75th EAAP Annual Meeting
1-5 September 2024 - Florence, Italy

ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

Animal and Food Genomics Group

Molecular phenotyping through metabolomic analyses in pig breeds

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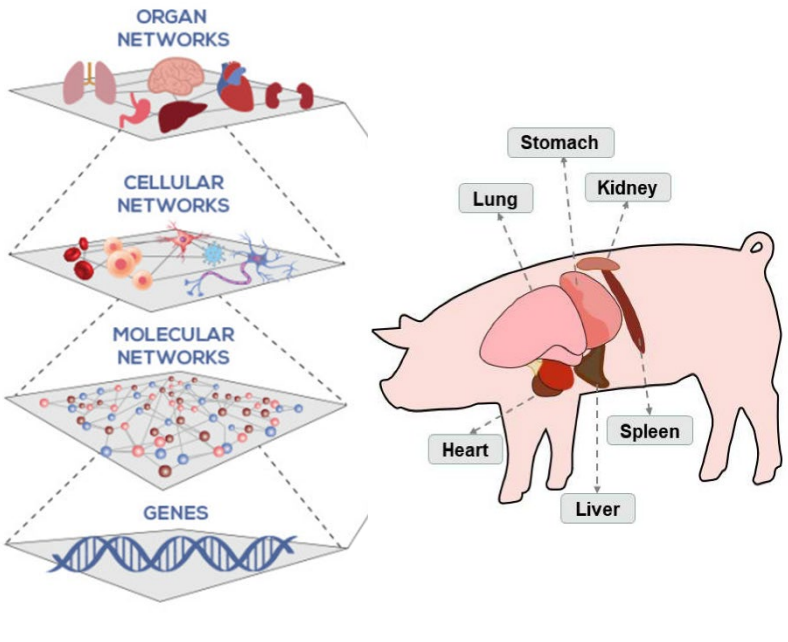
Biological complexity: an overview

The different layers of organization establish complex links allowing properties to emerge.

Complexity
Emergent properties

Functions
Processes
Pathways



DNA
RNA
Proteins
Biochemical
Biological Phenotype

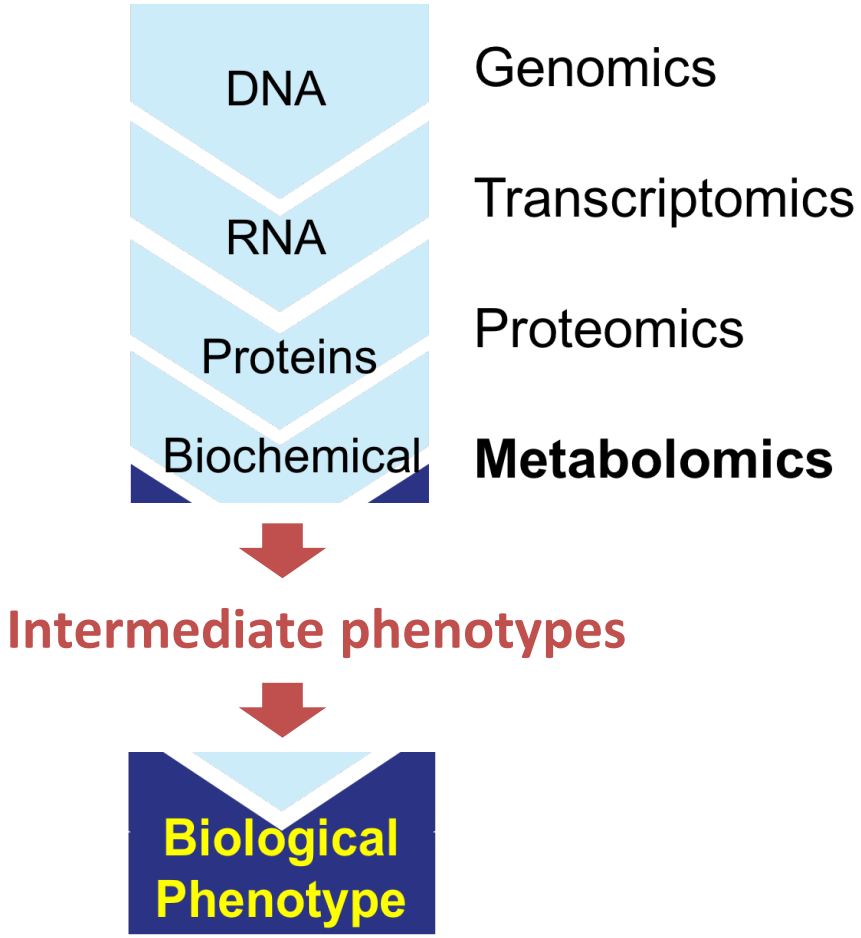
Genomics
Transcriptomics
Proteomics
Metabolomics

Multi-omics

Processes and pathways interact with each other, defining cell types, organs, and the organism as a whole.

Metabolomics as a molecular phenotyping approach

Metabolites are the end products of cellular processes.

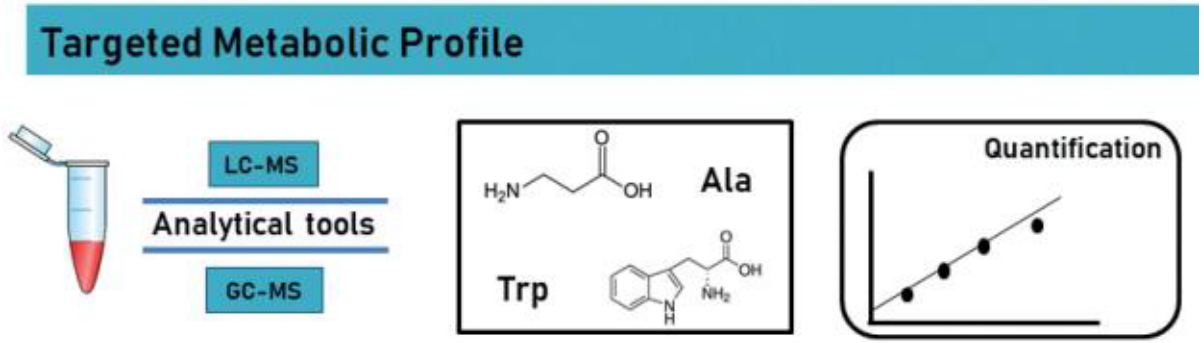


They can help define intermediate phenotypes, i.e. precursors to final phenotypes and complex traits

Metabolomics aims to characterize and quantify low molecular weight metabolites (<1.5 kDa) in a biological sample.

Metabolomics as a molecular phenotyping approach

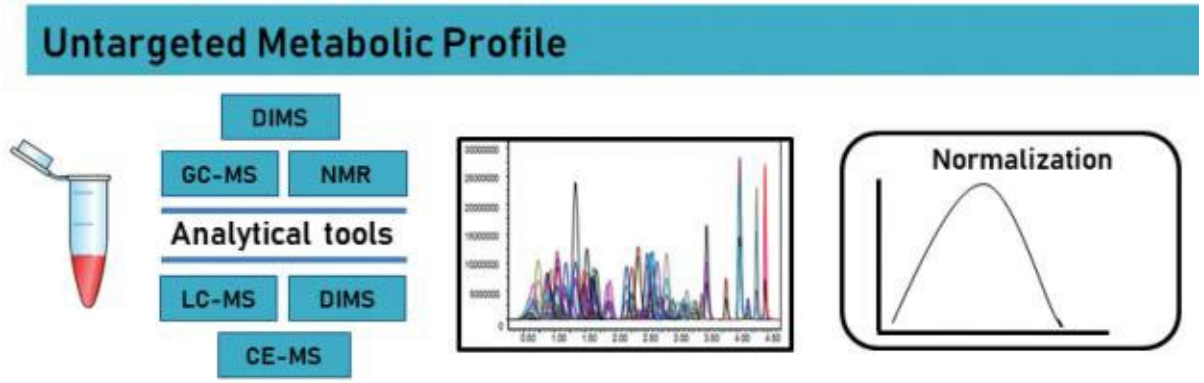
Main approaches:



50 - 200 Metabolites

biocrates
The future of research and health

- Low detection limit
- Quantitative
- Limited targets
- Requires standards



~1000 Metabolites

Metabolon
SMALL MOLECULES, BIG INSIGHTS

- Comprehensive
- High throughput
- Unexpected/novel metabolites
- Semi-quantitative
- Unknown compounds

