dimension into account. Univariate statistical analysis was carried out using GraphPad Prism 9.0, and the results are presented as the mean ± standard error of the mean (SEM). The statistical significance of the difference in the metabolite samples’ concentrations between two different groups was evaluated by parametric (unpaired t-tests with Welch correction) or non-parametric (Mann–Whitney tests) tests. The normal distribution was verified according to D’Agostino and Pearson’s test.

2.8. Polyamine Assay

The total polyamine content in muscle biopsies from the VPG and CG was measured by using a fluorimetric assay kit (Abcam, Cambridge, UK), according to the manufacturer’s protocol. Briefly, muscle biopsies were homogenized on ice using a Dounce homogenizer and centrifuged at 10,000 × g for 5 min at 4 °C to collect the supernatants containing the metabolites. The samples were treated with a sample clean-up reagent and then filtered through a 10 kDa spin column to avoid common metabolites other than polyamines interfering with the assay. The assay kit includes a selective enzyme mix that acts on polyamines to generate hydrogen peroxide and includes a specific fluorometric probe to detect a fluorescence signal proportional to the amount of polyamine after the reaction of the hydrogen peroxide produced. According to the manufacturer’s protocol, the samples were prepared in a black 96-well plate and the fluorescence was read using a Perkin Elmer Enspire plate reader (Perkin Elmer, Waltham, MA, USA) at 535 and 587 nm as the excitation and emission wavelengths, respectively. The total polyamine content was quantified
using a calibration curve established with known amounts of polyamine standards. The experiments were performed using five independent biological replicates, each with three technical repeats.

3. Results

3.1. Identification of Differentially Expressed Proteins in the Skeletal Muscle from Veteran Football Players (VPG) versus Untrained Subjects (CG)

To investigate whether lifelong football training affects the protein expression profiles in the skeletal muscles of the lower limb, a label-free differential proteomic study was carried out to analyze muscle biopsies from the *V. lateralis* of 18 healthy male volunteers, nine belonging to the veteran football player group (VPG) and nine healthy age-matched untrained subjects (control group, CG), enrolled at the University of Copenhagen. The two groups showed no differences in their anthropometric, biochemical and clinical parameters, but the BMI (24.7 ± 1.7 kg/m² vs. 29.6 ± 4.3 kg/m², p < 0.05) and body fat percentage (22.9 ± 6.5% vs. 33.4 ± 5.0%, p < 0.05) were significantly lower and the VO₂max (34.8 ± 1.5 mL/min/kg vs. 25.2 ± 3.1 mL/min/kg, p < 0.001) was significantly higher in the VPG than in the CG (Table 1). After extraction, muscle protein mixtures from the VPG were pooled and separated by monodimensional SDS-PAGE; the same procedure was applied to muscle protein mixtures from the CG (Figure S1). Coomassie-stained protein bands were excised, in-gel digested and analyzed by LC–MS/MS, and their raw data were further analyzed by the Proteome Discoverer platform to allow identification and quantification of the proteins. We identified 873 unique proteins, which are listed in Table S1 along with their MS details. To define a map of the differentially expressed species between the VPG and CG, a label-free quantitative analysis was performed based on the MS spectral counts of the identified proteins. We found 188 differentially expressed proteins in the VPG versus CG; Tables 2 and 3 list the 92 overexpressed and 96 underexpressed species, respectively. For each protein, the Uniprot accession code, the description, the gene name and the Rsc value are reported.

Table 2. Label-free quantitative analysis of proteins identified from *V. lateralis* muscle biopsies. Overexpressed proteins with Rsc ≥ 1.40 in the VPG vs. CG are shown.

| Accession | Description | Gene | Rsc |
|-----------|-------------|------|-----|
| Q9NQX4    | Unconventional myosin-Vc | MYO5C | 4.51 |
| Q9UNM6    | 26S proteasome non-ATPase regulatory subunit 13 | PSMD13 | 3.72 |
| P02774    | Vitamin D-binding protein | GC | 3.72 |
| Q9NX40    | OCIA domain-containing protein 1 | OCIAD1 | 3.43 |
| P04632    | Calpain small subunit 1 | CAPNS1 | 3.43 |
| P50991    | T-complex protein 1 subunit delta | CCT4 | 3.43 |
| Q8NBN7    | Retinol dehydrogenase 13 | RHD13 | 3.43 |
| Q5HYK3    | 2-Methoxy-6-polypropyl-1,4-benzoquinol methylase, mitochondrial | COQ5 | 3.43 |
| O75891    | Cytosolic 10-formyltetrahydrofolate dehydrogenase | ALDH1L1 | 3.43 |
| P11532    | Dystrophin | | 3.43 |
| O95182    | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 7 | NDUF47 | 3.07 |
| Q9UFN0    | Protein NipSnap homolog 3A | NIPSAP3A | 3.07 |
| P55039    | Developmentally regulated GTP-binding protein 2 | DRG2 | 3.07 |
| Q9NUJ1    | Mycophenolic acid acyl-glucuronide esterase, mitochondrial | ABHD10 | 3.07 |
| Q9H9P8    | L-2-hydroxyglutarate dehydrogenase, mitochondrial | L2HGDH | 3.07 |
| O43464    | Serine protease HTRA2, mitochondrial | HTRA2 | 3.07 |
| Q15818    | Inorganic pyrophosphatase | PPA1 | 3.07 |
| O15399    | Glutamate receptor ionotropic, NMDA 2D | GRIN2D | 3.07 |
| O60229    | Kalirin | KALRN | 3.07 |
| Q00G26    | Perilipin-5 | PLIN5 | 3.07 |
| Q15203    | Myosin-binding protein H | MYBPH | 3.07 |
| Q8N3L3    | Beta-taxilin | TXLNB | 2.58 |
| P49773    | Histidine triad nucleotide-binding protein 1 | HINT1 | 2.58 |
| P21964    | Catechol O-methyltransferase | COMT | 2.58 |
Table 2. Cont.

