Analysis of the metabolic switch induced by the spirulina peptide SP6 in high fat diet ApoE\(^{-/-}\) mice model: A direct infusion FT-ICR-MS based approach

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**ABSTRACT**

Atherosclerosis, dyslipidemia and hypertension are comorbid diseases often found in combination. Among different pharmacological approaches the employment of natural multifunctional peptides is an attractive option as side therapy. Mass spectrometry-based metabolomics provide valuable information on metabolic changes and can be useful to elucidate peptide pharmacodynamics. In this work we performed a preliminary investigation on the potential effect of a recently characterized *Spirulina platensis* peptide named SP6 (GIVAGDVPTI) on the modulation of metabolism in a high fat diet ApoE\(^{-/-}\) mice atherosclerotic model. A direct infusion Fourier transform ion cyclotron resonance mass spectrometry (DI-FT-ICR-MS) approach was used to elucidate polar and non-polar metabolites extracted by mice plasma following four weeks SP6 treatment. The method delivered fast analysis time, repeatability, high mass accuracy and resolution for unambiguous molecular formula assignment. Multivariate statistical analysis (PLS-DA) highlighted a clear class separation, revealing the alteration of numerous metabolites levels belonging to different classes. In particular sphingolipids, glycerophospholipids, TCA cycle intermediates, and amino acids, which are key players in the atherosclerotic process and progression, were upregulated in saline alone HFD ApoE\(^{-/-}\) group, while were sensibly decreased after treatment with SP6 peptide. These results could open the way to further, large-scale, investigation of SP6 peptide effects in the regulation of atherosclerotic disease development and progression, and show the potential of DI-FT-ICR as fast analytical tool to take snapshots of metabolic changes before moving to targeted MS-based approaches.

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**1. Introduction**

In the landscape of metabolic disorders, hypertension and dyslipidemia are comorbid pathologic conditions often found in combination, that are also associated to the development of endothelial disfunction. Several evidences demonstrate that long-term high-fat diet (HFD) induces hypertension, and impair NO release [1]. Moreover, clinical studies showed that hypertension is not only a well-known cardiovascular risk, but can contribute to the atherosclerosis development, and could play a strictly connected role in the exacerbation of this process, partially due to the synergy between elevated blood pressure and other atherogenic stimuli, inducing oxidative stress on the arterial vessels. [2]. In fact, an increase of reactive oxidative species (ROS) generation, results in higher oxidation rate of low-density lipoprotein (LDL) in atherosclerotic lesions [3]. The employment of natural compounds has experienced a boost in the last years as possible adjuvant or side approaches to pharmacological therapy for the prevention and treatment of multifactorial cardiovascular disorders. Bioactive compounds, especially those of plant or marine...
origin, have been recognized of several healthy properties, through their involvement in different biochemical pathways, and have found widespread adoption in novel products, such as nutraceuticals and functional foods [4]. Besides phytochemicals, bioactive peptides have garnered attention as novel therapeutic molecules. Peptides are usually encrypted in the sequence of respective proteins, and released after proteolysis events, which can occur in vivo or in vitro, and exploit their activity [5]. The interest towards natural peptides is related to several aspects, in fact they show low or absent side effects, are easily metabolized by endogenous enzymes, and can display very high specificity for their biological targets. Bioactive peptides from food and other natural matrices have shown therapeutic application against different and closely related pathologies such as diabetes, hypertension and dyslipidemia, which define the metabolic syndrome [6]. Microalgae have emerged as a rich source of peptides with different properties, and, in particular, for the prevention of cardiovascular disease [7]. In this regard, we recently characterized a novel decameric peptide from the gastro-intestinal digest of Spirulina platensis, named SP6 (GIVAGDVTP, allophycocyanin α-chain, f: 95–104), which showed very potent in vivo anti-hypertensive activity by exerting endothelium-dependent vasodilation via a PI3K (phosphoinositide-3-kinase)/Akt (serine/threonine kinase Akt) pathway, converging on nitric oxide (NO) release [8]. Bioactive peptides from natural sources are often labelled as “multifunctional”, due to combined effects such as hypotensive and hypocholesterolemic, as showed by milk, soybean and lupin protein derived peptides [9]. Food derived tripeptides Val-Pro-Pro and Ile-Pro-Pro have shown anti-obesity properties [10] while vasodilating dipeptides were reported to reduce atherosclerotic lesions in ApoE−/− models [11]. For the comprehension of the complex interconnected metabolic mechanisms in physiopathological conditions, mass spectrometry-based metabolomics has emerged as leading method to monitor multiple metabolite classes and is highly suited to highlight molecular changes associated to drug treatments or in disease progression and outcome [12]. The high complexity of metabolic profiling, and the presence of metabolites with different chemistry, polarity and concentration range, requires high resolving power, mass accuracy and wide dynamic range. In this regard, high resolution mass spectrometry (HRMS) in direct infusion (DI) and/or coupled to ultra high performance liquid chromatography (UHPLC-HRMS) is now the workhorse of metabolomics and lipidomics [13]. In particular, Fourier transform ion cyclotron mass spectrometry (FT-ICR-MS) offers ultra high resolution and mass accuracy, together with the ability to measure isotopic fine structure (ISF), leading to unambiguous identification of molecular formulas; DI-FT-ICR, in direct infusion mode is a powerful tool for rapid phenotyping studies [14]. The aim of this study was the application of a fast and accurate direct infusion nano-electrospray Fourier transform ion cyclotron mass spectrometry (DI-FT-ICR) approach, to reveal in vivo metabolic changes exerted by the spirulina peptide SP6 in the modulation of atherosclerosis progression in HFD ApoE−/− mice model. The analysis of plasma polar and non-polar metabolites following four weeks treatment with SP6 could open valuable insight of the molecular mechanisms modulated in the atherosclerotic process, paving the way to further investigation for its employment in interconnected cardiovascular diseases.