Source: De San-Martin et al. 2021
10.20945/2359-3997000000300

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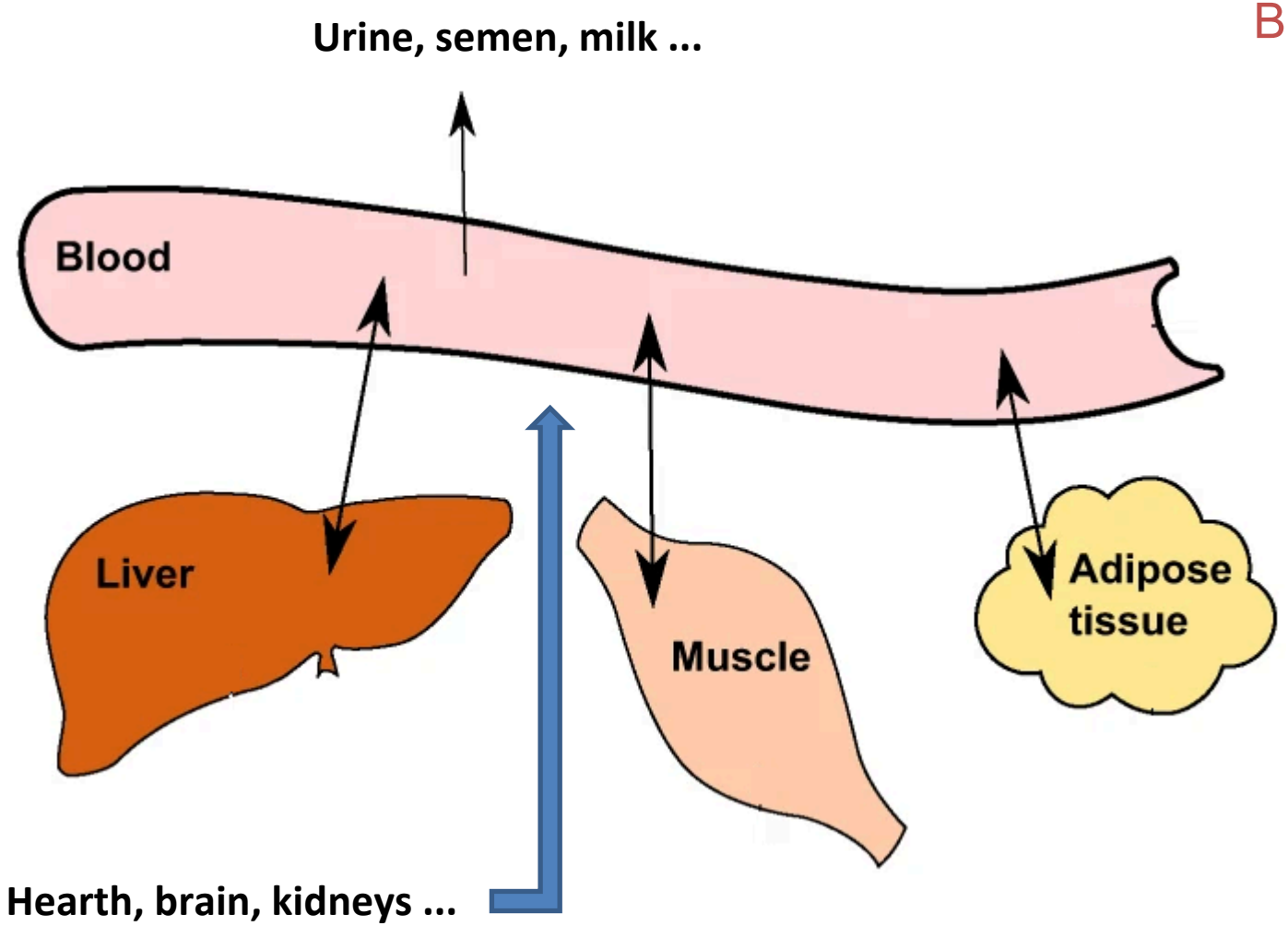
Biological matrices for metabolomics



Blood is one of the most widely used matrices

- Each organ is fed and drained by blood
- Blood is sensitive to the effects of health or disease, genetic variation, environment, nutrition, or the impact of toxicants
- It can be easily obtained.

Applications of metabolomic phenotyping: Pilot study on Italian heavy pigs

ELSEVIER

Animal
Volume 10, Issue 10, 2016, Pages 1741-1748

Metabolomics evidences plasma and serum biomarkers differentiating two heavy pig breeds

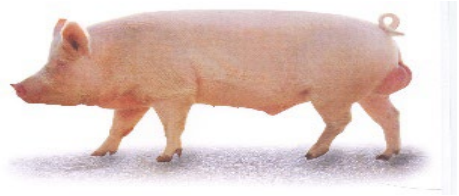
S. Bovo [1 a], G. Mazzoni [1 a], G. Galimberti [2], D.G. Calò [2], F. Fanelli [3], M. Mezzullo [3], G. Schiavo [1], A. Manisi [2], P. Trevisi [1], P. Bosi [1], S. Dall'Olio [1], U. Pagotto [3], L. Fontanesi [1]

- Biofluid: **Plasma and serum**
- Analysis : **Targeted**
- Comparison: **Between breeds (ILW vs IDU)**

Targeted metabolomics
Biocrates AbsoluteIDQ p180 Kit

Metabolite classes	No.	Biological relevance (selected)
Acylcarnitines	40	Energy metabolism fatty acid transport
Amino acids	21	Amino acid metabolism, **neurotransmitter metabolism**
Biogenic amines	19	**Neurological disorders** DNA stability, oxidative stress
Hexoses	1	Carbohydrate metabolism
Glycerophospholipids	90	
- lysoPhosphatidylcholine acyl – lysoPC a Cx:x	14	Degradation of phospholipids fatty acid profile
- Phosphatidylcholine diacyl – PC aa Cx:x	38	Dyslipidemia, Membrane composition and damage
- Phosphatidylcholine acyl-alkyl – PC ae Cx:x	38	
Sphingolipids	15	Signalling cascades membrane damage

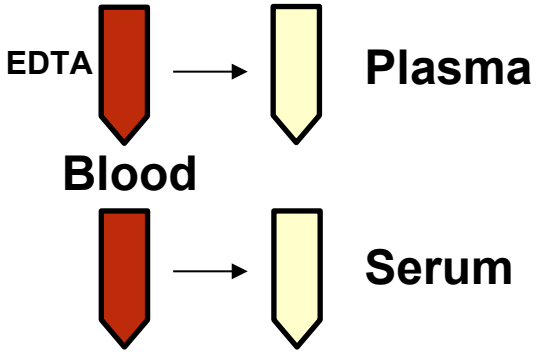
Applications of metabolomic phenotyping: Pilot study on Italian heavy pigs

