CLASSIFICATION OF CALVES BY THE METABOLIC PROFILE WITHOUT PREVIOUS HYPOTHESIS

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SUMMARY

In order to assess whether Metabolomics methodology could be applied to predict metabolic differences within a given set of animals without previous hypothesis for their classification, we applied multivariate statistical methods to data from UPLC-MS analysis of 16 calf plasma. Blood samples were taken at the age of 60 – 80 days.

For UPLC-MS analysis, metabolites were extracted with cold acetonitrile. An Acquity UPLC HSS T3 1.8 µm, 2.1 × 100 mm column with a pre-column (VanGuard 2.1 mm × 5 mm, 1.8 µm particle size) was used for the liquid-chromatography analysis (LC), which was performed in an Acquity™ Ultraperformance LC (UPLC®) from WATERS (Barcelona, Spain). Analyte detection was conducted in a mass spectrometer SYNAPT HDMS G2 (WATERS, Manchester, UK) fitted with an ESI source and time of flight analyser (ESI-QToF-MS) under positive ionization mode. The XS application of the MarkerLynx® software (WATERS, Manchester, UK) and the Metaboanalyst software (https://www.metaboanalyst.ca/) were used for statistical analysis of UPLC-MS data.

PLS-DA showed three separated groups could be considered. One group (A1) included only two animals, a second group (A2) included six animals, and a third group (B) included eight animals. R^2Y=0.72 and Q^2= 0.62 for component 1, and R^2Y=0.98 and Q^2=0.86 for component 2. Sample clustering with the Metaboanalyst software reported the same sample classification as with PLS-DA. According to the VIP score, several lysophosphatidylcholines and bile acids were shown as differential biomarkers. Further biochemical analysis showed that there were substantial differences between the animals of groups A (A1 + A2) and B in regard to the lipid metabolism. Further research will aim at determining whether the animal classification according to metabolomics may derive from genetic and dietetic factors regarding lipid metabolism. Ultimately, the lipid differences will be validated by Lipidomics and evaluated whether this feature has relevance for milk quality.

Introduction

Metabolomics is currently aimed at finding differential features after instrumental analysis (LC-MS for instance) of a raw material between samples previously ascribed to diverse groups. However, there may be phenotypic differences between the experimental individuals that lead to outliers and erroneous biomarkers after they are ascribed to the different groups. Therefore, we aimed at checking whether phenotypic differences may arise if a chemometric comparison between samples is run without previous hypothesis, that is comparing all individual samples against blanks without sample ascription to any group.

In this study, we have assessed how chemometrics classified calves from two groups (E0 vs CTRL) without previous hypothesis and how biomarkers risen from this classification changed after calves were ascribed to the two experimental groups.

Materials and methods

Raw material: Plasma from 16 calves grown under the same conditions. Two groups: E0 vs CTRL. E0 received a dietary supplement. Blank: water

Instrumental analysis: samples were analysed by UPLC-MS after deproteinization with a 3× volume of acetonitrile (ACN), centrifugation and concentration to the initial plasma volume.

Equipment: An Acquity® UPLC system coupled to a SYNAPT® HDMS G2 QToF mass spectrometrer (Waters, Manchester, UK). Data were acquired with the MassLynx software.

Chemometrics: UPLC-MS data were processed with the MarkerLynx software (Waters) to render the feature (retention time_m/z) array. PCA and OPLS-DA multivariate statistical analysis were conducted with the eXtended Statistics application (Umetrics, Sweden) available with the MarkerLynx software and MetaboAnalyst (https://www.metaboanalyst.ca/). Data directly acquired were normalized to sum and Pareto scaled. Important features rendered by the NON-HYPOTHESIS analysis and the experimental groups were compared afterwards. Only features whose *m/z* value matched reliable metabolites in the METLIN, HMDB and KEGG databases were accepted.

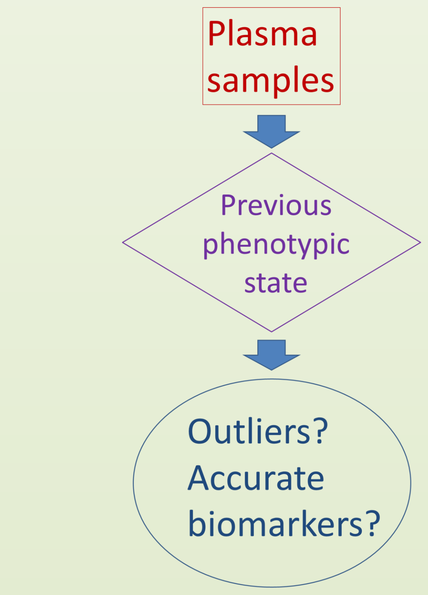

Fig. 1. Does the underlying phenotype influence group individual classification and biomarkers?