2. Materials and methods

2.1. Animal experiment

The data that support the findings of this study are available from the corresponding authors on reasonable request. Synthesis and purification of SP6 peptide is described in detail in supporting material. All animal studies were performed in accordance with approved protocols by the IRCCS Neurormed Animal Care Review Board and by the Istituto Superiore di Sanità and were conducted according to EU Directive 2010/63/EU for animal experiments. ApoE−/− mice (Charles River Laboratories, Sant’Angelo Lodigiano, Italy) were born at the expected mendelian ratios, developed normally, and were maintained in a ventilated environment. All animals were randomly divided into the control group treated with saline solution and group treated with peptide SP6 (5 mg/Kg) by daily gavage administration. Mice were fed up to 10 weeks with normal rodent chow (4.5 % fat; Ralston Purina Co.), and subsequently switched to the Western diet-high fat diet (HFD) (Complete feed for Rodents Purified Diet 60 % ENERGY FROM FATS – Mucedola) at 11 weeks for 1 week. Subsequently, in the next week they have been treated by gavage with saline solution (N = 4) or with SP6 (N = 5) daily for 4 weeks.

2.2. Mice monitoring and samples collection

All mice have been subjected to constant monitoring of systolic, diastolic blood pressure and heart rate during all observation period by non-invasive tail-cuff method using the BP-2000 instrument (Visitech systems, Apex, NC, U.S.A) as previously described [8]. Moreover, mice were weighed throughout the observation period using electronic veterinary weighing scale (Soehnle Industrial Solutions, Backnang, Germany).

At the end of treatment (4th week), blood was collected from the heart through cardiac puncture of isoflurane-anesthetized mice in heparinized tube, and rapidly centrifuged at 2200 rpm for 15 min to obtain plasma samples.