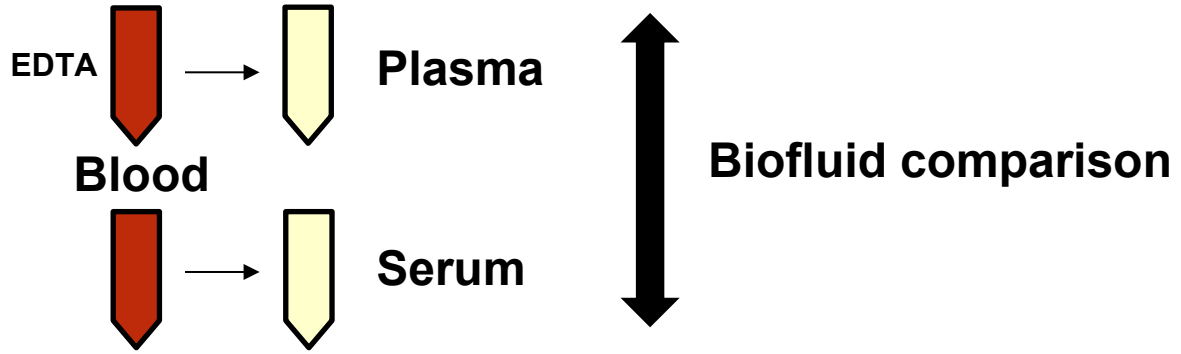


Breed comparison

12 Italian Large White
(6M + 6F)

12 Italian Duroc
6M + 6F

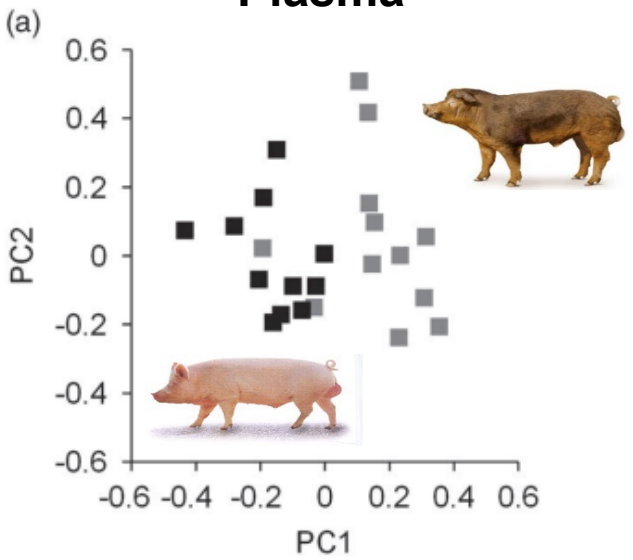




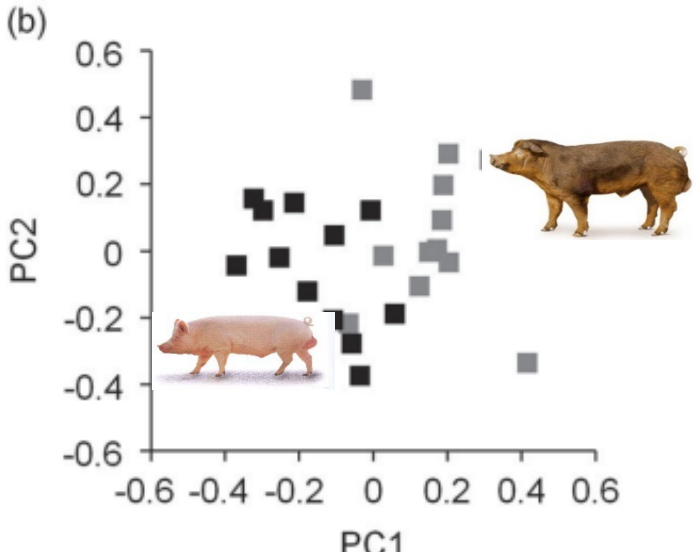
Applications of metabolomic phenotyping: Pilot study on Italian heavy pigs

Sparse Partial Least Square Discriminant Analysis (sPLS-DA)

Plasma



Serum



7 metabolites discriminate between breeds

Table 2 *Metabolites selected by sPLS-DA that were stable and significant in plasma and serum (P < 0.20)*

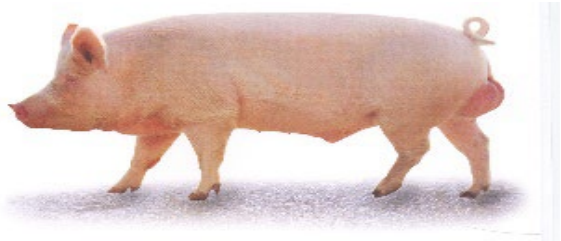
			ID				ILW				Stability[2]		Significance[3]		
Biofluid	Metabolic class	Metabolite[1]	$Mean_R$	SD_R	$Mean_r$	SD_r	$Mean_R$	SD_R	$Mean_r$	SD_r	*t*	P_{st}	*t*	P_{si}	Direction[4]
Plasma	Sphingomyelins	SM (OH) C14:1	1.69	0.18	0.14	0.18	1.40	0.50	−0.14	0.15	24	0.037	0.9066	0.000	ID
	Biogenic Amines	Ac.Orn	6.93	3.62	−3.42	3.84	14.11	3.07	3.42	3.01	24	0.064	−1.0855	0.001	ILW
		Kynurenine	0.48	0.25	−0.18	0.27	1.01	0.27	0.18	0.21	24	0.066	0.1169	0.177	ILW
Serum	Sphingomyelins	SM (OH) C14:1	1.74	0.21	0.15	0.20	1.37	0.18	−0.15	0.21	24	0.029	0.2798	0.008	ID
		SM (OH) C16:1	2.43	0.39	0.22	0.34	1.83	0.29	−0.22	0.34	22	0.084	0.2957	0.012	ID
		SM C16:0	74.36	7.12	4.77	6.42	61.78	5.13	−4.77	6.41	24	0.021	0.3759	0.002	ID
	Biogenic Amines	Ac.Orn	6.24	3.48	−3.15	3.77	12.98	2.36	3.15	2.38	24	0.052	−1.1162	0.001	ILW

Scaling up

Scaling up the analysis using **untargeted** metabolomics on around **1000 samples**