| Accession | Description                                      | Gene          | Rsc  |
|-----------|--------------------------------------------------|---------------|------|
| P61353    | 60S ribosomal protein L27                         | RPL27         | 2.58 |
| Q15404    | Ras suppressor protein 1                          | RSU1          | 2.58 |
| Q9UPY8    | Microtubule-associated protein RP/EB family member 3 | MAPRE3        | 2.58 |
| Q8WU1     | Protein THEM6                                     | THEM6         | 2.58 |
| O9608     | Mitochondrial import receptor subunit TOM40 homolog | TOMM40        | 2.58 |
| Q9NQ50    | 395 ribosomal protein L40, mitochondrial          | MRPL40        | 2.58 |
| O00743    | Serine/threonine-protein phosphatase 6 catalytic subunit | PPP6C        | 2.58 |
| P15880    | 40S ribosomal protein S2                          | RPS2          | 2.58 |
| Q5T3H0    | G patch domain-containing protein 4               | GPATCH4       | 2.58 |
| Q7Z3D6    | UPF0317 protein C1orf159, mitochondrial           | DGLUCY        | 2.58 |
| P54868    | Hydroxymethylglutaryl-CoA synthase, mitochondrial | HMGCS2        | 2.58 |
| Q13555    | Calcium/calmodulin-dependent protein kinase type II subunit gamma | CAMK2G        | 2.58 |
| Q96N2     | GPI transamidase component PIG-T                 | PIGT          | 2.58 |
| Q92523    | Carnitine O-palmitoyltransferase 1, muscle isoform | CPT1B         | 2.58 |
| Q13554    | Calcium/calmodulin-dependent protein kinase type II subunit beta | CAMK2B       | 2.58 |
| Q96AQ6    | Pre-B-cell leukemia transcription factor-interacting protein 1 | PBX1P1        | 2.58 |
| P43243    | Matrin-3                                         | MATR3         | 2.58 |
| P07384    | Calpain-1 catalytic subunit                       | CAPN1         | 2.58 |
| Q02641    | Voltage-dependent L-type calcium channel subunit beta-1 | CACNB1       | 2.58 |
| Q9NV11    | Fanconia anemia Group I protein                   | FANCI         | 2.58 |
| O14936    | Peripheral plasma membrane protein CASK           | CASK          | 2.58 |
| P39059    | Collagen alpha-1 (XV) chain                      | COL15A1       | 2.58 |
| Q92900    | Regulator of nonsense transcripts 1               | UFP1          | 2.58 |
| Q9Y490    | Talin-1                                          | TLN1          | 2.58 |
| Q8NEZ4    | Histone-lysine N-methyltransferase 2C             | KMT2C         | 2.58 |
| Q9NZJ6    | Hexaprenyldihydroxybenzoate methyltransferase, mitochondrial | COQ3          | 2.38 |
| O94874    | E3 UF1M1 protein ligase 1                         | UFL1          | 2.38 |
| Q5T440    | Putative transferase CAF17, mitochondrial         | IBA57         | 2.38 |
| Q15642    | Cdc42-interacting protein 4                       | TRIP10        | 2.38 |
| Q16795    | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex subunit 9, mitochondrial | NDUFA9        | 2.20 |
| Q9H2U2    | Inorganic pyrophosphatase 2, mitochondrial        | PPA2          | 2.13 |
| Q9H799    | Cilogenesis and planar polarity effector 1        | CPLANE1       | 2.13 |
| P02675    | Fibrinogen beta chain                            | FGB           | 2.13 |
| P40123    | Adenyl cyclase-associated protein 2               | CAP2          | 2.13 |
| P42765    | 3-Ketoacyl-CoA thiolase, mitochondrial            | ACAA2         | 2.10 |
| Q9HC38    | Glyoxalase domain-containing protein 4            | GLOD4         | 1.85 |
| P61019    | Ras-related protein Rab-2A                       | RAB2A         | 1.85 |
| P35270    | Sepiapterin reductase                            | SPR           | 1.85 |
| P67809    | Nuclease-sensitive element-binding protein 1      | YBX1          | 1.85 |
| Q9H6K4    | Optic atrophy 3 protein                          | OPA3          | 1.85 |
| Q15124    | Phosphoglucomutase-like protein 5                 | PG5           | 1.85 |
| Q02224    | Centromere-associated protein E                   | CENPE         | 1.85 |
| P45954    | Short/branched chain-specific acyl-CoA dehydrogenase, mitochondrial | ACADSB       | 1.85 |
| Q9H0P0    | Cytosolic 5'-nucleotidease 3^                           | NT5C3A        | 1.76 |
| P10809    | 60 kDa Heat shock protein, mitochondrial           | HSPD1         | 1.76 |
| P56134    | ATP synthase subunit f, mitochondrial              | ATP5J2        | 1.64 |
| O75150    | E3 ubiquitin-protein ligase BRE1B                 | RNF4          | 1.64 |
| Q15746    | Myosin light chain kinase, smooth muscle          | MYLK          | 1.64 |
| P42704    | Leucine-rich PPR motif-containing protein, mitochondrial | LPPRRC       | 1.64 |
| P55397    | Succinyl-CoA ligase (ADP/GDP-forming) subunit alpha, mitochondrial | SUCLG1       | 1.57 |
| P62701    | 40S ribosomal protein S4, X isoform               | RPS4X         | 1.54 |
| Q0VFZ6    | Coiled-coil domain-containing protein 173         | CCDC173       | 1.48 |
| P46778    | 60S ribosomal protein L21                         | RPL21         | 1.48 |
| P07741    | Adenine phosphoribosyltransferase                 | APRT          | 1.48 |
| Q02338    | D-beta-hydroxybutyrate dehydrogenase, mitochondrial | BDH1          | 1.48 |
| O75915    | PRA1 family protein 3                             | ARL6IP5       | 1.48 |
| P62081    | 40S ribosomal protein S7                          | RPS7          | 1.48 |
| Q13557    | Calcium/calmodulin-dependent protein kinase Type II subunit delta | CAMK2D       | 1.48 |
### Table 2. Cont.

