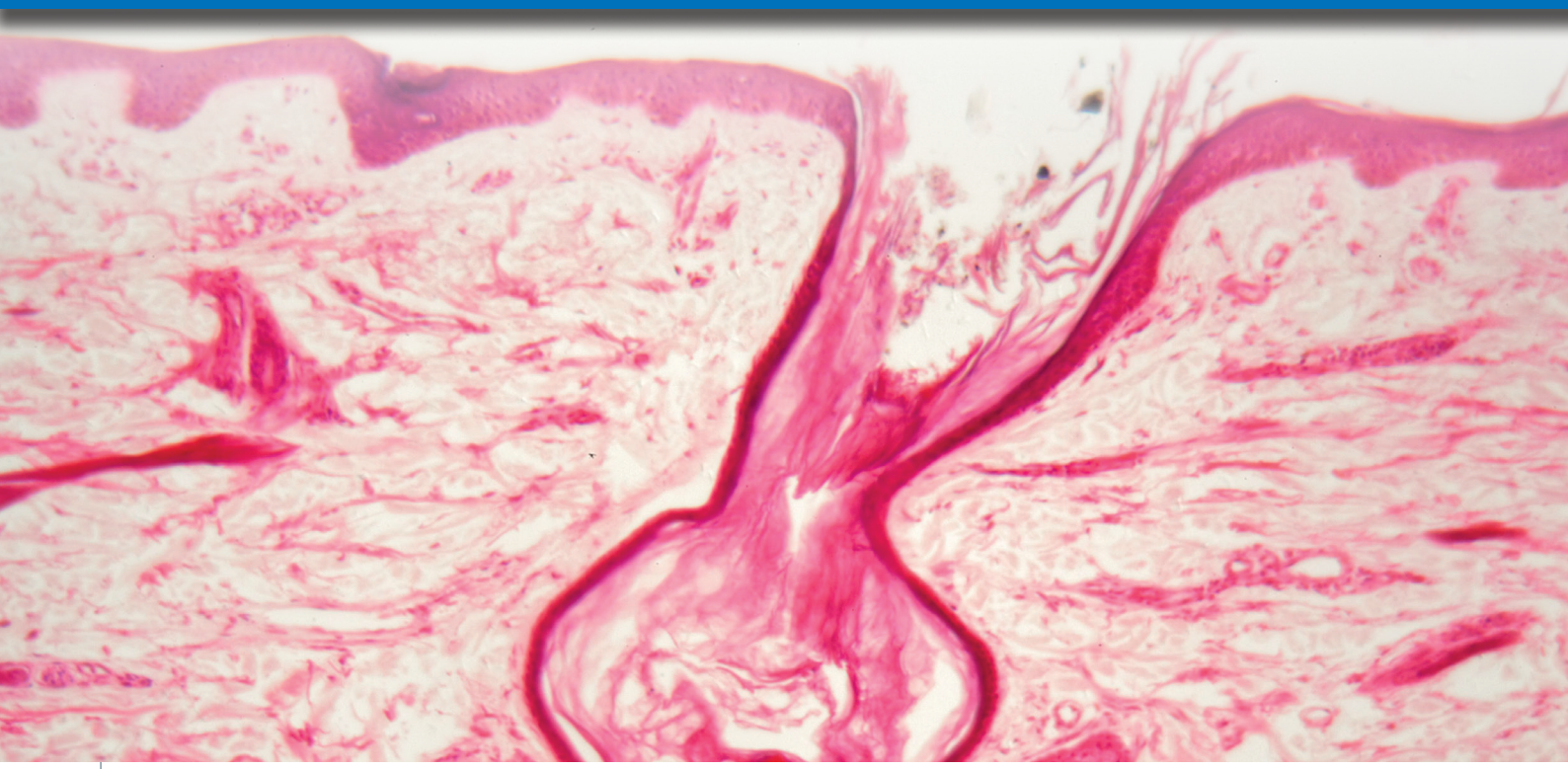

Analysis of metabolic changes in murine hair follicles treated with Procyanidin-B2 rich nutraceuticals by DI-MRMS

Known for anti-inflammatory and antioxidant properties, nutraceuticals enriched in Procyanidin-B2 promote hair growth both in vitro and in vivo. However, the metabolic changes associated with the treatment have not been elucidated.