2.3. Immunofluorescence analysis

Immunofluorescence analyses of aortic arch from ApoE−/− mice treated with saline solution or SP6 for 4 weeks were performed on snap-frozen segment in OCT embedding medium. Frozen sections (10 μm) were treated with blocking solution (2% donkey serum, 1.5 % BSA, 0.5 % fish gelatin) for 45 min and immunostained overnight at 4 °C in blocking solution with antibodies anti-α-SMA (1:200, abcam) and with anti-CDS68+ (1:200; abcam) and revealed with appropriate secondary antibodies (Rhodamine-Red anti-mouse IgG; Jackson ImmunoResearch; Alexa Fluor 488 anti-sheep IgG; Molecular Probes, Invitrogen). Nuclei were counterstained with DAPI. The images were acquired using a fluorescence THUNDER Imaging Systems (Leica, Bucinasco, Milan, Italy) microscope.

2.4. Sample preparation

Polar metabolites and lipids were extracted following the Matyash protocol with slight modifications [15]. Briefly 20 μL of plasma were thawed on ice and 225 μL of ice cold MeOH were added, samples were vortexed for 10 s, subsequently 750 μL of cold MTBE were added and the solution was mixed in a thermonix® (Eppendorf) for 6 min at 4 °C. Subsequently 188 μL of H2O were added and samples were vortexed for 20 and finally centrifuged at 14,680 rpm, for 5 min at 4 °C for phase separation. The two phases were recovered separately and dried under nitrogen. The non-polar extract was solubilized in 400 μL of 5 mM CH3COONa, MeOH/DCM 90/10 v/v, while the polar extract was solubilized in 200 μL of 1:1 ACN/H2O 0.1 % HCOOH. A pooled quality control (QC) sample was prepared for both polar and non-polar analysis. This was achieved by pooling an aliquot of plasma from each sample until the same volume used for extraction and subsequently extracted as described above. Unless otherwise described, all solvents and
additives were LCMS grade and purchased by Merck (Darmstadt, Germany).

2.5. DI-FT-ICR analysis

Analyses were performed in direct infusion nano-electrospray by an automated multisample chip-based nESI sample ionization platform TriVersa NanoMate (Advion BioSciences Ltd, Ithaca NY, U.S.A), which was operated with the following parameters: gas pressure (nitrogen) was 0.3 psi, spray voltage 1.45 kV, sample volume was 5 μL, sample plate temperature was set to 10 °C, 5 μm nominal internal diameter nozzle chip was used. Data were acquired on a SolarIX XR 7T (Bruker Daltonics, Bremen, Germany). The instrument was tuned with a standard solution of sodium trifluoroacetate (NaTFA). Mass Spectra were recorded in broadband mode in the range 150–1500 m/z for lipids, whereas 90–800 m/z was used for polar metabolites, with an ion accumulation of 10 ms, 32 scans were acquired using 2 million data points (2 M), with a resolution of 150,000 at m/z 400. Drying gas (nitrogen) was set at 2 mL/min, with a drying temperature of 150 °C. Funnel amplitude was set to 90 V (polar metabolites) or 100 V (lipids), transfer was set at 0.6 MHz, and TOF 0.7 s. Both positive and negative ESI ionization were employed in separate run. Five replicates of each injection were carried out. The instrument was controlled by Bruker FTMS Control (Bruker).