| Accession | Description | Gene       | Rsc  
|-----------|-------------|------------|------
| Q9Y512    | Sorting and assembly machinery component 50 homolog | SAMM50 | 1.48 |
| Q00539    | Heterogeneous nuclear ribonucleoprotein U | HNRNPU | 1.48 |
| P07919    | Cytochrome b-c1 complex subunit 6, mitochondrial | UQCRH | 1.48 |
| P62899    | 60S ribosomal protein L31 | RPL31 | 1.48 |
| Q99733    | Nucleosome assembly protein 1-like 4 | NAP1L4 | 1.48 |
| Q9UKU7    | Isobutyryl-CoA dehydrogenase, mitochondrial | ACAD8 | 1.48 |
| P15088    | Mast cell carboxypeptidase A | CPA3 | 1.48 |
| Q96A26    | Protein FAM162A | FAM162A | 1.48 |
| Q15555    | Microtubule-associated protein RP/EB family member 2 | MAPRE2 | 1.48 |
| P21817    | Ryanodine receptor 1 | RYR1 | 1.44 |

*Rsc represents the log$_2$ ratio between the protein expression level of the muscle biopsies of VPG and those of the CG. Proteins with Rsc $\geq 1.40$ or $\leq -1.40$ were considered to be differentially expressed.*

### Table 3. Label-free quantitative analysis of proteins identified from *V. lateralis* muscle biopsies.

Underexpressed proteins with Rsc $\leq -1.40$ in the VPG vs. CG are shown.