Italian Large White

- **727** samples



243 Castrated males

484 Entire gilts (females)

Comparison

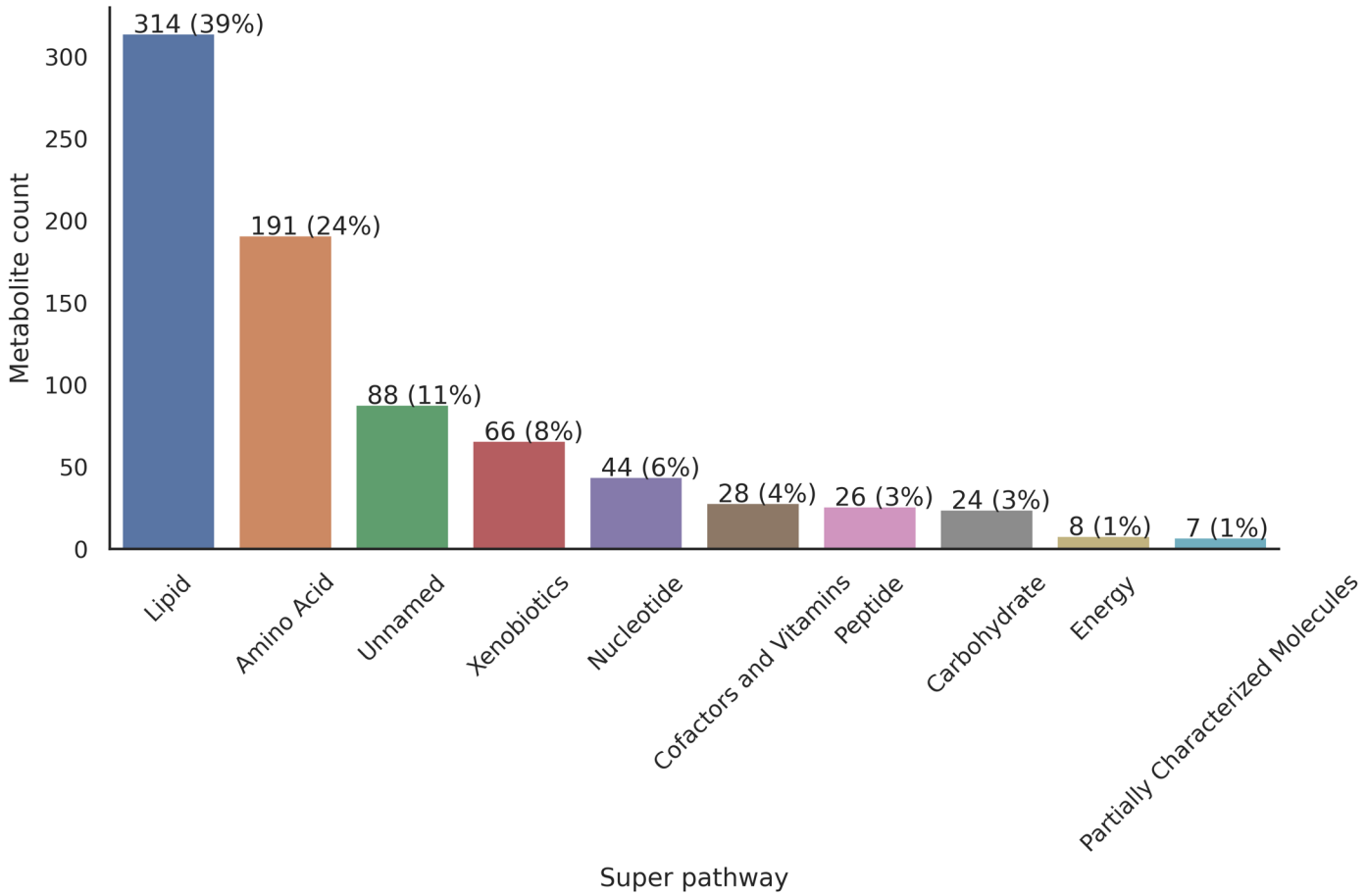
Italian Duroc

- **271** samples

78 Castrated males

193 Entire gilts (females)

Approach: Untargeted metabolomics



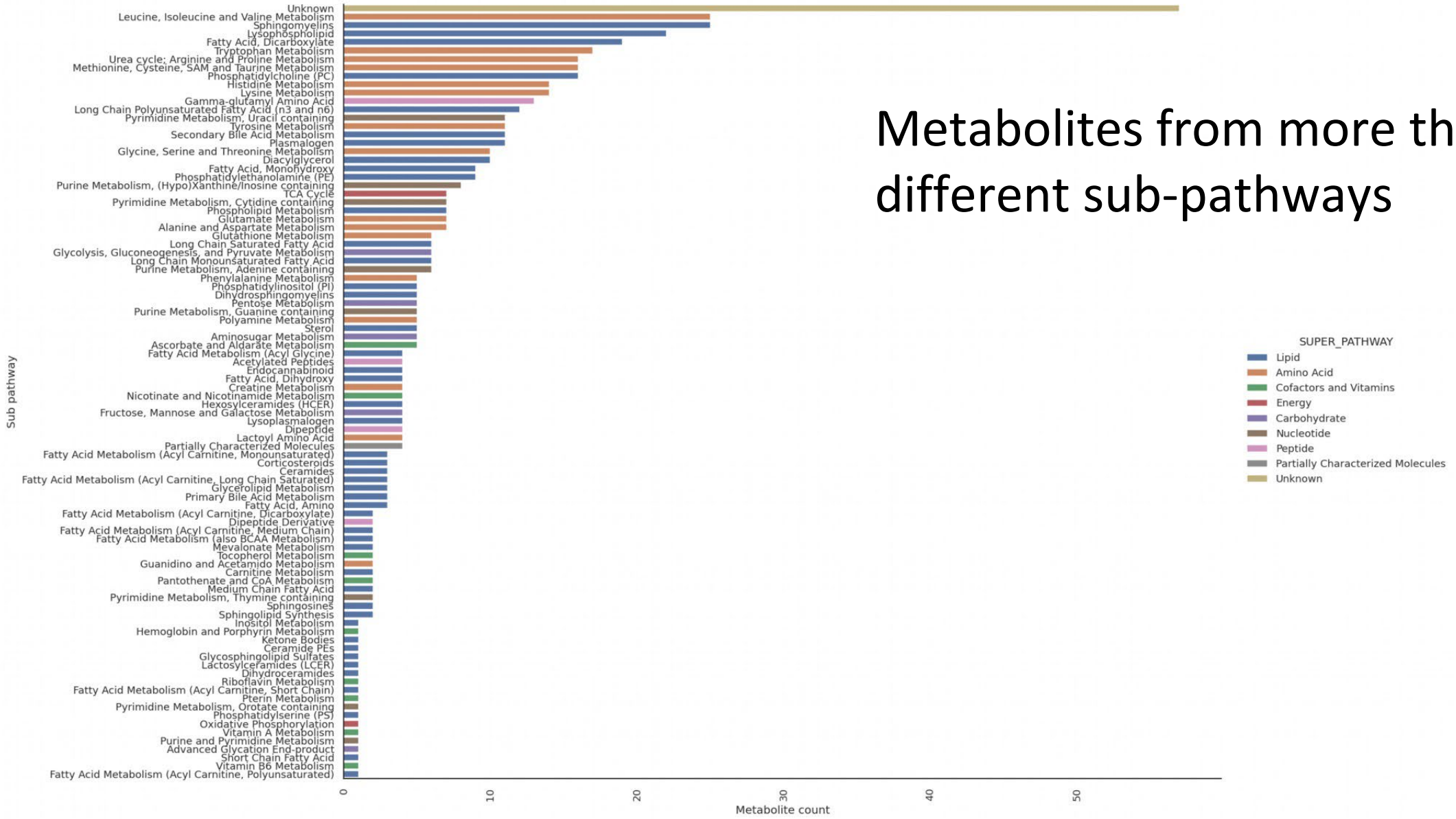
Biofluid: **Plasma**

Platform: Metabolon
SMALL MOLECULES, BIG INSIGHTS™

796 metabolites measured:

- 642 Endogenous
- 88 Unnamed
- 66 Xenobiotic

Approach: Untargeted metabolomics



Metabolites from more than 60 different sub-pathways

Quality control

- Internal QC practices implemented by the data providers:
  - QC-normalization
  - Anchor sample merging
- Additional QCs (within breed) :
  - Identified and removed outlier metabolite values (> 5 times outside the interquartile range)
  - Removed metabolites with > 25% of missing values or outliers
  - Excluded Xenobiotic metabolites from the analysis

580 metabolites passed all QCs and were used in the analysis

Missing data imputation

Missing metabolite values imputed with MICE (Missing data Imputation with Chained Equations)

- Imputation algorithm: Predictive Mean Matching (PMM)
- Predictors: Top 10 correlated metabolites (Endogenous only)
- 5 MICE runs generating 5 datasets each => to account for the random component

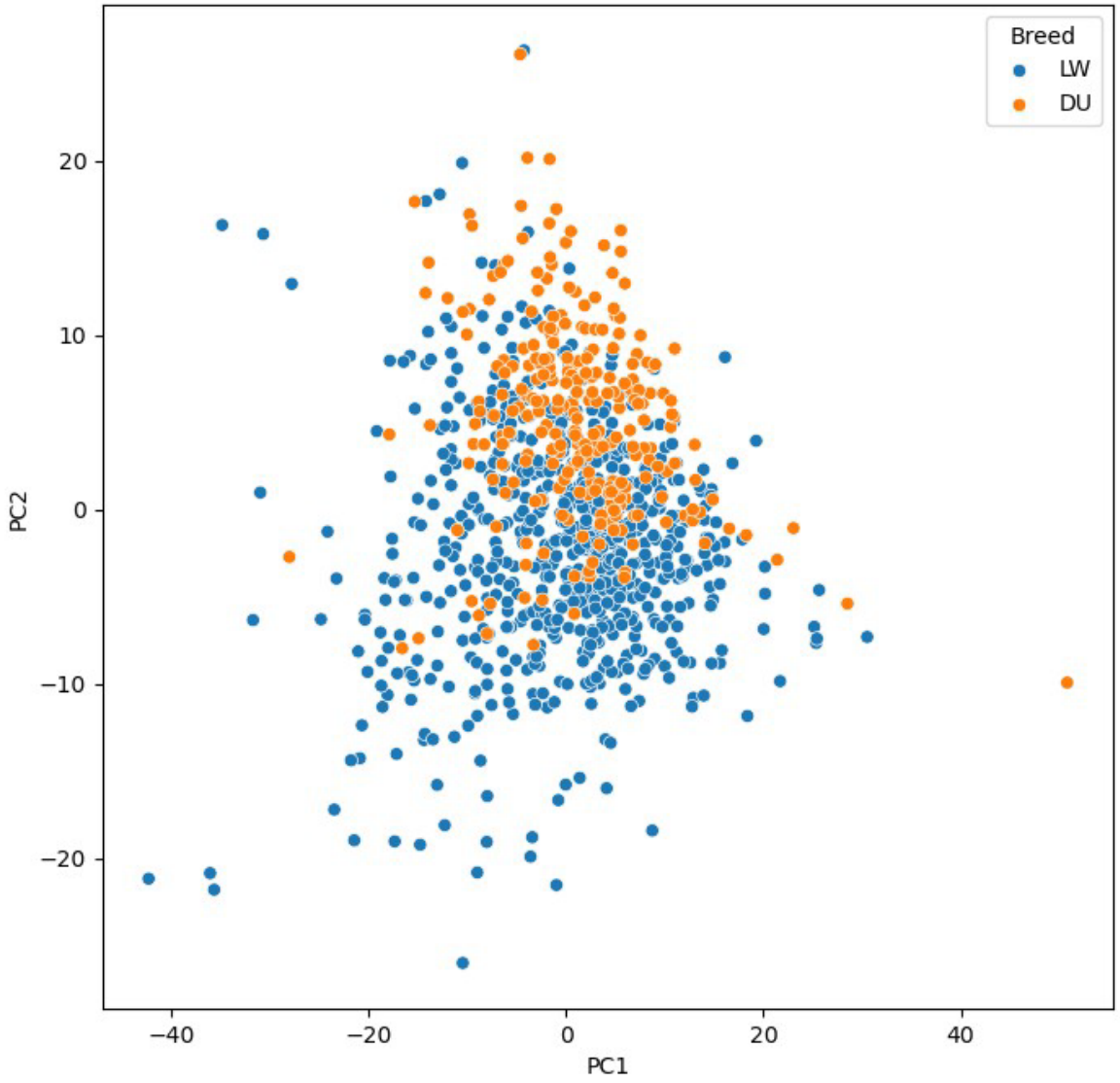
Total of **25 imputed datasets**

Removal of confounding effects

Confounding effects removed with a linear model:

- Slaughter date
- Weight of the animal
- Sex

Principal component analysis



PCA on all 580 metabolite residuals analyzed after missing data imputation

PC2 shows the most difference between the two breeds

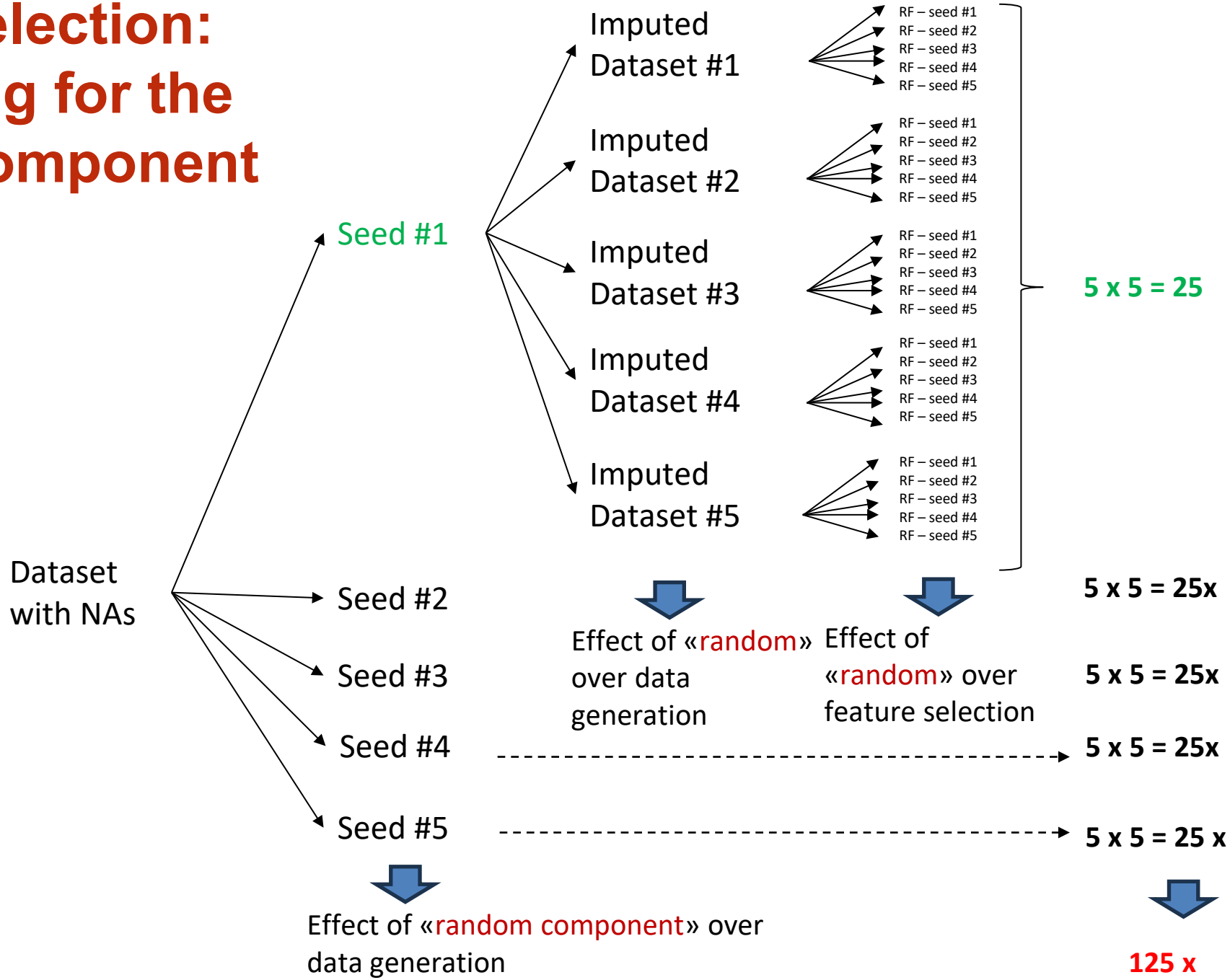
Detection of differentially abundant metabolites

Two methods applied in this study:

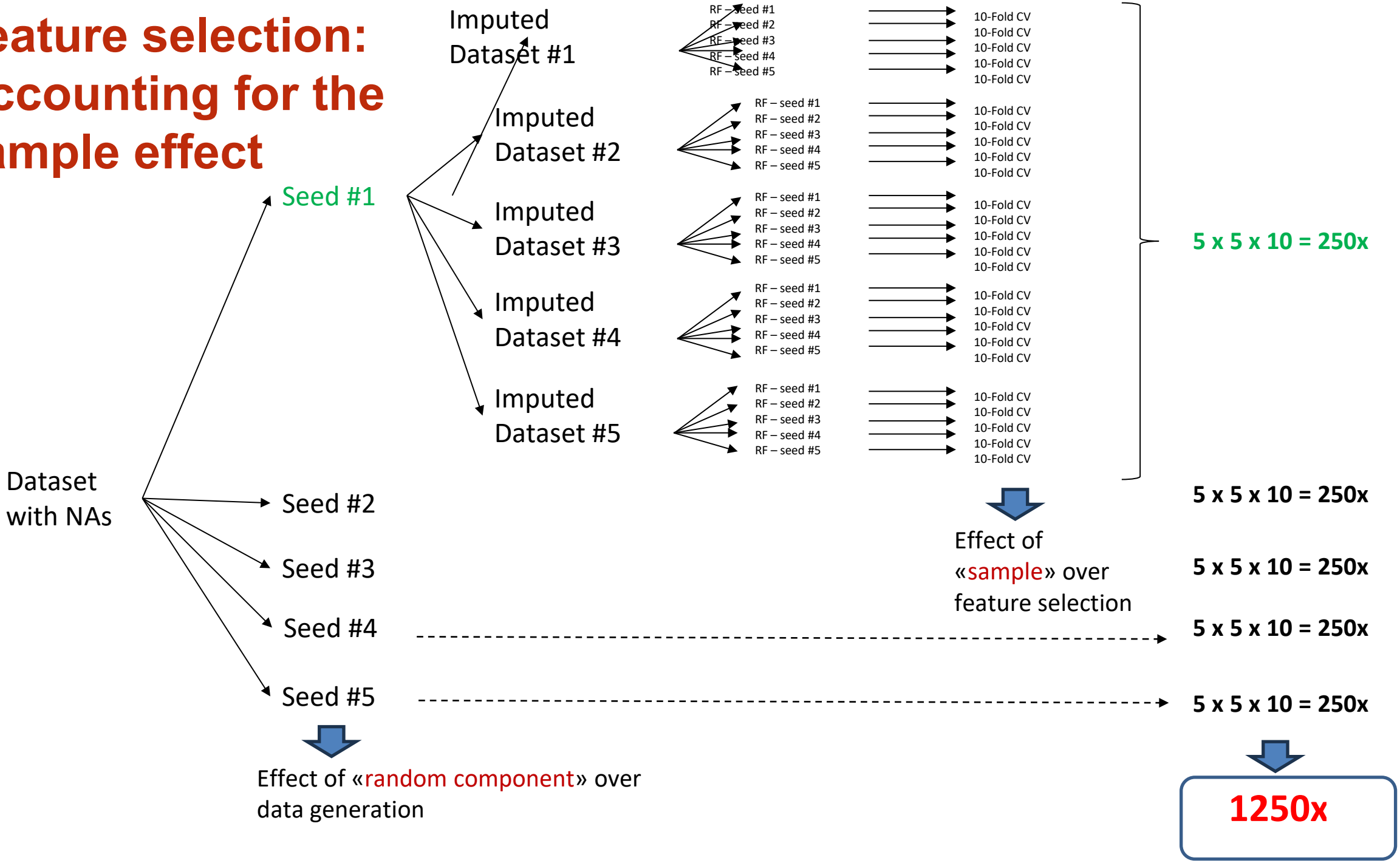
- Sparse Partial Least Squares Discriminant Analysis (**sPLS-DA**): multivariate method best suited for analysing multicollinear data with a high number of features

- **Boruta:** All-relevant feature selection method based on Random Forest classification (machine learning)