2.6. UHPLC-HRMS/MS analysis

Two UHPLC-HRMS/MS methods (reversed phase and hydrophilic interaction liquid chromatography) were employed to enforce DI-FT-ICR results and avoid metabolite misannotation. Analyses were performed on a Thermo Ultimate RS 3000 coupled online to a Q-Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). equipped with a heated electrospray ionization probe (HESI II). For Lipid analysis the separation was performed with an Acquity UPLC CSH C18 column (100 × 2.1 mm; 1.7 μm) protected with a VanGuard CSH precolumn (5 × 2.1 mm; 1.7 μm) (Waters, Milford, MA, U.S.A). The column temperature was set at 55 °C, a flow-rate of 0.4 mL/min was used, mobile phase consisted of (A): ACN/H2O with 10 mM CH3COONa and 0.1 % HCOOH 60:40 (v/v) and (B): IPA/ACN with 0.1 % HCOOH 90:10 (v/v). The following gradient has been used: 0 min, 40 %B, 2 min, 43 %B, 2 min, 43 %B, 10 min, 60 %B, 12 min, 75 %B, 13 min, 99 %B hold for 2 min, returning to 40 %B in 0.1 min. The separation of polar metabolites was performed in HILIC mode, with an Acquity BEH Amide (100 × 2.1 mm; 1.7 μm) protected with a VanGuard amide precolumn (5 × 2.1 mm; 1.7 μm) (Waters). The column temperature was set at 40 °C, and the flow-rate was 0.350 mL/min. The mobile phase was (A): 0.1 % HCOOH in H2O (v/v) and (B): 0.1 % HCOOH in ACN (v/v). The following gradient was employed: 0 min, 99 %B, 1 min, 99 %B, 10.50 min, 20 %B, 11 min, 20 %B, returning to 99 % in 0.1 min. 2 μL were injected in both RP and HILIC. Full MS (Lipids: 300–1500 m/z, polar metabolites: 50–800 m/z) and data-dependent MS/MS were performed at a resolution of 35,000 and 15,000 FWHM respectively, a fixed normalized collision energy (NCE) value of 30 was used. Both ESI+ and ESI− were used, in separate run. Source parameters: Sheath gas pressure, 45 arbitrary units; auxiliary gas flow, 15 arbitrary units; spray voltage, +/-3.5 kV; capillary temperature, 280 °C; auxiliary gas heater temperature, 350 °C. Three replicate of each
sample were performed, QC were randomly inserted in the batch to monitor system stability over time.

2.7. Data processing

DI-FT-ICR data analysis was performed with MetaboScape (v. 5.0, Bruker). The first step is the creation of a matrix (bucket table) using the T-ReX 2D algorithm. The T-ReX 2D algorithm for feature extraction from FT-ICR single spectra extracts m/z / intensity pairs (peaks) from acquired raw data and subjects them to deisotoping in order to create features consisting of isotope patterns. Subsequently, spectra alignment, filtering, normalization were performed. The spectra were processed in positive mode using H⁺ as the primary ion, Na⁺ and NH₄⁺ as a potential adducts, while in negative mode H⁻ was set as the primary ion and Cl⁻ as a potential adduct. For metabolite annotation, assignment of the molecular formula was performed for the detected features using Smart Formula™ (SF), isotopic fine structure (IFS) and data recalibration. The bucket table was annotated with a list of metabolites and lipids obtained respectively from the HMDB (https://hmdb.ca/) and LIPIDMAPS database (www.lipidmaps.org). Annotation was performed with 0.2 ppm (narrow) or 1 ppm (wide) mass tolerance and a mSigma value below 200. Molecular formula were manually inspected taking into account the most probable adduct form. For UHPLC-HRMS processing Thermo RAW, data files were converted to ABF format using Reifys Abf (Analysis Base File) converter, subsequently data alignment and metabolite annotation were performed by MS-DIAL software (v. 4.16, http://prime.psc.riken.jp/comps/mstdial/main.html) Detailed parameters for peak picking, alignment and annotation are reported in supporting material. Metabolites were putatively annotated on level 2 Metabolomics Standard Initiative (MSI level 2) with accurate mass/isotopic fine structure or spectral similarity, for some case where isomeric overlap occurred and/or in absence of reference MS/MS spectra, metabolites were annotate on level 3 and thus assigned to a biochemical class [16].

