MICROB-PREDICT

MICROBiome-based biomarkers to PREDICT decompensation of liver cirrhosis and treatment response

D3.4 Report on Metabolomic data

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1. Executive Summary

Metabolomic analyses were performed on the serum, urine and stool samples from the MUCOSA-PREDICT samples (300 samples from 94 patients collected at three different time-points) using liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS). This resulted in 3 annotated peak tables (one per biological matrix) with statistical analyses, that have been transmitted to WP4. A total of 459 metabolites were detected in the three biological matrices, with a greater number of metabolites detected in urine and feces (~330), compared to serum samples (~220). Metabolomic signatures discriminating patients with acute decompensation (AD) with or without acute-on-chronic-liver failure (ACLF) were obtained with the three biological matrices. However, the best performance in terms of discrimination between AD and ACLF was obtained with the serum signature, whereas data from urine and fecal samples had no added value in terms of sample classification. Furthermore, metabolomic analyses enabled to obtain serum signatures that discriminate between stable and unstable decompensated patients, and also enabled prognosis of AD patients who will declare ACLF.

Metabolomic analyses were also used to establish whether or not it could be possible to obtain a prognostic signature regarding the response to a treatment of AD patients with albumin. To this end, we focused on samples collected at inclusion and on patients that did not yet receive albumin at the sampling time. We failed to obtain statistically significant biomarkers by this mean. However, a statistically relevant model was built on the 224 annotated serum metabolites by analyzing data corresponding to patients treated by albumin and being either responders (R) or non-responders (NR), leading to a signature of 9 metabolites able to discriminate between R and NR patients. We then analyzed plasma samples from the ANSWER cohort (a clinical trial investigating the efficacy of albumin administration in cirrhotic patients with AD) in order to replicate/confirm these results. However, no statistically significant biomarkers were obtained in these conditions. Only two metabolites, namely Confidential information exhibit similar concentrations trends (although not statistically significant) as those obtained with serum samples of the MUCOSA-PREDICT samples.

2. Background and rationale

Acute decompensation (AD) of cirrhosis and its progression to acute-on-chronic liver failure (ACLF) are associated with intense systemic inflammation, multiple organ dysfunctions, and a major risk of short-term mortality. There is a need to develop tools to stratify patients in order to improve their management, predict their response to treatments, and reduce the mortality observed in decompensated patients and ACLF. Metabolomics has already proved its relevance to the field with the identification of a 38-metabolite blood fingerprint specific for ACLF whose intensity correlates with systemic inflammation and which revealed mitochondrial dysfunction in peripheral organs[1].

In the frame of WP3, we aimed at performing metabolomic analyses on serum, urine and stool samples in order to assess whether or not ACLF development could be predicted in patients with decompensated cirrhosis, and whether or not patients having unstable decompensated

cirrhosis could be discriminated from those that are stable, especially thanks to the detection of microbioal metabolites. We also aimed at evaluating the possibility of detecting metabolite signatures that could be prognostic of albumin response.

3. Samples

CEA has received:

- 308 serum, 305 urine and 309 stool samples from the MUCOSA-PREDICT cohort, which have been drawn from 94 patients with decompensated cirrhosis with and without ACLF at three different time-points.

- 126 plasma samples obtained from 63 patients with decompensated cirrhosis, administered with albumin, and enrolled in the ANSWER study[2].

4. Experimental methods

Metabolites were extracted from serum samples following protein precipitation using methanol, as previously described.[1,2] Resulting extracts were then analyzed by liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) using an Dionex Ultimate chromatographic system (Thermo Fisher Scientific, Courtaboeuf, France) coupled to a Q-Exactive mass spectrometer from Thermo Fisher Scientific fitted with an electrospray (ESI) source and operating in the positive and negative ion modes for metabolite separations on C18 and ZIC-pHILIC columns, respectively. LC-HRMS conditions were exactly those previously described by our group[3] Data acquisition was performed in a single batch. A quality control sample (QC) was obtained by pooling 20 µL of each sample to be studied. QC samples were injected at regular intervals every 10 samples throughout the experiments to evaluate the signal variations of any metabolite.

Data processing as well as statistical analysis was first performed automatically using the Workflow4metabolomics (W4M) platform for computational metabolomics[5]. Resulting features were then annotated by using our spectral database according to accurate measured masses and chromatographic retention times[3, 6, 7]. Metabolite signals were then filtered according to the following criteria: (i) the correlation between QC dilution factors and areas of chromatographic peaks (filtered variables should have coefficients of correlation above 0.7 to account for metabolites occurring at low concentrations and which are no longer detected in the most diluted samples) (ii) repeatability (the coefficient of variation obtained on chromatographic peak areas of QC samples should be below 30%), and (iii) ratio of chromatographic peak areas of biological to blank samples above a value of 3. Chromatographic peaks of the annotated metabolites were manually reviewed in QC samples using the XCalibur software (Thermo Fisher Scientific).