| Accession | Description | Gene       | Rsc  
|-----------|-------------|------------|------
| P51911    | Calponin-1 | CNN1 | -4.78 |
| Q01995    | Transgelin | TAGLN | -4.60 |
| P02790    | Hemopexin | HPX | -3.83 |
| Q14847    | LIM and SH3 domain protein 1 | LASP1 | -3.65 |
| Q9UNZ2    | NSFL1 cofactor p47 | NSFL1C | -3.65 |
| P13798    | Acylamino-acid-releasing enzyme | APEH | -3.44 |
| P48147    | Prolyl endopeptidase | PREP | -3.44 |
| P02679    | Fibrinogen gamma chain | FGG | -3.20 |
| P62280    | 40S ribosomal protein S11 | RPS11 | -3.20 |
| Q2TBA0    | Kelch-like protein 40 | KLHL40 | -3.20 |
| Q9Y6B6    | GTP-binding protein SAR1b | SAR1B | -2.91 |
| Q15370    | Transcription elongation factor B polyepitope 2 | TCEB2 | -2.91 |
| Q969G5    | Protein kinase C delta-binding protein | PRKCDBP | -2.91 |
| P02743    | Serum amyloid P-component | APCS | -2.91 |
| P21980    | Protein-glutamine gamma-glutamyltransferase 2 | TGM2 | -2.91 |
| Q13526    | Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 | PIN1 | -2.91 |
| P6CW22    | 40S ribosomal protein S17-like | RPS17L | -2.91 |
| O15145    | Actin-related protein 2/3 complex subunit 3 | ARPC3 | -2.91 |
| Q13561    | Dynactin subunit 2 | DCTN2 | -2.91 |
| P31153    | S-adenosylmethionine synthase isoform type-2 | MAT2A | -2.91 |
| P22661    | Protein-L-isolaaspartate(D-aspartate) O-methyltransferase | PCMT1 | -2.84 |
| P49189    | 4-Trimethylaminobutyraldehyde dehydrogenase | ALDH9A1 | -2.68 |
| P56556    | NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6 | NDUF6 | -2.55 |
| O14602    | Eukaryotic translation initiation factor 1A, Y-chromosomal | EIF1AY | -2.55 |
| P31942    | Heterogeneous nuclear ribonucleoprotein H3 | HNRNP | -2.55 |
| P30566    | Adenylosuccinate lyase | ADSL | -2.55 |
| Q9COG0    | Zinc finger protein 407 | ZNF407 | -2.55 |
| Q81UG5    | Unconventional myosin-XVIIIb | MYO18B | -2.55 |
| Q9G2ZZ1   | N-alpha-acyltransferase 50 | NAA50 | -2.55 |
| Q00765    | Receptor expression-enhancing protein 5 | REEP5 | -2.55 |
| P62491    | Ras-related protein Rab-11A | RAB11A | -2.55 |
| P62195    | 26S protease regulatory subunit 8 | PSMC5 | -2.55 |
| P01011    | Alpha-1-antichymotrypsin | SERPINA3 | -2.55 |
| Q16853    | Membrane primary amine oxidase | AOC3 | -2.55 |
| P36776    | Lon protease homolog, mitochondrial | LONP1 | -2.55 |
| Q7LX3     | Serine/threonine-protein kinase TAK1 | TAO1 | -2.55 |
| P78527    | DNA-dependent protein kinase catalytic subunit | PRKDC | -2.55 |
| P51884    | Lumican | LUM | -2.29 |
| P61026    | Ras-related protein Rab-10 | RAB10 | -2.24 |
| P20774    | Mimecan | OGN | -2.19 |
| Accession | Description | Gene | Rsc |
|-----------|-------------|------|-----|
| O94760    | N(G),N(G)-dimethylarginine dimethylaminohydrolase 1 | DDAH1 | −2.06 |
| P38646    | Stress-70 protein, mitochondrial | HSPA9 | −2.06 |
| P20618    | Proteasome subunit beta type-1 | PSMB1 | −2.06 |
| P61254    | 60S ribosomal protein L26 | RPL26 | −2.06 |
| Q15185    | Prostaglandin E synthase 3 | PTGES3 | −2.06 |
| P07203    | Glutathione peroxidase 1 | GPX1 | −2.06 |
| Q961U4    | Alpha/beta hydrolase domain-containing protein 14B | ABHD14B | −2.06 |
| Q9H1H3    | Methyltransferase-like protein 7A | METTL7A | −2.06 |
| P55042    | GTP-binding protein RAD | RRAD | −2.06 |
| P50454    | Serpin H1 | SERPINH1 | −2.06 |
| Q99807    | Ubiquinone biosynthesis protein COQ7 homolog | COQ7 | −2.06 |
| P54619    | 5'-AMP-activated protein kinase subunit gamma-1 | PRKAG1 | −2.06 |
| O43765    | Small glutamine-rich tetratricopeptide repeat-containing protein alpha | SGTA | −2.06 |
| Q9BUB7    | Transmembrane protein 70, mitochondrial | TMEM70 | −2.06 |
| P27169    | Serum paraoxonase/arylesterase 1 | PON1 | −2.06 |
| Q00059    | Transcription factor A, mitochondrial | TFAM | −2.06 |
| Q99536    | Synaptic vesicle membrane protein VAT-1 homolog | VAT1 | −2.06 |
| P54837    | 26S protease regulatory subunit 6B | PSMC4 | −2.06 |
| P51888    | Prolargin | PRELP | −2.06 |
| Q9Y230    | RuvB-like 2 | RUVBL2 | −2.06 |
| P16930    | Fumarylacetoacetase | FAH | −2.06 |
| P48637    | Glutathione synthetase | GSS | −2.06 |
| P39148    | Stress-induced-phosphoprotein 1 | STIP1 | −2.06 |
| Q8NB59    | Thioredoxin domain-containing protein 5 | TXNDC5 | −2.06 |
| O00148    | ATP-dependent RNA helicase DDX39A | DDX39A | −2.06 |
| Q9HCC0    | Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial | MCCC2 | −2.06 |
| Q9N2N4    | EH domain-containing protein 2 | EHD2 | −2.06 |
| P23246    | Splicing factor, proline- and glutamine-rich | SFQP | −2.06 |
| Q9NUB1    | Acetyl-CoA synthetase 2-like, mitochondrial | ACS2 | −2.06 |
| Q9P1V8    | Sterile alpha motif domain-containing protein 15 | SAMD15 | −2.06 |
| P00747    | Plasminogen | PLG | −2.06 |
| O14795    | Protein unc-13 homolog B | UNC13B | −2.06 |
| Q702N8    | Xin actin-binding repeat-containing protein 1 | XIRP1 | −2.06 |
| Q8HNW7    | Retinitis pigmentosa 1-like 1 protein | RP1L1 | −2.06 |
| Q16787    | Laminin subunit alpha-3 | LAMA3 | −2.06 |
| P07385    | 120-kDa Decorin | DCN | −1.94 |
| P21291    | Cysteine and glycine-rich protein 1 | CSR1 | −1.89 |
| P20073    | Annexin A7 | ANXA7 | −1.89 |
| P62993    | Growth factor receptor-bound protein 2 | GRB2 | −1.86 |
| P52895    | Aldo-keto reductase family 1 member C2 | AKR1C2 | −1.79 |
| O43488    | Aflatoxin B1 aldehyde reductase member 2 | AKR7A2 | −1.69 |
| Q9BX13    | Cytosolic 5'-nucleotidase 1A | NT5C1A | −1.67 |
| P61106    | Ras-related protein Rab-14 | RAB14 | −1.61 |
| P62277    | 40S ribosomal protein S13 | RPS13 | −1.61 |
| Q15084    | Protein disulfide-isomerase A6 | PDIA6 | −1.61 |
| P16083    | Ribosylidyhydrocinnaminate dehydrogenase (quinone) | NQO2 | −1.61 |
| P47985    | Cytochrome b-c1 complex subunit Rieske, mitochondrial | UQCRFS1 | −1.53 |
| P05254    | Prelamin-A/C | LMNA | −1.53 |
| P24844    | Myosin regulatory light polypeptide 9 | MYL9 | −1.51 |
| P09382    | Galectin-1 | LGALS1 | −1.51 |
| P28070    | Proteasome subunit beta type-4 | PSMB4 | −1.51 |
| P62269    | 40S ribosomal protein S18 | RPS18 | −1.51 |
| P14543    | Nidogen-1 | NID1 | −1.51 |
| P13716    | Delta-aminolevulinic acid dehydratase | ALAD | −1.46 |