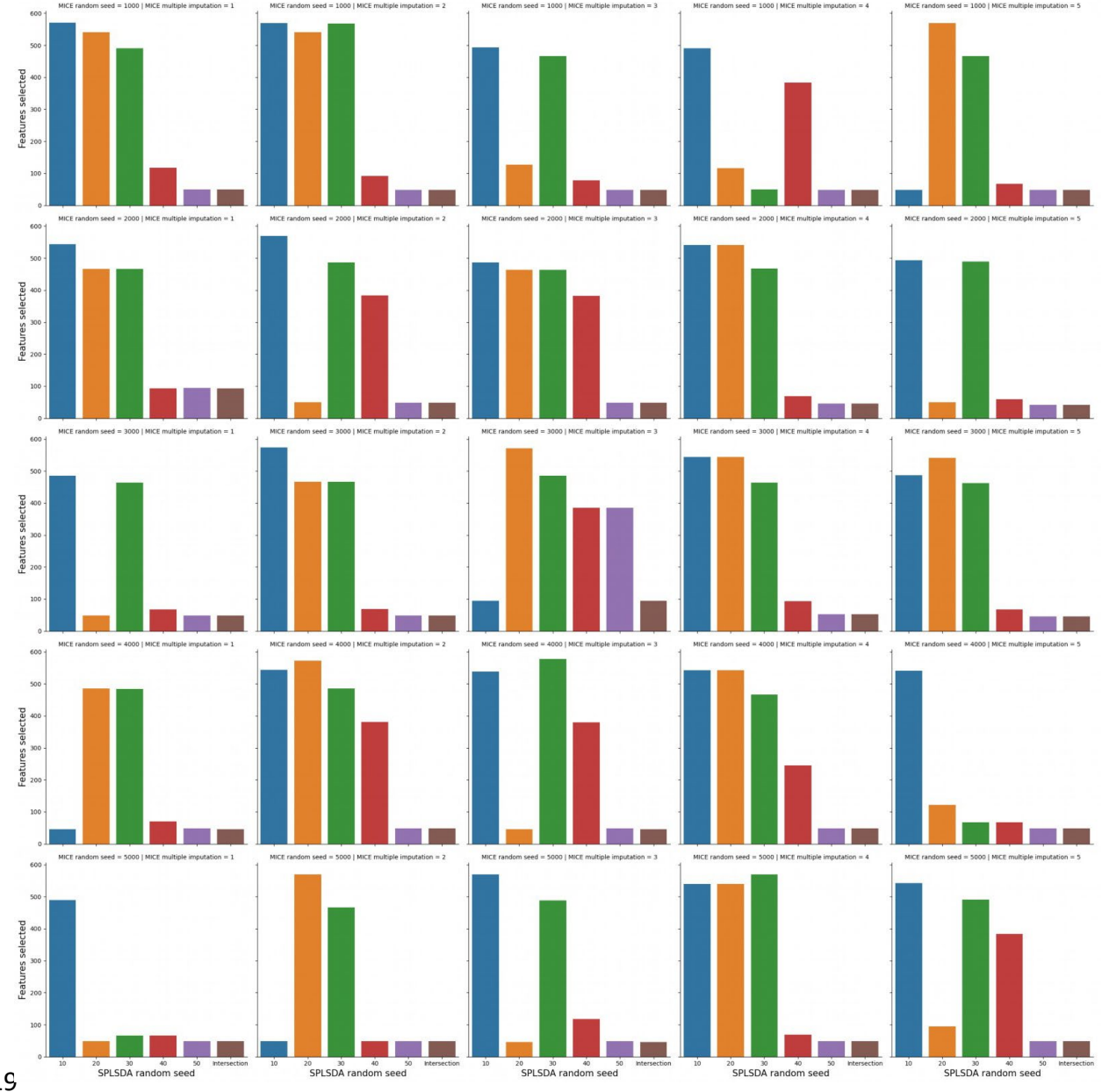
Feature selection: Accounting for the random component



Feature selection: Accounting for the sample effect



Feature selection results - sPLS-DA

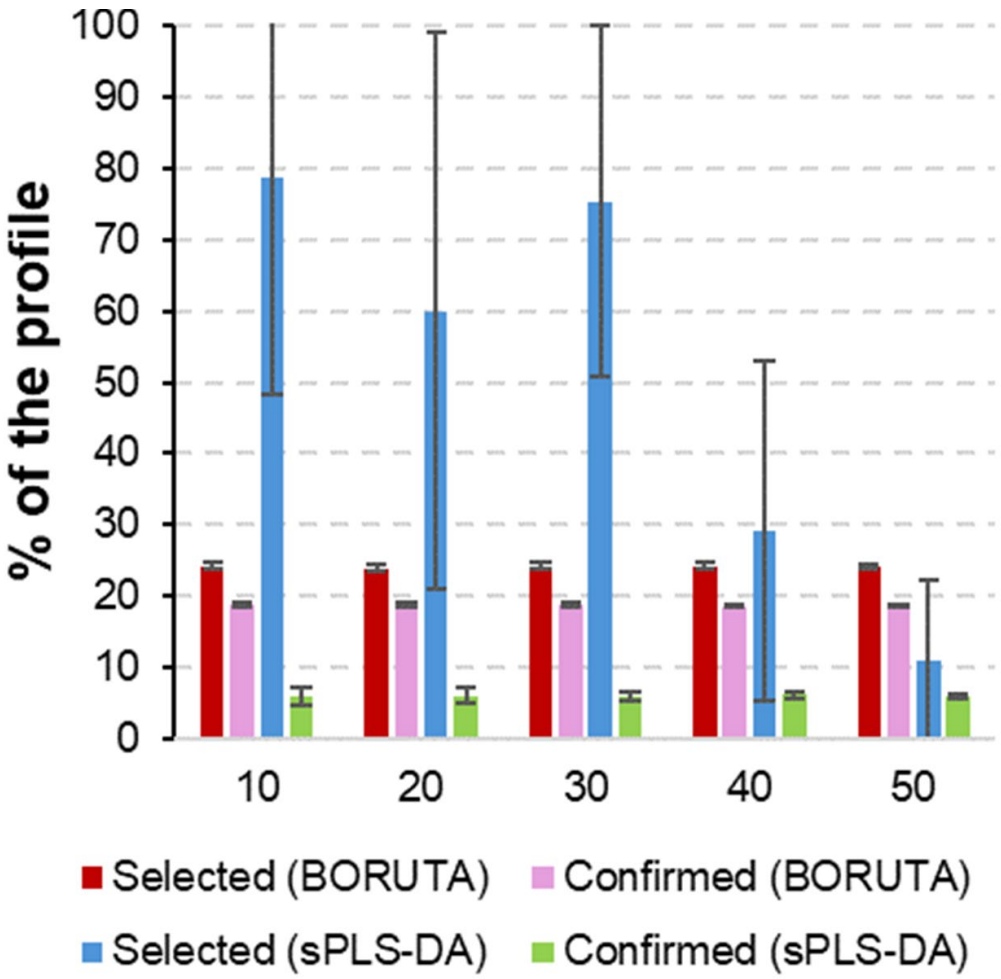


- Each bar represents a feature selection iteration
- sPLS-DA is unstable across runs => random component
- 31 metabolites are selected in all iterations and CVs

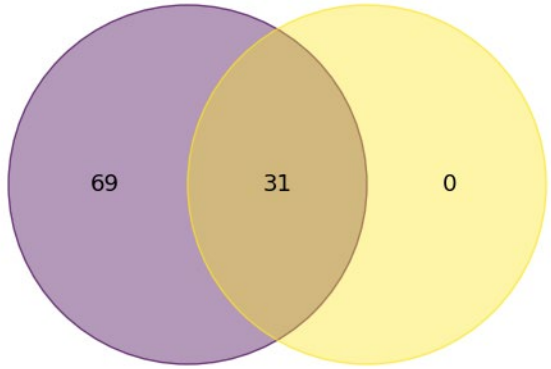
Feature selection results - Boruta

- Boruta is mostly stable
- 100 metabolites are selected in all iterations and CVs

Comparison between Boruta and sPLS-DA results



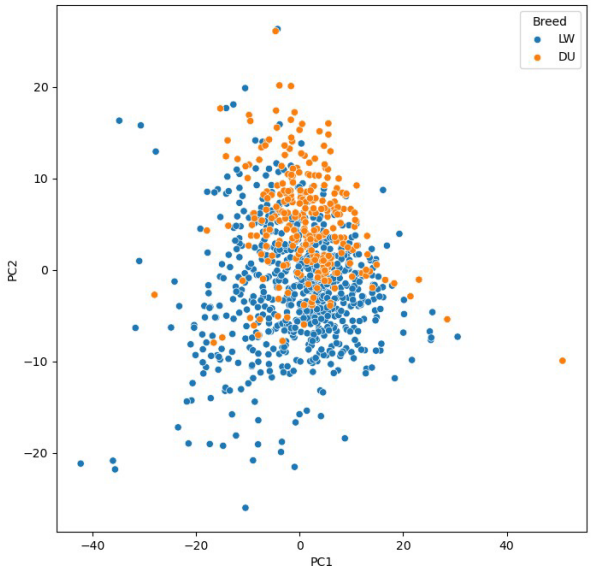
- All 31 metabolites selected by sPLS-DA are also selected by Boruta
- 69 metabolites are selected only by Boruta



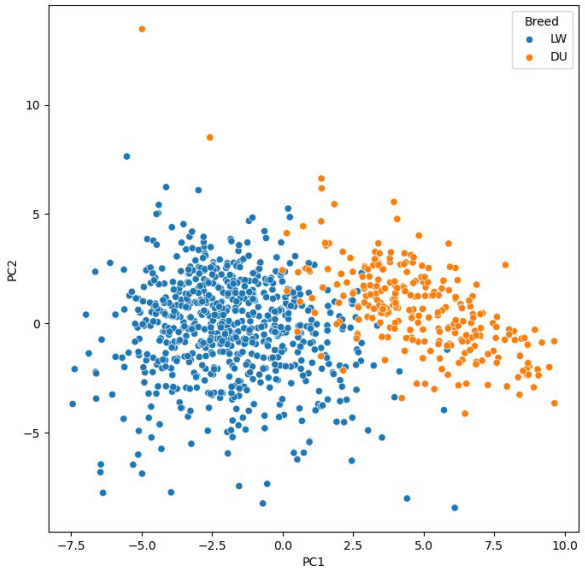
- Total of 100 metabolites selected
- All 7 metabolites from the pilot study were selected again