2.8. Statistical analysis

Statistical analysis of systolic, diastolic blood pressure and heart rate was performed using two-way ANOVA followed by Bonferroni’s multiple comparisons test. Two-way ANOVA followed by Sidak’s multiple comparisons test was used for weight comparison. Unpaired t-test was used for α-SMA and CD68 comparisons between groups. For DI-FT-ICR-MS and UHPLC-HRMS/MS univariate and multivariate statistical analysis were conducted with normalized and pareto-scaled HRMS data using MetaboAnalyst (v. 4.0, http://www.metaboanalyst.ca), data from both ionization polarities were treated simultaneously. The normalized variables obtained from data processing were visualized in a Volcano Plot to show which variables present a stronger combination of fold change (FC) and statistical significance (p-value) from a Student t-test, features with fold change >1, <−1 and p value <0.05 were considered statistically significant. Principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) was used to visualize discrimination among the two groups, the validity and robustness of the PLS-DA model was evaluated using the coefficient R² (model-fit) and the coefficient Q² (predictive ability), respectively, using the 5-fold internal cross-validation method. The loading plot was used to identify significant metabolites responsible for maximum separation in the PLS-DA score plot, and these metabolites were ranked according to their variable influence on projection (VIP) scores. Metabolic pathways and Whisker box plot were created and visualized using Metaboanalyst.

3. Results and discussion

3.1. SP6 treatment in HFD ApoE−/− mice reduces aorta monocytes/macrophages infiltration without affecting hemodynamic parameters

In a previous paper [8] we characterized a novel allophycocyanin derived peptide, named SP6, that at the dose of 10 mg/kg of SP6 reduced systolic blood pressure in an experimental model.
of arterial hypertension. Considering the important hemodynamic effect evoked by acute high-dosage treatment with SP6, here we chronically treated HFD ApoE knockout mice with a lower-dosage (5 mg/kg) of SP6, which, while is not sufficient to modulate blood pressure, is still associated with a significantly improved vascular function in mesenteric arteries [8]. In this case we monitored its possible action of systolic, diastolic blood pressure and heart rate during all six weeks of gavage administration. As showed in Fig. 1, the monitoring of all three cardiovascular parameters measured revealed that chronic treatment with a dosage of 5 mg/kg/day for 42 days did not significantly change blood pressure (Fig. 1a, 1b) and heart rate (Fig. 1c) compared to control animals treated with saline alone. Interestingly, the weight of the animals progressively increases with the weeks of high-fat diet (Fig. 1d), in line with what has already been reported in the literature, thus confirming the metabolic effect of HFD. Furthermore, we have stained mice aortic arch with the important muscular marker of vascular re-organization, alpha-smooth muscle actin (α-SMA), and with monocytes/macrophages marker CD68 to evaluate the possible impact of SP6 treatment on this phenomenon (Fig. 1e, f). Interestingly, while the mean-area-quantification of α-SMA did not show significant differences between saline and SP6 group, the area-quantification of CD68 signal highlights an important reduction of the red positive area, thus suggesting a potential beneficial effect of SP6 on the mechanisms that regulates the monocytes/macrophages vessels accumulation. It is well-known that one of the first processes that acts as key factor of the atherosclerotic event, is represented by the recruitment of monocytes/macrophages to the vessels wall, which favors the lipid accumulation and inflammation [17], despite this, the complex molecular mechanisms involved in plaque progression still remain obscure [18]. Based on these results, to elucidate the possible modulation of molecular pathways by SP6 and translate into useful information for pharmacodynamic comprehension, we moved to evaluate the metabolomic changes by an untargeted DI-FT-ICR-MS platform.