*Rsc represents the log2 ratio between the protein expression level of muscle biopsies of the VPG and those of the CG. Proteins with Rsc ≥ 1.40 or ≤ −1.40 were considered to be differentially expressed.*
3.2. Functional Annotation, Biological Network and Pathway Analyses of Differentially Expressed Proteins in Skeletal Muscle from VPG versus CG

To investigate the molecular basis related to healthy longevity in the skeletal muscle from the VPG versus CG, under- and overexpressed protein datasets were analyzed separately by bioinformatic tools. Table 4 lists the most significant Gene Ontology (GO) terms belonging to the BP (biological processes) category related to the two subsets obtained by querying the DAVID platform. Among the overexpressed proteins in skeletal muscle from the VPG versus the CG, the most represented functional categories were “mitotic cell cycle”, “calcium ion transport” and “energy derivation by oxidation of organic compounds”; whereas “protein complex assembly”, “positive regulation of ubiquitin–protein ligase activity” and “organic acid catabolic process” were all categories that were significantly enriched in the underexpressed protein subset. Differentially expressed proteins were also analyzed by STRING software version 11.5 to unveil the relevant biological networks and non-redundant biological processes in both protein subsets. The STRING output revealed three and five relevant subnetworks among the over- and underexpressed proteins in the VPG vs. CG, respectively (Figure 1). In agreement with the DAVID analysis (Table 4), the STRING subnetworks of overexpressed proteins contained nodes belonging to the following biological processes: “nuclear-transcribed mRNA catabolic process”, “regulation of calcium ion transport” and “oxidation–reduction process” (Table 5, Figure 1A). Similar to the DAVID analysis, the underexpressed protein nodes in the STRING analysis were also related to “protein-containing complex subunit organization” and “post-translational protein modifications” (Table 5, Figure 1B). Interestingly, 26S proteasome subunits (PSMB4, PSMC5, PSMB1, PSMC4), which were underexpressed proteins in the VPG vs. CG, were significantly represented in the BP term “positive regulation of ubiquitin-protein ligase activity” according to DAVID (Table 4) and highly interconnected in the STRING subnetwork related to “post-translational protein modifications” (Figure 1B). When the Reactome database was queried, these four 26S proteasome subunits matched the “regulation of ornithine decarboxylase (ODC)” pathway (p-value = 1.13 × 10⁻¹⁰, FDR 5.40 × 10⁻⁹), a cellular process affecting the endogenous biosynthesis of polyamines.

Figure 1. STRING network analysis of overexpressed (A) and underexpressed (B) proteins in skeletal muscle from the VPG vs. CG. The node colors correspond to the enriched categories with a FDR ≤ 0.05.

