Elucidation of metabolic changes in HFD-ApoE$^{-/-}$ model by SP6 peptide: A flow injection analysis magnetic resonance mass spectrometry (FIA-MRMS) study

Natural peptides have emerged as an attractive option for the treatment of cardiovascular diseases. A novel peptide from *Spirulina Platensis* (SP6) was evaluated for its potential anti-atherosclerotic effect in a high fat diet ApoE$^{-/-}$ mice model using a metabolomics approach.

Abstract

The employment of natural peptides in the treatment of cardiovascular diseases is an attractive option to pharmacological therapy. In this study, a novel antihypertensive peptide from *Spirulina Platensis* (SP6) was employed to evaluate its potential anti-atherosclerotic effect in high fat diet ApoE$^{-/-}$ mice model. Untargeted metabolomics and lipidomics was performed by a Flow Injection Analysis Magnetic Resonance Mass Spectrometry (FIA-MRMS) approach, resulting in high mass resolution and mass accuracy as well as repeatability and fast analysis time. Results showed a distinct metabolic switch, with the positive modulation of several key markers of atherosclerosis progression, such as sphingolipids and glycerophospholipids, amino acids and tricarboxylic acid (TCA)

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Authors: Eduardo Sommella[1], Anastasia Kalli[2], Fabrizio Merciai[1,3], Albino Carrizzo[4,5], Marina Sala[1], Pietro Campiglia[1,6];
[1] Department of Pharmacy, University of Salerno, Fisciano, SA, Italy; [2] Bruker Scientific LLC, Billerica, Massachusetts 01821, United States; [3] PhD Program in Drug Discovery and Development, University of Salerno, Fisciano, SA, Italy; [4] Department of Medicine and Surgery (A.C., C.V.), University of Salerno, Baronissi SA, Italy; [5] IRCCS Neuromed, Loc. Camerelle, Pozzilli, IS, Italy; [6] European Biomedical Research Institute of Salerno, Salerno, Italy

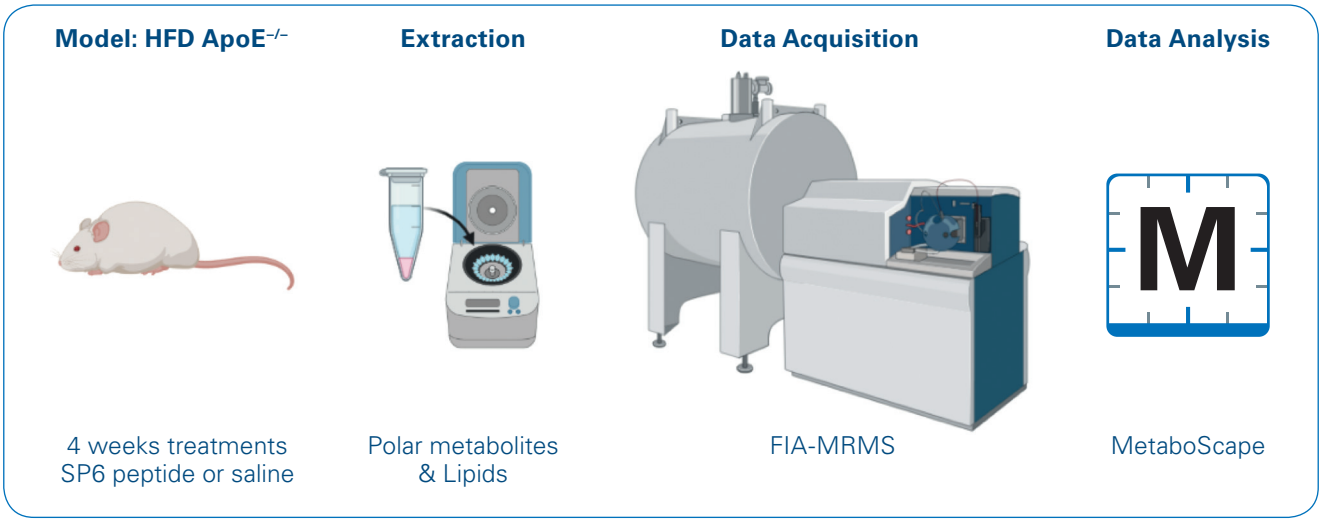

Figure 1. Workflow for the extraction and analysis of metabolites and lipids from high fat diet ApoE$^{-/-}$ mice atherosclerotic model.

cycle intermediates. The FIA-MRMS approach has been proven to be a fast and effective tool for large scale investigation of SP6 effects in large cohorts.