Principal Component Analysis on selected features

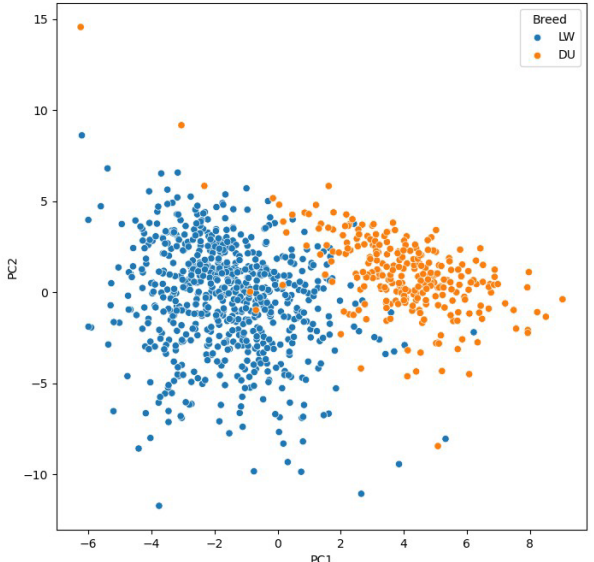
a)



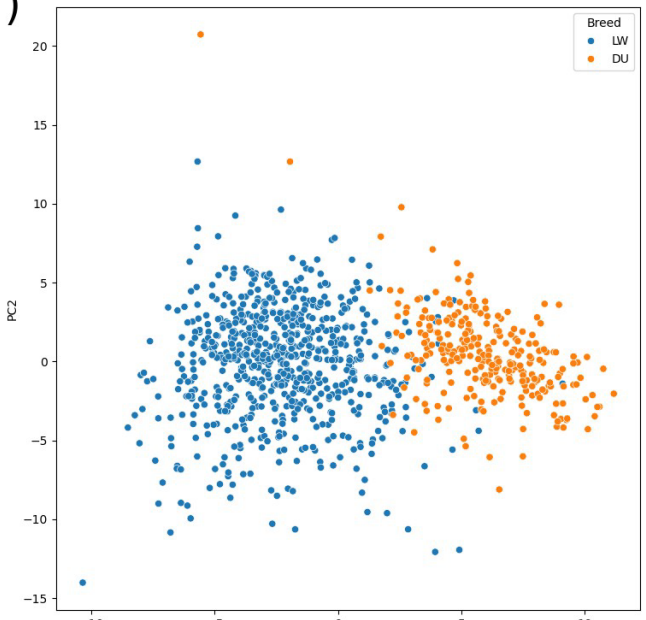
b)



c)



d)



a) All 580 metabolites

b) 31 metabolites selected by both methods

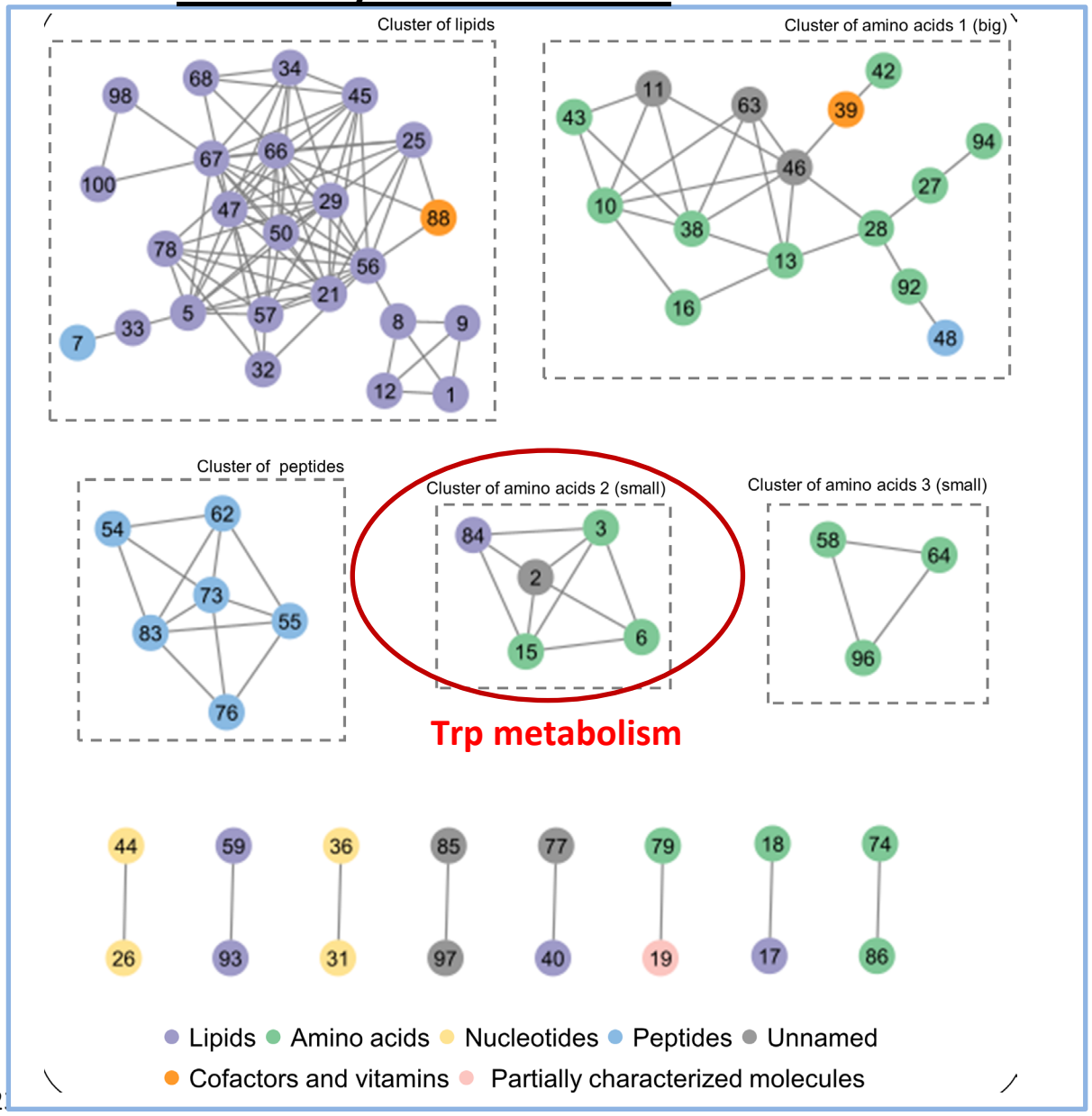
c) 69 metabolites selected by Boruta only

d) All 100 metabolites selected by Boruta

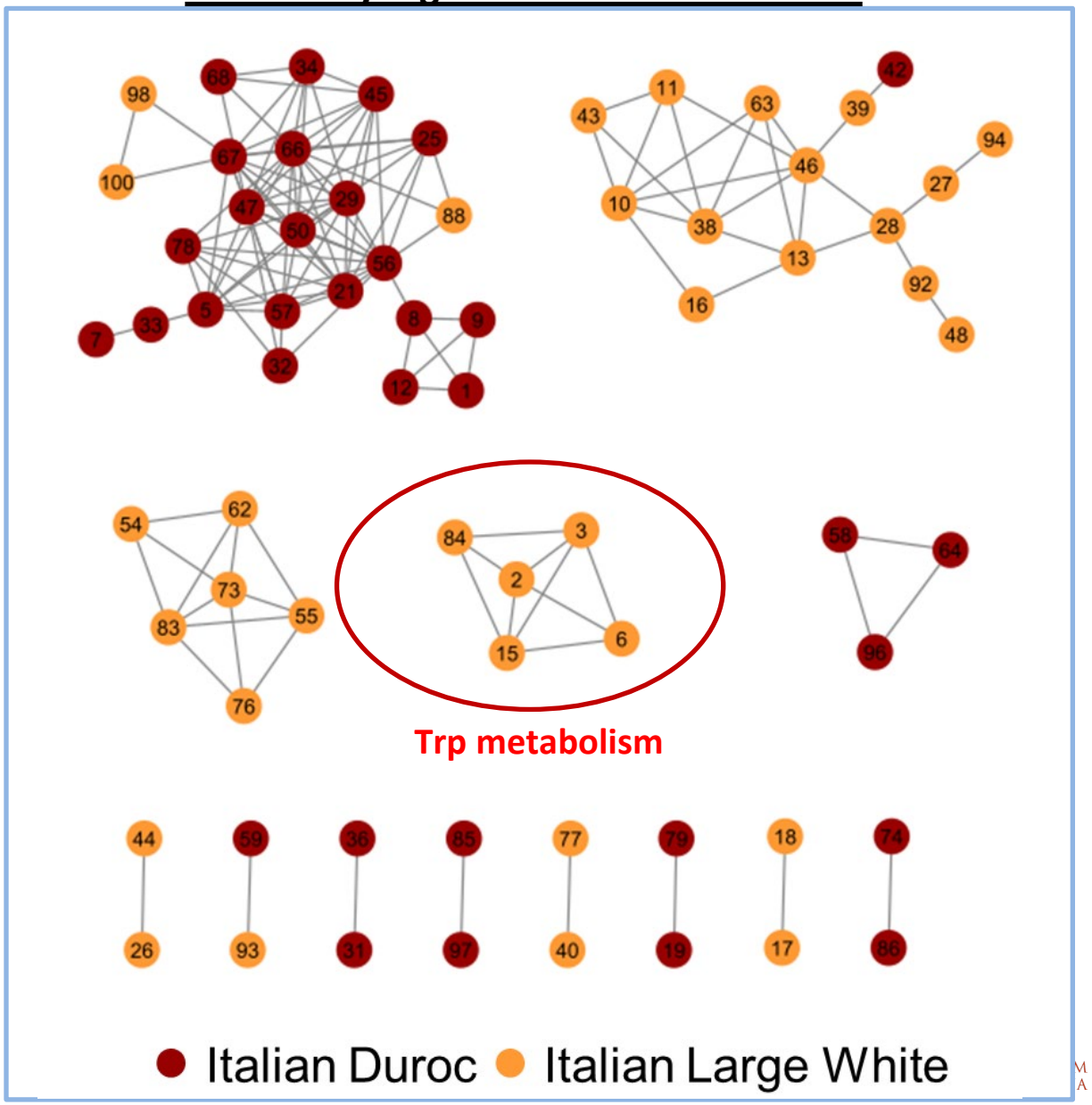
The selected metabolites show a clear distinction between the two breeds