Abstract

In this study, direct infusion magnetic resonance mass spectrometry (DI-MRMS) was employed to understand the metabolic shift produced by treatment with Procyaindin-B2 nutraceuticals (Annurca apple extract) in murine models. DI-MRMS allowed the identification of several metabolites using ultra-high mass accuracy and fast analysis time, glutaminolysis, pentose phosphate pathway, glutathione, citrulline and nucleotide synthesis derived metabolite were detected. The metabolic profile revealed that the treatment with Procyanidin-B2 results in the early exit of hair follicles from telogen phase and increased keratin biosynthesis.

Keywords: Metabolomics, Metabolite, MetaboScape, MRMS, solariX

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Introduction

Patterned hair loss affects around 50% of the adult population worldwide. Independent of age and gender, this condition exerts a profoundly negative impact on people's quality of life and is frequently associated with depression, especially when occurring at an early age. Among pharmacological treatments, Finasteride and Minoxidil are FDA approved drugs. However, Finasteride and Minoxidil activity reaches a plateau within two years of usage and both drugs produce adverse effects on patients. In the past decade an increasing number of reports have proven nutritional and antioxidant therapies to be an effective and safe treatment as an option for hair loss. Among these, nutraceuticals enriched in Procyanidin-B2 (Figure 1), a dimeric Procyanidin, such as Annurca apple extract (AAE), have been recently shown to promote hair growth and induce anagen phase in humans. This has resulted in the increased usage of these types of nutraceuticals for hair growth over the last several years. However, studies reveal this mechanism is far from being complete. Here, the metabolic profile of AAE treatment on mice hair follicles was studied to give new insights into the promotional growth effects of Procyanidin-B2.

Methods

Animals

Wild-type C57/BL6 mice (7 weeks old, postnatal day 42) were used in all experiments to test the effect of cosmetic foam containing AAE. All animals received humane care and were maintained in separate cages at 22°C – 24°C and fed a general rodent diet. Differently from other published protocols, here animals were left unshaved and topically treated with 2 cm^3 of the indicated cosmetic foam for 4 weeks, twice a week. Only male animals were used in this study. All animal experiments were performed in compliance with ethical guidelines and approved by the University of Naples Federico II.

Extraction

Mice tissues were rinsed and kept in PBS immediately after tissue excision. Hair shafts were plunked out with sterile tweezers and immediately covered with a solution of PBS at room temperature. To allow detachment of hair follicle cells, plunked hair shafts were incubated for 15 minutes in PBS supplemented with 5 mM EDTA. Hair shafts were then removed and the remaining hair follicle cells centrifuged for 5 minutes at 500 rpm.

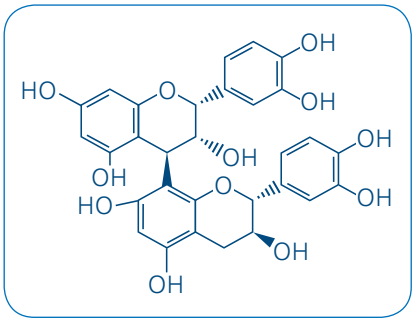

Figure 1: Structure of Procyanidin-B2

The cell pellets were washed twice in PBS and homogenized in 1 ml of pre-chilled methanol/water 80:20 solution and finally centrifuged at 10,000 g for 10 min at 4°C. The resulting supernatants were collected and transferred into new Eppendorf tubes and stored at -80°C.

MS analysis

Analyses were performed by direct infusion electrospray ionization using a Hamilton syringe (250 µL) at a flow rate of 2 µL/min. Data were acquired on a MRMS solariX XR 7T. The instrument was tuned and calibrated with a standard solution of NaTFA. Mass spectra were recorded in broadband mode in the range m/z 100-1500 with an ion accumulation time of 20 ms. 32 single scans were added for the final mass