Introduction

Metabolic disorders such as hypertension and dyslipidemia are comorbid pathologic conditions often found in combination and characterized by a deep connection of altered molecular pathways. The employment of natural compounds in combination to pharmacological treatment can be an attractive option as preventive therapy. Natural peptides are among the most employed compounds. In this regard a novel antihypertensive decameric peptide has been recently characterized from the gastrointestinal digest of *Spirulina platensis* microalgae [1]. To understand the molecular pathways that are influenced by bioactive compounds, metabolomics has emerged as a leading approach. High resolution mass spectrometry (HRMS) is the workhorse for metabolomics applications, and MRMS due to its high mass accuracy and resolution, facilitates phenotyping studies. In this regard, the objective of this work was to investigate the effect of the SP6 peptide in a mice model of atherosclerosis (high fat diet ApoE$^{-/-}$) and evaluate the altered molecular pathways by a FIA-MRMS approach, termed MRMS aXelerate®. Results showed evidence of a distinct modulation of key molecular analytes involved in the disease development and open the way to further large-scale studies.

Methods

Animal treatment

All animal studies were performed in accordance with approved protocols by the IRCCS Neuromed 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 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 week 11 for 1 week. Subsequently, in the next week they were treated by gavage with saline solution (N = 4) or with SP6 (N = 5) daily for 4 weeks. 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.

Sample preparation

Polar metabolites and lipids were extracted according to the Matyash protocol [2]. The workflow is shown in Figure 1.

FIA-MRMS analysis and processing

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, USA), 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 7T solariX XR. 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 accu-

mulation of 10 ms, 32 scans were acquired using 2 million data points (2 M). Drying gas (nitrogen) was set at 2 mL/min, with a drying gas temperature of 150°C. Funnel amplitude was set to 90 V (polar metabolites) or 100 V (lipids), ion transfer was set to 6 MHz, and TOF 0.7 ms. Both positive and negative ESI ionization were employed in separate experiments. Data analysis was performed with MetaboScape® (v.5.0). For metabolite annotation, assignment of the molecular formula was performed for the detected features using smartFormula (SF), isotopic fine structure (ISF) and data recalibration. The bucket table was annotated with a list of metabolites and lipids obtained from the HMDB and LIPIDMAPS database, respectively. Annotation was performed with 0.2 ppm (narrow) or 1 ppm (wide) mass tolerance and a mSigma value below 200.

Results

The FIA-MRMS method used an automated nanoelectrospray direct infusion system. The sample analysis time was less than 2 min including sample draw, analysis time and tip change. Mass accuracy values were on average 0.10 and 0.22 ppm for the detected polar metabolites and lipids respectively, which reflects the extreme mass accuracy of the MRMS platforms that, together with high