Network analysis of selected metabolites

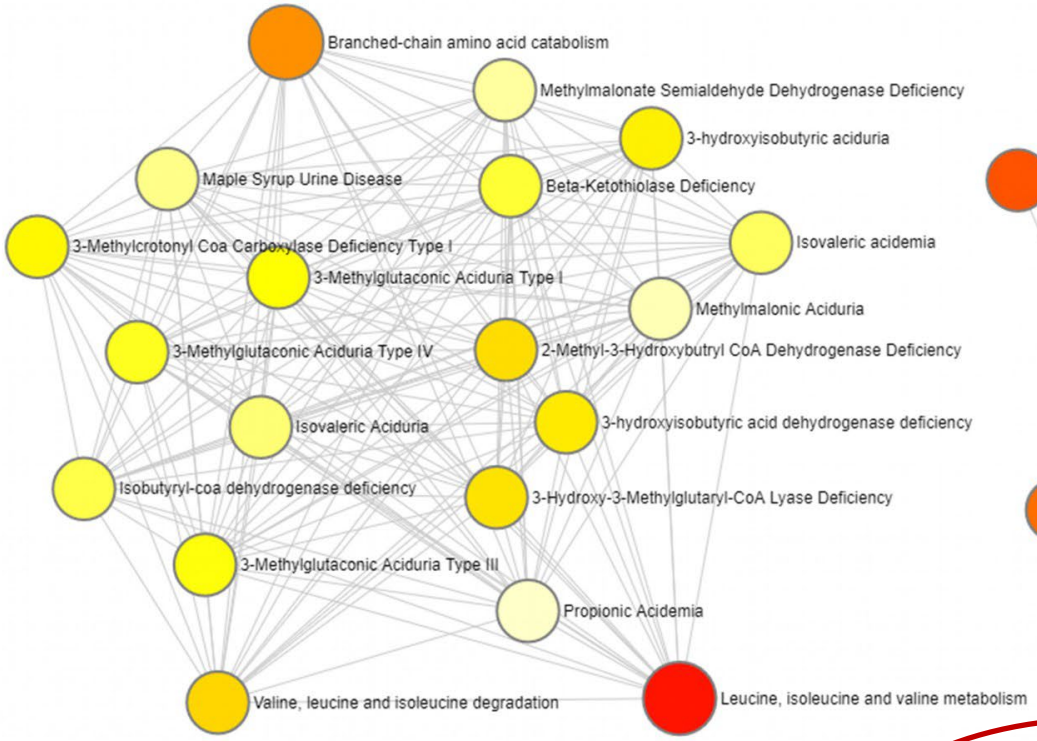
Colored by metabolite class

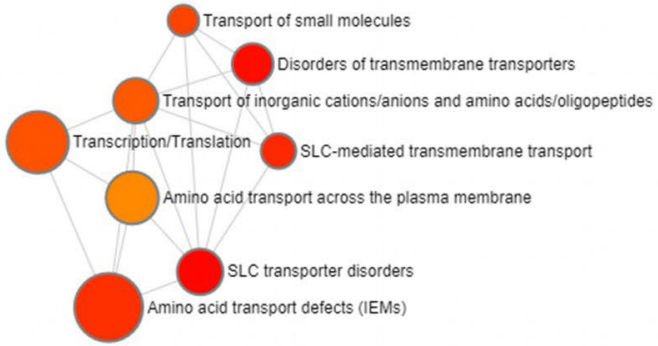


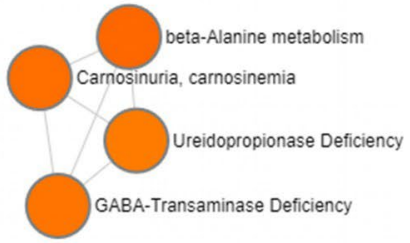
Colored by higher abundance in breed

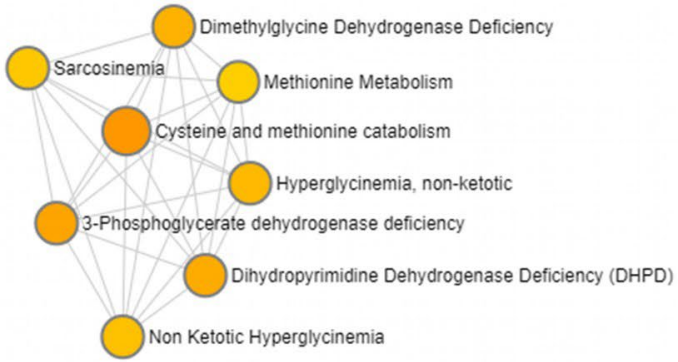


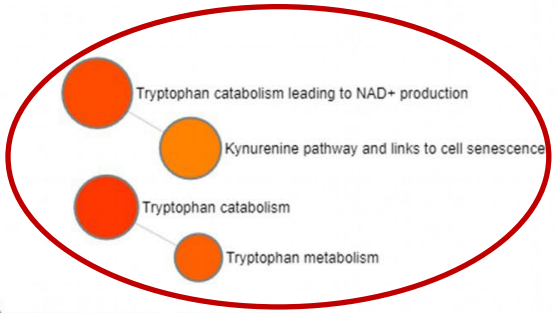
Pathway analysis of selected metabolites



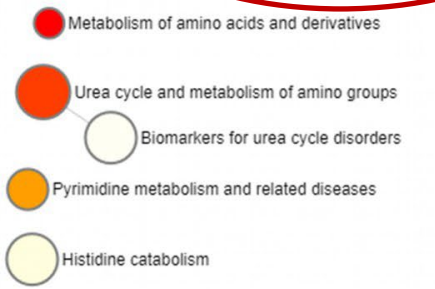








Tryptophan metabolism pathway



Conclusions

- We identified several metabolites that could be used as biomarkers to study the biological basis of complex traits in pigs
- We observed intrinsic differences between breeds at the metabolomic level -> due to the **genetic background**

- Five metabolites from the **tryptophan** metabolism pathway discriminate between breeds
- Further investigation on the genetic background determining these differences is warranted
- Metabolomics offers a promising approach for molecular phenotyping

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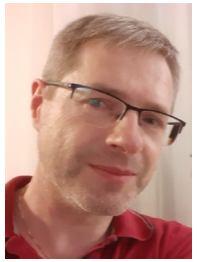

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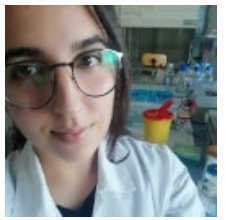
Data analysis



Valeria Taurisano
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Matteo Bolner
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Data analysis

Acknowledgments

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DELLA SOVRANITÀ ALIMENTARE
E DELLE FORESTE

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SUIS.2 Suinicoltura Italiana Sostenibile

PigPhenomics

Thank you !
Questions ?

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75th EAAP Annual Meeting
1-5 September 2024 - Florence, Italy

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Molecular phenotyping through metabolomic analyses in pig breeds

Matteo Bolner[1], Samuele Bovo[1], Giuseppina Schiavo[1], Giuliano Galimberti[2], Francesca Bertolini[1], Anisa Ribani[1], Maurizio Gallo[3], Stefania Dall'Olio[1], and Luca Fontanesi[1]

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