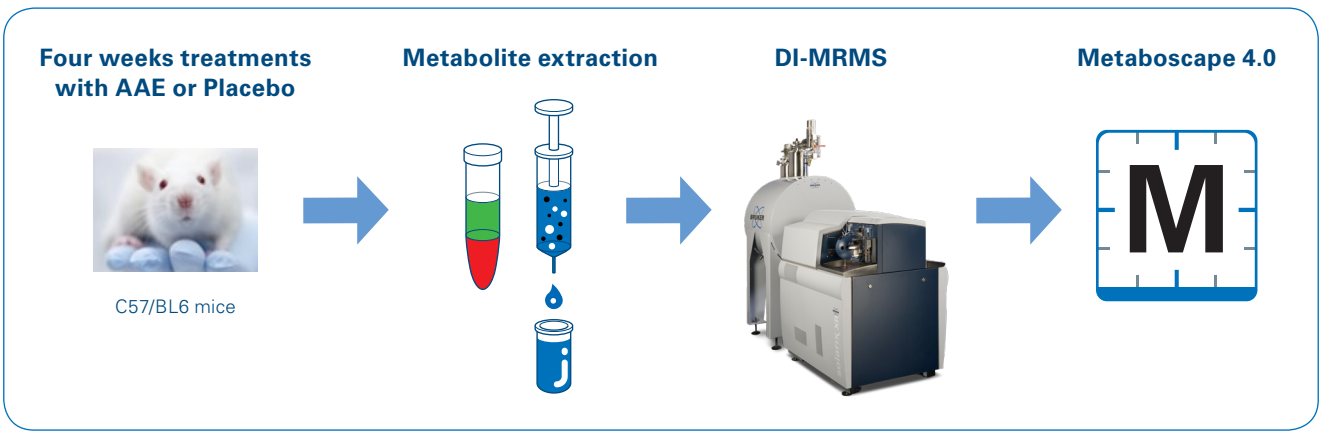

Figure 2: Workflow for analysis of the metabolite profile of mice hair follicles using DI-MRMS

spectrum. Data were acquired with 2 million data points. Nebulizing (N_2) and drying gases (N_2) were set at 1 and 4 L/min, respectively, using a drying gas temperature of 200°C. Spectra were acquired in positive and negative ion mode. The measurements were performed in five replicates (Figure 2).

Data processing

Peak alignment and tentative assignment of compounds was performed in MetaboScape 4.0 based on accurate mass measurements and a HMDB plasma analyte list. The feature calculation was performed with a mass resolution of 1 mDa. A bucket filter of 75% was used for replicate measurements and the values of the calculated features were recalibrated with accurate masses of compounds known in plasma. The accuracy of the isotopic with a maximum mSigma value of 50 was used for feature assignment. Statistical analysis with significant results were performed with Statistica® using two-way Anova and Bonferroni post tests.