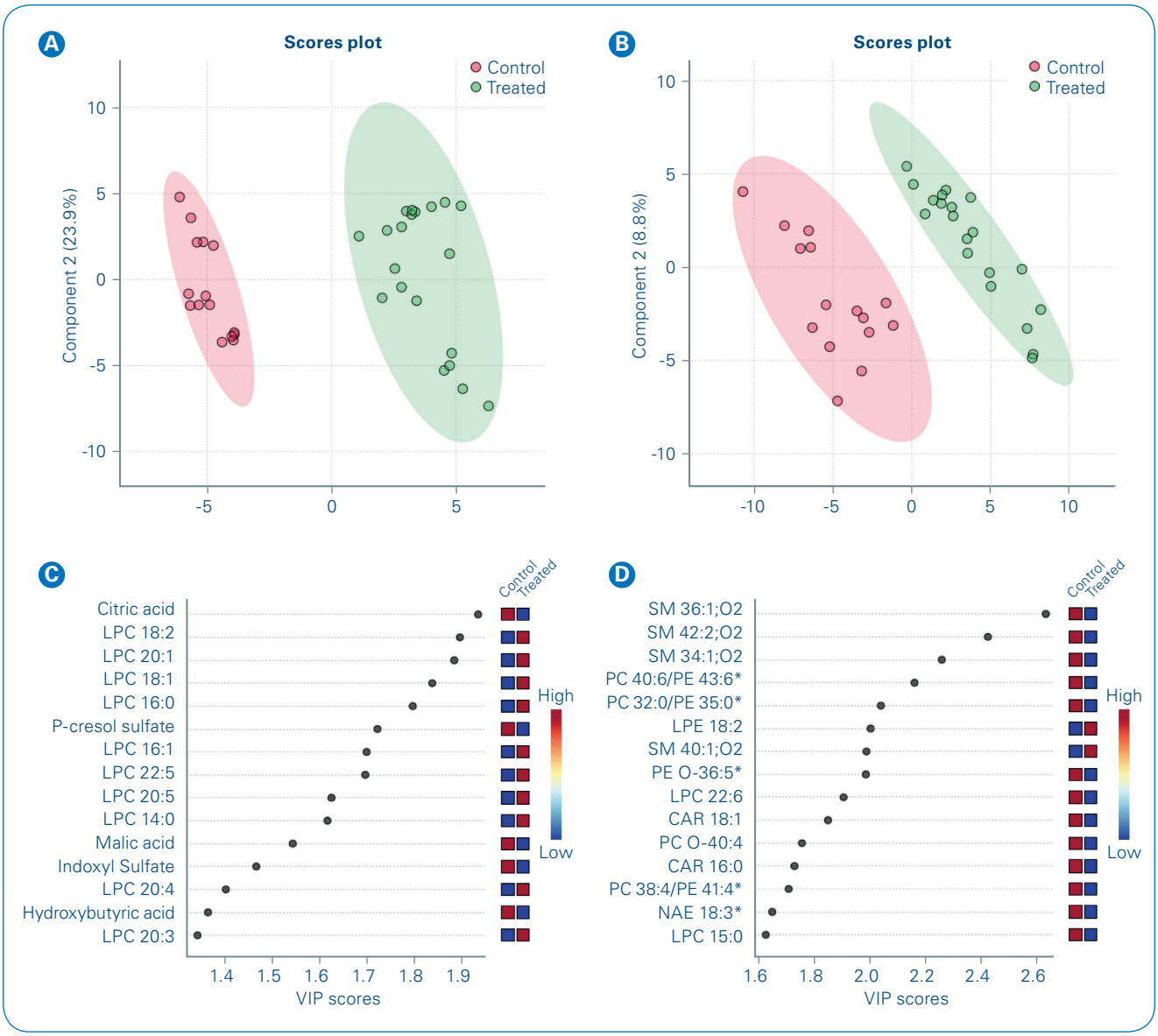

*Figure 2. PLS-DA score plot of polar (A) and lipid (B) plasma extracts in HFD ApoE$^{-/-}$ mice treated with saline alone (red circle) and those treated with Spirulina peptide SP6 (green circle). The first 15 metabolites with the highest VIP scoring of both polar and lipid extracts, are shown in figure (C) and (D). Metabolite annotations displayed in 2C and 2D were confirmed by LC-MS/MS. * indicates compounds which MS/MS spectra were unavailable and/or cases in which molecular formula was shared among different metabolites. These were generally assigned to a class of compounds.*

Table 1. Summary of statistical relevant annotated metabolites derived from PLS-DA analysis.