3.2. DI-FTICR MS analysis

The DI-FT-ICR method used an automated nanoelectrospray direct infusion system and the whole analysis took roughly 1.50 min per sample comprising sample draw, analysis time and tip change. This considerably lower than UHPLC-HRMS/MS methods (RPLC: 19 min, HILIC: 16 min, figure S1), moreover, since no single chromatographic method is able to resolve both lipids and polar metabolites, usually RPLC and HILIC or capillary electrophoresis (CE) are used in combination [19], which can result in an exponential increase of the overall analysis time. FT-ICR spectra of polar and lipid extracts, with both ionization, are reported in Fig. 2a. Mass accuracy values were on average 0.10 and 0.22 for polar metabolites and lipids respectively, which reflect the extreme mass accuracy of FT-ICR measurement, that, together with high resolution and isotopic fine structure (ISF) ability, leads to unambiguous molecular formula assignment, also for compound with several heteroatoms, such as the metabolite indoxyl sulphate, whose signal enlargement, simulated iso-plot and molecular formula are depicted in Fig. 2b, resulting in a very low mass error (0.08 ppm) and a calculated resolution of 290.757. In this study QC samples were prepared and employed to monitor the repeatability of the DI-FT-ICR workflow and were run randomly during the batch. In this regard, the coefficient of variation (CV%) relative to peak intensity was comprised between 0.11 and 10.95 % for polar extract sample (relative to the m/z range 100–750), and between 0.59 and 11.19 % for lipid extract sample (relative to the m/z range 150–885) indicating an acceptable repeatability for both metabolite classes (supporting Table 1). Furthermore, a satisfactory clustering of QC samples can be appreciated from PCA scores plot (figure S2) of both metabolite and lipids datasets, indicating that the quality of data was acceptable for the next steps of the study.

3.3. Multivariate data analysis of plasma samples

Data preprocessing was based on sample filter to remove peaks that were not present in at least 80 % of a single group, maximum deviation for mass accuracy was set to 1 ppm for DI-FT-ICR annotation and molecular formula generation, table S1 reports the whole bucket table features after filtering, for lipids and metabolites in both ionization modes. The Volcano plot representation of significant metabolites (FC > 1 or < -1, p < 0.05) of saline and SP6 treated peptides is reported in figure S3. PLS-DA was used to investigate the metabolic changes in high fat diet ApoE−/− mice after the treatment with SP6 peptide with respect to those who received saline alone. Fig. 3a, d shows the results of PLS-DA model for plasma polar and lipids extracts, as can be appreciated, the two groups are clearly separated. In particular for polar sample the first component explains 24.5 % of the variance, while the second explains 23.9 %, instead, for lipid sample, values were 17.9 % and 8.8 % respectively, with class discrimination parameters R2: 0.98, 0.99, Q2: 0.95, 0.92, for polar (Fig. 3b) and lipid sample respectively (Fig. 3e), which indicate a robust multivariate model. Metabolites that contributed to the clustering and discrimination were extracted based on the variable importance of projection (VIP), which were generated after PLS-DA processing. The first 15 metabolites with highest VIP scoring of both polar and lipid extracts, are reported in Fig. 3c, f.

3.4. Annotation of statistically relevant metabolites

Table 1 reports the putative annotation of metabolites with VIP score >1.2 and p < 0.05 showed in Fig. 3c, f, which were considered to be most influential for the separation between saline group and those which received SP6 peptide administration. Despite DI-FT-ICR delivers ultra high mass accuracy, this data alone often cannot differentiate the large amount of isomers occurring in biological samples, and thus additional information, such as MS/MS fragmentation pattern comparison against a reference spectra, is mandatory for reliable metabolite annotations [20]. For this reason we performed in parallel two UHPLC-HRMS/MS distinct methods for lipids and polar metabolites; in particular all statistically relevant metabolites annotated by DI-FT-ICR method were checked by fragmentation pattern and comparison with reference MS/MS spectra, accurate mass, isotope pattern and retention time, obtained on the Q-Exactive platform. In the largest part it was possible to support the annotation of several compounds, such as acyl or alkyl lysophosphatidylcholines [21], differentiate sphingomyelins (SM) between ceramide phosphorylethanolamines (PE-Cer) with the former that are characterized by diagnostic ions at 184 and 264 m/z [22] while the latter (that were not detected) by the neutral loss of 141 m/z [23], or further enforce the annotation of polar metabolites. Clearly, for lipids assignment of double bond position and precise isomer annotation requires a more detailed analysis. HCD MS/MS spectra of some relevant metabolites are reported in Fig. 4a-d and figure S4. Where MS/MS data were not available, such as for the four metabolites labelled with asterisk in Table 1, they were tentatively assigned to chemical classes and reported with MSI level 3. Noteworthy UHPLC-HRMS/MS confirmed also the statistical trend observed for DI-FT-ICR method (figure S5).