### Table 4. Biological processes according to the results of the DAVID functional enrichment analysis of over- and underexpressed proteins in the VPG vs. CG.

| GO Terms                                                  | Proteins                                                                 | p-value   |
|-----------------------------------------------------------|--------------------------------------------------------------------------|-----------|
| Overexpressed proteins                                    |                                                                          |           |
| Mitotic cell cycle (GO: 0000278)                           | MAPRE3, PPP6C, CENPE, PSMD13, CAMK2G, MAPRE2, CAMK2B, CAMK2D             | 0.004     |
| Calcium ion transport (GO: 0006816)                        | CACNB1, CAMK2G, CAMK2B, CAMK2D                                           | 0.007     |
| Energy derivation by oxidation of organic compounds (GO: 0015980) | SUCLG1, UQCRH, NDUFA7, NDUFA9                                           | 0.043     |
| Underexpressed proteins                                    |                                                                          |           |
| Protein complex assembly (GO: 0006461)                      | APCS, GRB2, PRKAG1, ELOB, TMEM70, TFAM, FGG, LONP1, TGM2, ADSL, UNC13B | 9.22 × 10⁻⁴ |
| Positive regulation of ubiquitin–protein ligase activity (GO: 0051443) | PSMB4, PSMC5, PSMB1, PSMC4                                                | 8.48 × 10⁻⁴ |
| Organic acid catabolic process (GO: 0016054)                | DDAH1, MCCC2, PON1, FAH                                                     | 0.029     |
Table 4. Biological processes according to the results of the DAVID functional enrichment analysis of over- and underexpressed proteins in the VPG vs. CG.

| GO Terms                                      | Proteins                                      | p-Value |
|-----------------------------------------------|-----------------------------------------------|---------|
| **Overexpressed proteins**                   |                                               |         |
| Mitotic cell cycle (GO: 0000278)              | MAPRE3, PPP6C, CENPE, PSMD13, CAMK2G, MAPRE2, | 0.004   |
|                                               | CAMK2B, CAMK2D                                |         |
| Calcium ion transport (GO: 0006816)           | CACNB1, CAMK2G, CAMK2B, CAMK2D, RYR1          | 0.007   |
| Energy derivation by oxidation of organic     | SUCLG1, UQCRH, NDUFA7, NDUFA9                | 0.043   |
| compounds (GO: 0015980)                      |                                               |         |
| **Underexpressed proteins**                  |                                               |         |
| Protein complex assembly (GO: 0006461)        | APCS, GRB2, PRKAG1, ELOB, TMEM70, TFAM, FGG, | 9.22 × 10⁻⁴ |
|                                               | LONP1, TGM2, ADSL, UNC13B                    |         |
| Positive regulation of ubiquitin-protein ligase activity | PSMB4, PSMC5, PSMB1, P | 8.48 × 10⁻⁴ |
|                                               | SM4, PIN1                                    |         |
| Organic acid catabolic process (GO: 0016054)  | DDAH1, MCCC2, PON1, FAH                       | 0.029   |

Table 5. Biological processes according to the results of the STRING analysis of overexpressed and underexpressed proteins in the VPG vs. CG.

| GO Terms                                      | Proteins                                      | p-Value |
|-----------------------------------------------|-----------------------------------------------|---------|
| **Overexpressed proteins**                   |                                               |         |
| Nuclear-transcribed mRNA catabolic process    | RPL31, RPL21, RPL27, RPS2, UPF1, RPS7, RPS4  | 0.002   |
| (GO: 0000184)                                 |                                               |         |
| Regulation of calcium ion transport           | MYLK, CAMK2G, CAMK2B, CAMK2D                  | 0.032   |
| (GO: 0051924)                                 |                                               |         |
| Oxidation-reduction process (GO: 0055114)     | UQCRH, NDUFA7, NDUFA9, SUCLG1, ACACA2, ACADSB | 0.02    |
| **Underexpressed Proteins**                  |                                               |         |
| Protein-containing complex subunit organization (GO: 0043933) | TCEB2, RPS17, LUM, MRPL11, TMEM70, PTGES3, FGG | 0.0017 |
| Post-translational protein modifications (GO: 0043687) | PSMB4, PSMC5, PSMB1, PSMC4, RAB11A, FGG, P | 0.0061 |
|                                               | PDIA6, LGALS1, TCEB2                         |         |

3.3. Validation of Selected Differentially Expressed Proteins

To validate the data obtained from the label-free quantitative proteomics, we analyzed the expression of selected identified proteins by Western blotting (Figure 2). In particular, we focused on two proteins, carnitine O-palmitoyltransferase 1, muscle isoform (CPT1B), and mitochondrial 3-ketoacyl-CoA thiolase (ACAA2), both belonging to the fatty acid degradation pathway. In agreement with the proteomic analysis, Western blotting showed the overexpression of these proteins in skeletal muscle from the VPG vs. CG (Figure 2A). This finding was confirmed by a densitometric analysis that showed, in the case of the overexpression of CPT1B and ACAA2, a significant difference between the VPG and CG (p < 0.05).
A targeted metabolomic analysis was performed to characterize the muscle biopsies from the VPG and CG (Figure 3). A detailed list of the identified metabolites, including their names, abbreviations and analytical concentrations is reported in Supplemental Tables S1–S6.

To define the VPG’s specific metabolomic signature, the datasets were processed according to a supervised partial least squares-discriminant analysis (PLS-DA). The VPG’s and CG’s metabolomes clustered according to variance of Component 1 (15.4%) and Component 2 (6.9%) (Figure 3A). The most discriminant hits between the VPG and CG were defined by evaluating the variable importance in projection (VIP) (Figure 3B).