m/z	Ion type/ adduct	Assignments	Molecular Formula	Mass Error (ppm)	Biochemical Class/Sub class	VIP value	p.value
103.04007	$[M-H]^-$	Hydroxybutyric acid	$C_4H_8O_3$	0.03	Alpha hydroxy acids and derivatives	1.36	$3.30E^{-15}$
133.01424	$[M-H]^-$	Malic acid	$C_4H_6O_5$	-0.08	Beta hydroxy acids and derivatives	1.53	$5.20E^{-12}$
187.00704	$[M-H]^-$	p-Cresol sulfate	$C_7H_8O_4S$	-0.04	Arylsulfates	1.70	$4.70E^{-07}$
191.01970	$[M-H]^-$	Citric acid	$C_6H_8O_7$	-0.05	Tricarboxylic acids and derivatives	1.91	$3.00E^{-06}$
212.00228	$[M-H]^-$	Indoxyl sulfate	$C_8H_7NO_4S$	-0.14	Arylsulfates	1.45	$1.10E^{-05}$
322.27413	$[M+H]^+$	NAE 18:3*	$C_{20}H_{35}NO_2$	0.27	Fatty amides*	1.43	$1.15E^{-02}$
400.34224	$[M+H]^+$	CAR 16:0	$C_{23}H_{45}NO_4$	0.40	Fatty acyl carnitines	1.53	$6.65E^{-03}$
426.35789	$[M+H]^+$	CAR 18:1	$C_{25}H_{47}NO_4$	-0.21	Fatty acyl carnitines	1.55	$1.70E^{-04}$
478.29294	$[M+H]^+$	LPE 18:2	$C_{23}H_{44}NO_7P$	0.24	Glycerophosphoethanolamines	1.67	$3.30E^{-05}$
482.32422	$[M+H]^+$	LPC 15:0	$C_{23}H_{48}NO_7P$	0.19	Glycerophosphocholines	1.44	$1.23E^{-02}$
490.29033	$[M+Na]^+$	LPC 14:0	$C_{22}H_{46}NO_7P$	0.12	Glycerophosphocholines	1.60	$8.90E^{-04}$
516.30599	$[M+Na]^+$	LPC 16:1	$C_{24}H_{48}NO_7P$	-0.07	Glycerophosphocholines	1.68	$1.80E^{-03}$
518.32162	$[M+Na]^+$	LPC 16:0	$C_{24}H_{50}NO_7P$	-0.14	Glycerophosphocholines	1.78	$2.10E^{-03}$
542.32164	$[M+Na]^+$	LPC 18:2	$C_{26}H_{50}NO_7P$	-0.54	Glycerophosphocholines	1.87	$3.60E^{-03}$
544.33724	$[M+Na]^+$	LPC 18:1	$C_{26}H_{52}NO_7P$	-0.59	Glycerophosphocholines	1.81	$4.70E^{-03}$
564.30598	$[M+Na]^+$	LPC 20:5	$C_{28}H_{48}NO_7P$	0.17	Glycerophosphocholines	1.60	$1.00E^{-02}$
566.32163	$[M+Na]^+$	LPC 20:4	$C_{28}H_{50}NO_7P$	-0.08	Glycerophosphocholines	1.40	$6.90E^{-12}$
568.33711	$[M+Na]^+$	LPC 20:3	$C_{28}H_{52}NO_7P$	-0.25	Glycerophosphocholines	1.33	$1.80E^{-02}$
568.3400	$[M+H]^+$	LPC 22:6	$C_{30}H_{50}NO_7P$	-0.07	Glycerophosphocholines	1.59	$1.20E^{-04}$
572.36859	$[M+Na]^+$	LPC 20:1	$C_{28}H_{56}NO_7P$	-0.10	Glycerophosphocholines	1.86	$1.80E^{-02}$
592.33726	$[M+Na]^+$	LPC 22:5	$C_{30}H_{52}NO_7P$	-0.15	Glycerophosphocholines	1.68	$2.30E^{-02}$
703.57504	$[M+H]^+$	SM 34:1;O2	$C_{39}H_{79}N_2O_6P$	0.51	Sphingomyelins	2.00	$2.90E^{-06}$
724.52789	$[M+H]^+$	PE O-36:5*	$C_{41}H_{74}NO_7P$	0.80	Glycerophosphoethanolamines*	1.80	$2.50E^{-05}$
731.60637	$[M+H]^+$	SM 36:1;O2	$C_{41}H_{83}N_2O_6P$	0.23	Sphingomyelins	2.22	$8.70E^{-11}$
734.56966	$[M+H]^+$	PC 32:0/PE 35:0*	$C_{40}H_{80}NO_8P$	0.54	Glycerophospholipids*	1.85	$1.80E^{-06}$
787.66893	$[M+H]^+$	SM 40:1;O2	$C_{45}H_{91}N_2O_6P$	0.43	Sphingomyelins	1.69	$3.10E^{-05}$
810.60095	$[M+H]^+$	PC 38:4/PE 41:4*	$C_{46}H_{84}NO_8P$	0.51	Glycerophospholipids*	1.50	$6.65E^{-03}$
813.68478	$[M+H]^+$	SM 42:2;O2	$C_{47}H_{93}N_2O_6P$	0.76	Sphingomyelins	2.12	$2.50E^{-09}$
824.65303	$[M+H]^+$	PC O-40:4	$C_{48}H_{90}NO_7P$	0.57	Glycerophosphocholines	1.60	$1.40E^{-04}$
834.60101	$[M+H]^+$	PC 40:6/PE 43:6*	$C_{48}H_{84}NO_8P$	0.60	Glycerophospholipids*	1.90	$7.50E^{-07}$

*Metabolite annotations were confirmed by LC-MS/MS. * indicates compounds which MS/MS spectra were unavailable and/or cases in which molecular formula was shared among different metabolites. These were generally assigned to a class of compounds.*

resolution and isotopic fine structure (ISF) ability, leads to unambiguous molecular formula assignment. The coefficient of variation (CV%) relative to peak intensity was 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 a satisfactory repeatability for both metabolite classes. Data preprocessing was based on sample filter to remove peaks that were not present in at least 80% of a single group. Figures 2a and 2b show the results of PLS-DA model for plasma polar and lipids extracts, demonstrating that the two groups are clearly separated. Metabolites that contributed to the clustering and discrimination were extracted based on the variable importance in 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 Figure 2c and 2d. Different metabolite classes were found dysregulated, and in particular: hydroxyl and tricarboxylic-organic acids, amino acids, lysophosphatidylcholines, sphingomyelins, and other glycerophospholipids (Table 1). The treatment with SP6 was able to modulate the levels of these key metabolites which are involved in atherosclerotic plaque progression and development.

Conclusion

- The peptide SP6 was initially evaluated in a model of atherosclerosis.
- MRMS aXelerate® was used for untargeted metabolomics and lipidomics.
- The MRMS aXelerate® workflow delivers high mass accuracy, resolution, repeatability and fast analysis time.
- The modulation of key markers of atherosclerosis progression was demonstrated.
- The results open the way to a large-scale study of SP6 preventive treatment.

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Bruker Daltonics GmbH & Co. KG

Bremen · Germany
Phone +49 (0)421-2205-0

Bruker Scientific LLC

Billerica, MA · USA
Phone +1 (978) 663-3660

ms.sales.bdal@bruker.com – www.bruker.com