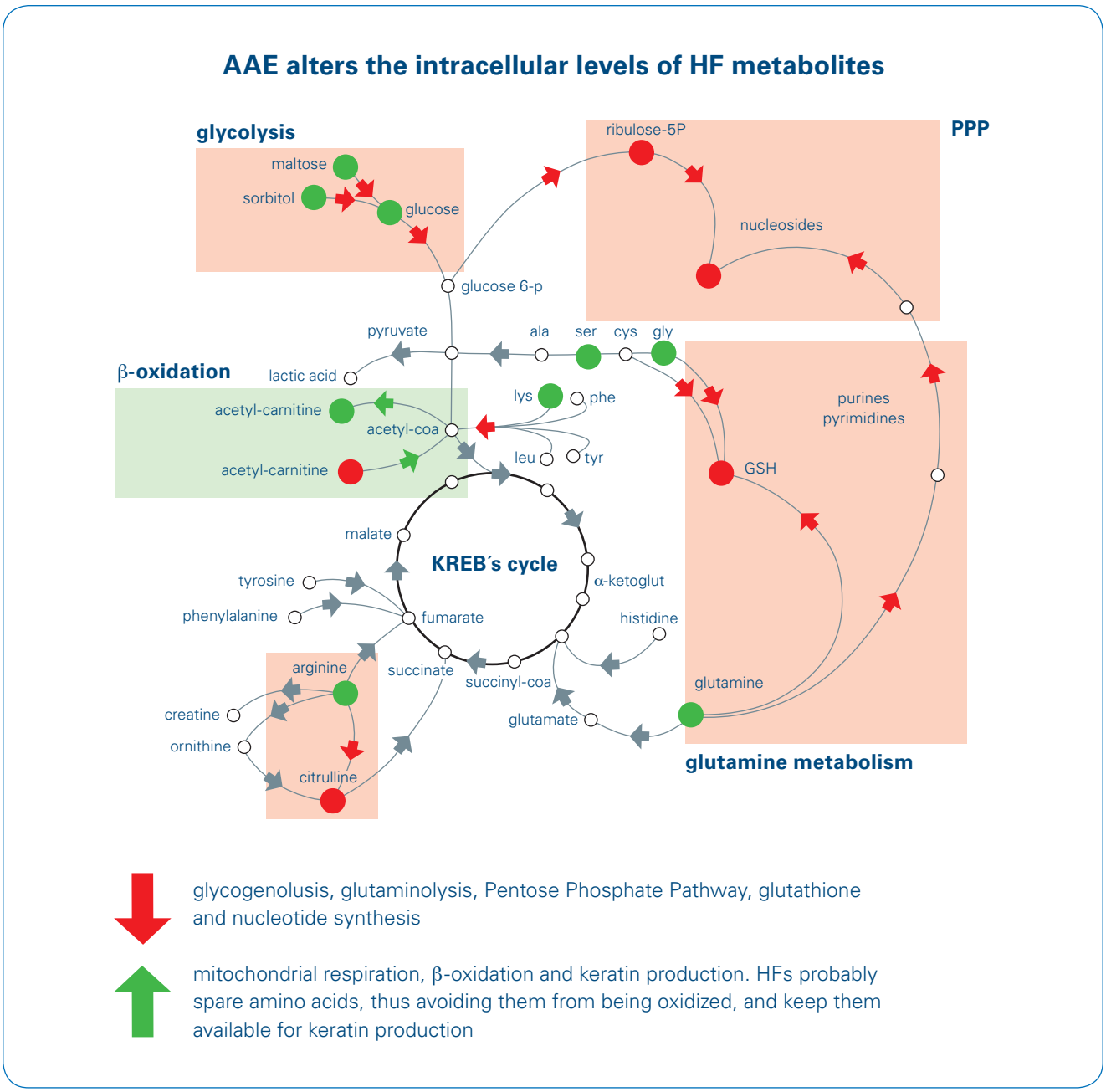

Figure 3: Modulation of mice hair follicles metabolites following treatment with apple Annurca foam

Metabolite	Pathway	m/z	Detected as	Mass error (ppm)
Glucose	Glycolysis	203.05265	$[M+Na]^+$	0.006
Lactic acid		113.02091	$[M+Na]^+$	-0.176
Maltose	Glycogenolysis	365.10543	$[M+Na]^+$	-0.012
Glutamine	Aminoacids	169.05836	$[M+Na]^+$	0.001
Arginine		197.10090	$[M+Na]^+$	0.029
Glutathione		306.07675	$[M-H]^-$	0.028
Citrulline		198.08495	$[M+Na]^+$	0.072
Adenosine	Nucleotides	290.08596	$[M+Na]^+$	0.028
Cytosine		266.07476	$[M+Na]^+$	0.015
Deoxy-Cytosine		250.07984	$[M+Na]^+$	0.044
Deoxy-Inosine		275.07507	$[M+Na]^+$	-0.091
Palmitoyl-carnitine	β-oxidation	422.32404	$[M+Na]^+$	0.148
Acetyl-carnitine		226.10501	$[M+Na]^+$	0.211

Table 1: List of relevant metabolites for treatment of hair follicles with apple Annurca foam detected by DI-MRMS