3.5. Plasma metabolic differences between HFD ApoE−/− and SP6 treated mice

Different metabolite classes were found dysregulated, and in particular hydroxyl and tricarboxylic-organic acids, amino acids, lysophosphatidylcholines, sphingomyelins, and other glyc-
erophospholipids. To visualize and identify the metabolic pathways influenced by the SP6 peptide administration, the HMDB identities of significant altered metabolites (p < 0.05) were imported in the pathway analysis tool of Metaboanalyst 4.0 to construct the metabolic maps which are reported in Fig. 5a, b. Among the pathways that showed significant perturbations were sphingolipids and glycerophospholipids metabolism, fatty acid biosynthesis, tricarboxylic acid cycle (TCA), and glutamate/glutamine metabolism.

3.6. Sphingolipids and glycerophospholipids metabolism

As can be appreciated from the box-plots in Fig. 5c, several sphingomyelins (SM) were increased in saline HFD ApoE−/− group, and, in this class, in particular: 34:1, 36:1, 42:2; these results are in accordance with several HFD ApoE−/− models [24,25]. Both in vitro and in vivo studies have demonstrated the links between SM and atherosclerosis, in fact, the inhibition of SM synthesis prevents the development of atherosclerotic lesions, and reduces atherosclerotic plaque area [26], moreover SM 34:1 and 42:2 have been positively correlated to mortality in vascular and metabolic diseases [27]. Interestingly, after the four weeks treatment with peptide SP6, the levels of all the corresponding sphingomyelins were decreased. Notably these were among the lipids with the highest statistical influence (VIP ≥ 2). Regarding glycerophospholipids, several metabolites were differently modulated in the saline alone HFD ApoE−/− group, in accordance with previous relevant findings on induced HFD obese mice models [28,29]. Plasma levels of numerous lysophosphatidylcholines (LysoPCs) were reduced in saline group (LysoPCs: 14:0, 15:0, 16:0, 16:1, 18:1, 18:2, 19:0, 22:5, Fig. 5c, Fig. 5f) which is in agreement with previous lipidomic analyses in HFD obese mice models [30]. Decreased LysoPCs levels have been also found in inflammatory status and their composition can play
Table 1

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<th>Ion type/Adduct</th>
<th>Name</th>
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<th>Molecular Formula</th>
<th>Biochemical Class/Sub class</th>
<th>VIP value</th>
<th>p.value</th>
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|indicates compounds which MS/MS spectra were unavailable and/or where molecular formula was shared among different metabolites, these were generally assigned to a class of compounds. Detailed MS/MS spectra of relevant metabolites are reported in Fig. 4 and S4, complete UHPLC-HRMS/MS data are reported in table S1.

Fig. 5. A-C: Pathway analysis of metabolic routes influenced by the administration of SP6 peptide in HDF ApoE-/- mice model. Pathway analysis was performed on all significantly altered metabolites (p < 0.05) between the two groups. Whisker box plots (red color: saline only group, green color: SP6 treated group) relative to sphingomyelins A), LysoPCs B), Tricarboxylic acids C), amino acids D).

potential protective role in cardiovascular diseases (CVDs), in particular LysoPCs 16:0, 18:0 and 18:2 have been negatively associated with CVDs mortality [27]. Opposite to saline group, the trend of the listed LysoPCs was increased in SP6 treated group, and these metabolites were among the main contributors to class separation.