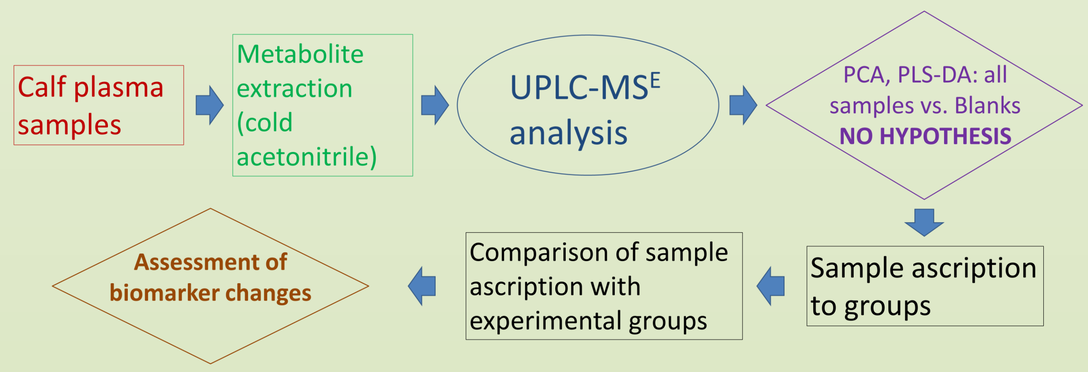

Fig. 2. Experimental procedure: testing by chemometrics with NO PREVIOUS HYPOTHESIS ON GROUP CLASSIFICATION OF SAMPLES

Results and discussion

- Three sample groups were shown in the non-hypothesis chemometric analysis (A1, A2 and B).
- Groups A1 and A2 accounted for 5 out of 8 samples of the CTRL experimental group
- Group B accounted for 6 out of 8 samples of E0 group.
- Metabolites with the highest score did not vary in the different chemometric comparisons, but others were shown to be different.
- Important metabolites show main differences between groups A1, A2 and B rely on lipid metabolism.
- Genetic and gut microbiome are hypothesized to be the factors leading to sample unmatching between non-hypothesis and experimental group classification.
- Further research will aim at understanding whether the phenotypic differences have relevance for milk quality.

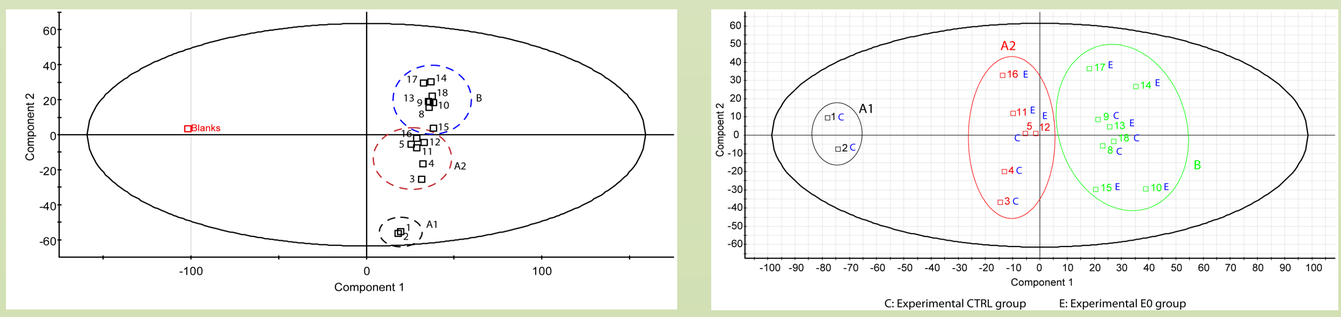

Fig. 3. Sample grouping according to principal component analysis (PCA) without previous hypothesis (XS application of MarkerLynx).