In detail, the relative abundance of metabolites such as C16:1OH, C16:1, C14OH, C16, C18:1OH, C14:2 and Orn were able to discriminate between the analyzed groups according to a VIP > 1.5. Interestingly, the Euclidean distance-based hierarchical clustering of the identified and quantified metabolites visualized in the heatmap (Figure 3C) exhibited a clear distinct pattern in the metabolites’ abundance between the two groups, VPG and CG, with the exception of VPG1. The metabolite concentrations were ranked by their t-test (p < 0.05) results.

In order to highlight individual metabolite profiles between the VPG and CG, a univariate binary comparison was performed according to a volcano plot. Figure 3D summarizes the significant differences in the abundance of muscle metabolites between the two groups. The volcano plot analysis revealed that 7/50 and 9/50 metabolites increased and decreased, respectively, in the muscle samples from the VPG and CG.
Finally, the normal distribution of the metabolite concentrations was verified, and significant differentially profiles were evaluated in the different groups. In detail, metabolites such as Orn, Phe, Gly, Met, Arg were found to be increased in the VPG vs. CG; conversely, C16, C5DC, C14OH, C16:1, C8dc, Cit, C12Oh, C14:2 and C18 were found to be decreased in the VPG vs. CG (Figure 3E).

**Figure 3.** Metabolomic discriminant analysis of the VPG. (A) A supervised partial least squares-discriminant analysis (PLS-DA) was performed using the metabolome dataset. Muscle metabolite
concentrations were log(10)-transformed and Pareto-scaled. A clear separation according to Component 1 (15.4%) and Component 2 (6.9%) was obtained. (B) The 30 most discriminant features were identified according to the variable importance in projection (VIP) score (>1.0). (C) Hierarchical cluster analysis and heatmap visualization of the top 40 members of the lipid dataset (y-axis), ranked by the t-test (p < 0.05) results are shown. (D) Volcano plot analysis, showing the metabolites’ abundance in the VPG versus CG. The relative abundance of each metabolite was plotted against its statistical significance as the fold change (log2 ratio) and –log10(p-value). Red and green dots indicate significantly decreased and increased metabolites. Black dots indicate the metabolites identified in the dataset for which the relative abundance was not significantly changed between the VPG and CG. (E) Plots showing the different metabolite concentrations (means ± SEM) in the VPG versus CG. The significant difference in the metabolite concentrations was evaluated by performing a parametric t-test with Welch’s correction in normally distributed datasets and the Mann–Whitney test in non-normally distributed datasets (* p < 0.05, ** p < 0.01, **** p < 0.0001, ns = not significant). The normal distribution was verified according to D’Agostino and Pearson’s test.

3.5. Polyamine Biosynthesis in Skeletal Muscle from Veteran Football Players (VPG) versus Untrained Subjects (CG)

The Orn/Cit profiles determined by a metabolomic approach, and the bioinformatic output obtained from the Reactome database suggested that we should verify the total polyamine content in the muscle biopsies from the VPG and CG by using a fluorimetric assay. As shown in Figure 4, the total polyamine content in the muscle was 39% higher in the VPG in comparison with the CG (p < 0.05).

Figure 4. Determination of total polyamine content in skeletal muscle from the VPG vs. CG using a fluorimetric assay. Fluorescence signals proportional to the polyamine concentration in the muscle biopsies were obtained from three technical measures in five independent biological replicates. Results are represented as the mean ± SD. * p < 0.05.