3.7. TCA cycle, amino acids and other metabolites

TCA cycle, the terminal of fat oxidation, is dysregulated in diet induced obesity and diabetic models, and results in inflammation process and oxidative stress [31]. TCA cycle related metabolites citric, malic and lactic acid levels were higher in the saline ApoE-/-
group (Fig. 5c), which correlates with similar results in rabbit atherosclerotic models, where the increased levels of TCA intermediates translates into enhanced aerobic fatty acid oxidation and anaerobic pathways for ATP production in macrophage-rich atherosclerotic arteries [32]. Contrariwise their levels were reduced in the SP6 treated group. Among amino acids, glutamate and glutamine have been often recognized as markers of atherosclerosis development and CVD risk. r-glutamate alteration has been associated to pro-atherogenic effects, through an increased triglycerides accumulation in macrophages [33], whereas glutamine has been linked with clinical evidences of atherosclerosis, since it has been associated with higher risk for both plaque development and increased intima–media thickness [34]. Glutamate and glutamine levels were upregulated in the saline group, while they were both decreased in SP6 group (Fig. 5c). Among other altered metabolites, particularly interesting are the two uremic toxins indoxyl sulphate and p-cresyl sulphate. In fact, these two metabolites have been associated to several diseases such as endothelial dysfunction, atherosclerosis and hypertension and have been found upregulated in plasma and urine of obesity models [35]. Both p-cresol and indoxyl sulphate levels were higher in ApoE−/− saline alone group, while reduced in SP6 (Fig. 5, figure S6). Overall, the metabolic profile carried out by DI-FT-ICR points out a significant change of several metabolites after four week treatment with SP6 peptide, resulting in the modulation of key markers of atherosclerosis and dyslipidemia, and the crosstalk with multiple molecular pathways. Clearly, being this a pilot study, the obtained findings should be considered in the context of study limitations, such as a small sample size, and the need of further histological and biochemical investigations, such as correlation with lipoproteins, triglycerides and cholesterol levels, as well as analysis of other vessels for plaque evaluation and immunological studies. Nevertheless, these results show a distinct significant metabolic response induced by SP6 treatment, that, besides its hypotensive and vasodilating potential at higher doses, could be a promising alternative for adjuvant or preventive therapies in atherosclerosis at lower dosage. The analytical approach used allows to take a rapid and accurate “snapshot” of both polar and non-polar metabolite changes in very short time, and overcomes the limitation of LC–MS methods, such as longer analysis time and drift of retention times. DI-FT-ICR could be easily adapted for larger sample numbers, which will employ other animal models, diet conditions and comparison with drugs employed in cardiovascular diseases, which are necessary to explore the pharmacodynamics of SP6 peptide, before moving to UHPLC-HRMS/MS methods for confirmation of metabolite annotation or targeted LC–MS/MS approaches for quantification purposes.

4. Conclusions

In this study, a fast and accurate direct infusion FT-ICR based metabolic profiling was applied for the preliminary evaluation of the effect of the novel spirulina peptide SP6 in a model of atherosclerosis, namely high fat diet ApoE−/−. DI-FT-ICR analysis with a short analysis time of 1.50 min per sample delivered high mass accuracy and resolution, together with good repeatability. The statistical analysis showed robustness and allowed to elucidate a significant modulation of different metabolic routes caused by the administration of SP6 peptide, comprising of key metabolites which are involved in atherosclerotic plaque progression and development. These preliminary results encourage to extend the investigation of the possible employment of SP6 peptide in the prevention and treatment of multifactorial cardiovascular diseases.


M.N. Barber, S. Riis, C. Yang, P.J. Meikle, M. Staples, M.A. Febbraio, C.R. Bruce, Plasma Lysophosphatidylcholine Levels Are Reduced in Obesity and Type 2 Diabetes, PloS One 7 (2012) e41456.