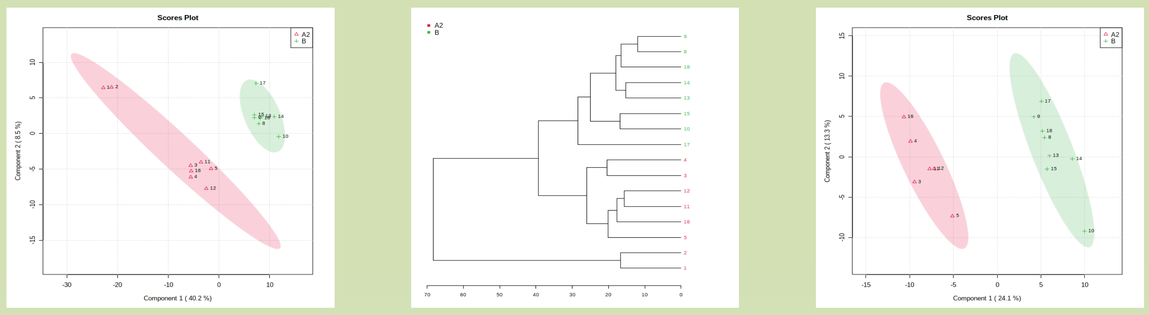

Fig. 4. Sample grouping according to partial least squares discriminant analysis (PLS-DA) without previous hypothesis (MetaboAnalyst software). Grouping is equivalent to that obtained with PCA. Left panel: samples of groups A1 and A2 were joined in one group. Central panel: dendogram considering samples 1 and 2 within the group A2. Right panel: samples 1 and 2 were excluded.

Table 1. Ranking of the VIP scores for the different biomarkers arisen in the comparisons according to sample ascription without previous hypothesis and to groups CTRL and E0.

	Ranking according to VIP score			
Feature Metabolite	(A1+ A2) vs B No hypothesis	A2 vs B No hypothesis	E0 vs CTRL	E0 vs CTRL – unmatching samples
3.90_496.2408 LPC(16:0)	1 & 2 (two features)	1 & 2 (two features)	2 & 3 (two features)	1 & 2 (two features)
0.73_203.0535 Succinylacetoacetate	3	3	1	3
3.33_468.3087 LPC(14:0)	4	5	5	5
0.98_120.0817 Unidentified	5	4	-	-
0.70__216.9234 Unidentified	6	7	11	12
2.24_105.0346 Malonate?	7	9	15	11
3.23_500.3042 Taurochenodeoxycholate	8	8	4	4
3.43_464.2832 Unidentified	9	-	10	6
2.16_188.0712 Indoleacrylic acid	10	10	-	-
2.93_440.2769 LPC(12:0)	11	12	14	-
3.41_512.3364 LPS(O-18:0)	15	-	-	-
3.48_494.3243 LPC(16:1)	-	6	13	13
3.56_416.3159 N-linoleoyl-dopamine	-	15	-	-
2.80_516.2997 Taurocholic acid	-	-	6	7
2.80_498.2887 LPS(16:0)	-	-	7	9
3.33_518.3236 LPC(18:3)	-	-	8	8
0.75_383.1169 Flavonoid	-	-	12	-

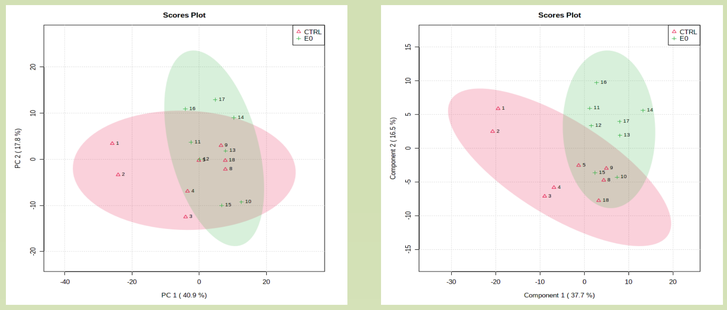

Fig. 5. PCA (left) and PLS-DA (right) of groups CTRL and E0 (MetaboAnalyst)

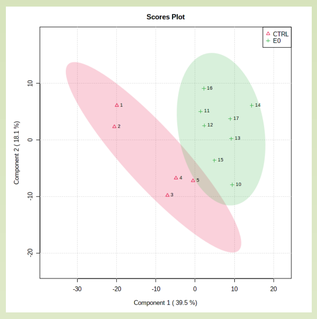

Fig. 6. PLS-DA of groups CTRL and E0 after excluding mismatching samples.

Bibliography

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