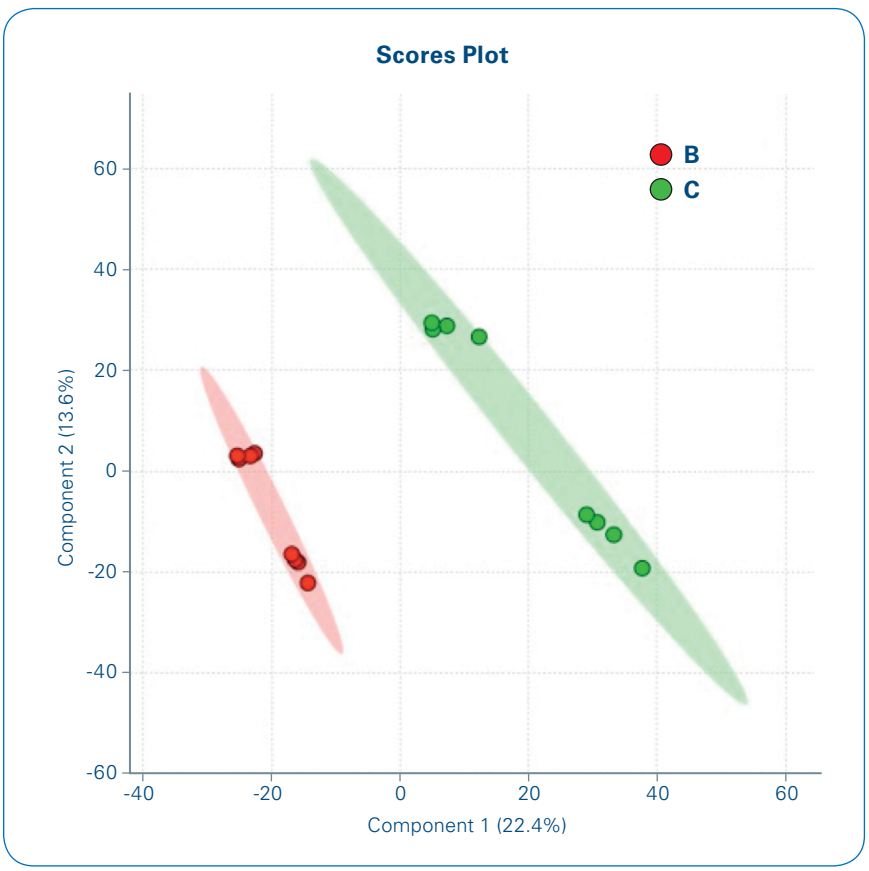

Figure 4: PLS-DA scores plot of control hair follicle (red) vs treated (green) with apple Annurca foam

Results

C57/BL6 mice were topically treated with a foam supplemented either with AAE or with a placebo. After 4 weeks of treatment, mice (11 weeks old) were sacrificed and their dorsal skin was excised. Skin biopsies were embedded in paraffin and prepared for histology. The metabolic content of hair follicle cells plucked out by mice were treated topically with AAE and analyzed by DI-MRMS mass spectrometry (workflow is shown in Figure 2). Complex profiles were obtained in positive and negative ion mode. The ultra-high mass accuracy (average mass error of only 0.166 ppm, Table 1), exact isotopic distribution and comparison with available standards ensured identification with high confidence. By screening intracellular metabolites with similar alteration tendency in all the AAE treated mice, glutaminolysis, pentose phosphate pathway (PPP), amino acid oxidation, mitochondrial β-oxidation as well as

Arginine metabolites became our focus. Significant elevation of glutamine and glycine as well as the increase in the intracellular level of the PPP intermediate Ribulose 5P together with the reduction of the intracellular level of nucleotides and deoxy-nucleotides suggest that AAE cause a reduction in the utilization of glucose and glutamine for PPP (Figure 3). This is a metabolic pathway that correlates with nucleotide bio-synthesis in hair follicles. The reduced intracellular level of glutathione also confirmed that the catabolism of glutamine is halted in AAE treated hair follicles. It can be concluded from PLS DA scores plot in Figure 4 that the hair follicles treated with AAE were well separated from the mice hair follicles treated with placebo. This verifies the observed results of the regulation of the metabolites shown in Table 1.

Overall, considering the results of SEM data (not shown here) and the metabolite profiles we could suggest that AAE diverts the intra-cellular metabolism of hair follicles from mainly set on PPP to a pool of selected amino acids to be used for keratin biosynthesis.

Conclusions

- Direct infusion MRMS can be used for fast and reliable metabolite profiling of hair follicle cells treated with Annurca apple extract.
- Several metabolites involved in different pathways could be detected and identified by DI-MRMS.
- A metabolic shift of hair follicle cells towards production of keratin was elucidated.
- A further test on a larger population is needed, as well as the employment of different Procyanidin rich extracts.

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