4. Discussion

In-depth knowledge of the molecular mechanisms that govern the aging process in the skeletal muscle are instrumental to better counteract the structural and functional decay that occurs in the elderly [34,35]. Several studies have analyzed, at the molecular level, the effects of PA on different systems/organs, including the skeletal muscle, in the young, adults and older people [22,25,26,35,36]. Here, we characterized muscle biopsies from the V. lateralis of male subjects aged over 65 yrs by a multi-omics approach in order to gain an insight into the molecular mechanisms characterizing successful aging through lifelong football training. The anthropometric, biochemical and clinical data of the two groups under investigation differed in their VO2 max, which was significantly higher in the VPG, and in the BMI and body fat percentage, which were significantly lower in the VPG. The proteomic approach identified 188 differentially expressed proteins between the two groups, 40 of which were from the mitochondria. Mitochondrial species were mostly upregulated (75%), similar to the results of previous reports [35,36]; among them, half were from the mitochondrial membrane. These proteins covered a variety of mitochondrial functions, as already described for high-functioning octogenarian master athletes [35].
and supported the established knowledge that regular PA improves mitochondrial function [37–41], counteracting the decline in muscle strength and mass and in neuromuscular control [42,43]. In fact, the exercise-mediated coordinated expression of mitochondrial species activates mitochondrial biogenesis, which affects several functions within the organelle and triggers several signaling pathways [44,45] that are able to induce specific adaptations to the mechanical stimuli; among these, the increased oxidative capacity is a well-recognized hallmark of PA-mediated adaptation in the mitochondria [41,46,47]. Accordingly, by using different bioinformatic tools, in the overexpressed subset of this study, mitochondrial proteins involved in the oxidative metabolism were found, such as NDUFA7, NDUFA9, SUCLG1, UQCRH, ACA2, ACADSB and ATP5J, confirming the previous findings. Moreover, the increase in the cytosolic Ca\(^{2+}\) concentration determined the overexpression of PGC-1\(\alpha\) [25] and of the beta, delta and gamma subunits of CaMK2, a Ca\(^{2+}\)/calmodulin-dependent protein kinase, which is involved in contraction-induced signaling [46], which favors lipid oxidation and supports skeletal muscles in using FAs as energetic substrates [45]. In agreement with such findings, in our dataset, we also found the overexpression of CPT1B, a rate-limiting enzyme in lipid oxidation, and the underexpression of the mitochondrial acetyl-CoA synthetase 2 (ACSS2), an enzyme involved in FA biosynthesis. A similar difference in expression was reported by Joseph et al. [48] in the skeletal muscle of rats following exercise, demonstrating the crucial role played by CaMK2 in enhancing the oxidative capacity of the mitochondria by both promoting the expression of enzymes involved in the oxidation of FAs and reducing the expression of those that catalyze lipid synthesis [48]. All together, these data indicate that the rate of FA beta oxidation was very high in the VPG compared with the CG, caused by the increased amount of the acyltransferase CPT1B, which promotes the entry of long-chain FAs into the mitochondria, and of ACADSB, ACAD8 dehydrogenases and ACA2 thiolase, which are directly involved in the mitochondrial oxidative process. The proteomic picture was also supported by the metabolomic analysis, in which unsaturated, branched and hydroxylated ACs, considered to be intermediate metabolites of FA beta oxidation, were significantly reduced in the VPG compared with the CG. As a matter of fact, the increased mitochondrial oxidative capacity in the VPG resulted in the rapid disposal of lipide intermediates due to the more efficient FA beta oxidation, whereas the lack of any structured PA in the CG contributed to the accumulation of FA metabolites. Such results confirmed that lifelong football training is able to enhance the muscles’ oxidative capacity in the elderly [25] by promoting lipids as preferential energetic substrates and hence determining a healthier body composition and metabolic profile.

Another interesting result obtained from our multi-omics approach is related to the regulation of ODC pathway, which emerged as significant process in the bioinformatic analysis comparing the proteomic data of the VPG and CG. ODC catalyzes the first rate-limiting step of putrescine synthesis, the first member of the polyamine family, under the stimulus of several growth factors, including exercise-sensitive circulating species. Putrescine, synthesized from the amino acid ornithine, is converted by the S-adenosylmethionine decarboxylase in spermidine and then in spermine [49–51].

Polyamines are small aliphatic polycations, the concentration of which is tightly regulated in their biosynthesis, catabolism and transport. They are involved in cell growth, proliferation and differentiation, and also in aging, metabolic diseases, cancer and neurodegenerative disorders; they also have the function of stabilizing the DNA and modulating some membrane receptor complexes [49,50,52].

Polyamines play several roles in the cell, aimed at protecting against oxidative stress by regulating the expression of proteins involved in the response to an oxidizing agent/condition [53–58]; they also act as ROS scavengers at a physiological pH [59–61], with spermine being the most effective at protecting DNA from oxidative stress [59]. Information on the role of exercise-induced polyamines in the skeletal muscle is still scarce. According to Turchanowa et al., polyamines are apparently involved in the oxidative metabolism of skeletal muscles, as their concentrations increased after resistance and/or
endurance exercises [52,62]. Interestingly, in our experimental dataset, metabolomic data on the amino acids showed that in the VPG, ornithine concentrations were higher and, concurrently, citrulline concentrations were lower in respect to the CG’s muscle biopsies, supporting the hypothesis that ornithine is committed to polyamine biosynthesis through ODC’s activity. The fluorimetric assay confirmed that the total polyamine content in skeletal muscles from the VPG was higher than in the CG. Such a finding reinforces the putative involvement of polyamines in muscle growth and hypertrophy [49], and it encourages further investigation into the role of these exercise-sensitive metabolites.

5. Conclusions

This multi-omics study characterized the protein and metabolic profile from muscle biopsies of the VPG vs. CG. The study showed that mitochondrial biogenesis is effectively triggered in subjects who practice lifelong football training by means of the activation of CAMKII signaling and a very efficient mitochondrial oxidative process of FA; in addition, the concentration of small molecules, such as polyamines, which are known to be ROS scavengers and to have anti-inflammatory properties, was increased in the veterans compared with the controls. These results suggest that lifelong football training, as structured PA, significantly influences the expression of proteins and metabolites involved in successful aging and help reduce the risk of the onset of NCDs in the elderly.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ijerph192315835/s1. Figure S1: SDS-PAGE analysis of proteins in the skeletal muscle from veteran football players (VPG) versus untrained subjects (CG). MW: molecular weight protein standard. Table S1: Metabolites’ abbreviations and extended names. Table S2: Amino acid concentrations (µM) in muscle samples. Table S3: Metabolite concentrations (µM) in muscle samples. Table S4: Unsaturated AC concentrations (µM) in muscle samples. Table S5: Branched AC concentrations (µM) in muscle samples. Table S6: Hydroxylated AC concentrations (µM) in muscle samples.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE [31] partner repository with the dataset identifier PXD037792.

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