Confirmation of metabolite annotation was accomplished by LC-MS/MS experiments using a Dionex Ultimate chromatographic system combined with a Q-Exactive mass spectrometer (Thermo Fisher Scientific). Non-resonant collision-induced dissociation conditions using higher-energy C-trap dissociation (HCD) were used. To be identified, ions had to match at least two orthogonal criteria (among accurate measured mass, isotopic pattern, MS/MS spectrum,

and retention time) to those of an authentic chemical standard analyzed under the same analytical conditions, as proposed by the Metabolomics Standards Initiative[8].

The resulting datasets consisted of a matrix containing m/z and retention time values of features together with their concentrations expressed in arbitrary units (i.e. areas of chromatographic peaks).

Regarding statistical analyses, the following workflow was used:
- The raw peak areas were log10 transformed before any test.
- Univariate tests were performed using Kruskal-Wallis and the p-values were adjusted for multiple comparison using the False Discovery Rate.
- For ACLF/AD, UDC/SDC and preACLF/AD comparisons, a linear mixed model was used to account for the unbalanced design with different numbers of samples per patient and avoid pseudo-replication. The following formula was used: y = GROUP + (1|PATIENT), where "GROUP" is a fixed effect of the factor under study and "PATIENT" is a random effect corresponding to the patient ID. The fixed effects means for each group were estimated using the estimated marginal means method with the 'emmeans' r package.
- When possible, predictive models were built with four classifiers by using the R package 'caret'. The data were centered and scaled beforehand. A cross-validation was done ten times using the 10-fold methods (10 times 10 x partitions of the data in test and training sets). The hyper-parameters were tuned to maximize accuracy. The resulting AUC-ROC curves are shown for each repeats of the 10-fold cross-validation. The Importance of variables were calculated using classifier-specific methods (see here for details). The following classifiers were used:

RF: Random Forest,
PLS: Partial Least Square,
KNN: k-Nearest Neighbors,
SVM: Support Vector Machines with Linear Kernel

5. Results

5.1. Overview of the metabolites detected in serum, urine and stool samples

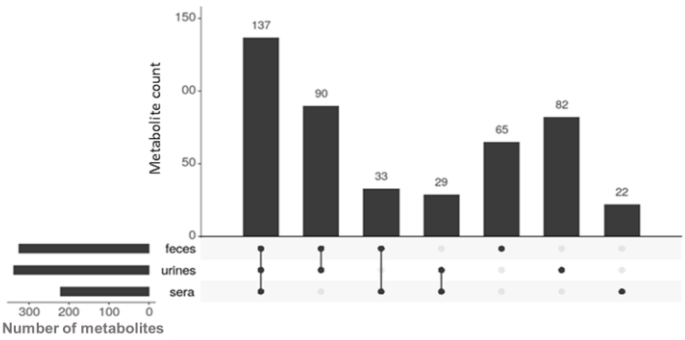


Figure 1: Overview of the metabolites detected in samples of the MUCOSA-PREDICT cohort

A total of 459 metabolites were detected in the three biological matrices. As displayed in the Figure 1, a greater number of metabolites were detected in urine (338 metabolites) and fecal (326 metabolites) samples compared to serum (222 metabolites) samples. 137 metabolites are shared by the 3 biological matrices, corresponding to 60% of serum metabolites and 40% of fecal and urinary metabolites.

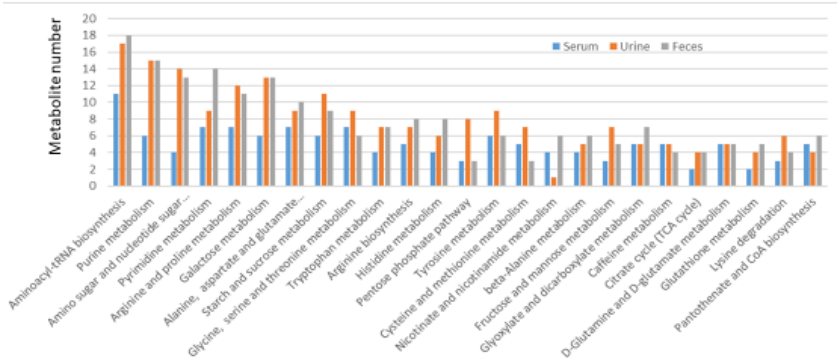


Figure 2: Pathway enrichments related to the metabolites detected in the samples of the MUCOSA-PREDICT cohort

As displayed in Figure 2, metabolites are of various chemical origins and metabolic pathways, including amino-acid metabolism, short chain fatty acids, aliphatic and aromatic organic acids, nucleotidic bases, nucleosides, vitamins, sugars. Pathway enrichment data indicate that the main pathways are present in the 3 matrices. However, some are more represented in certain biological media, as it is the case for histidine and pyrimidine metabolism in stool samples, for example.

5.2. Stratification of cirrhotic patients with stable or unstable decompensation and having or not ACLF.

5.2.a. Metabolomic signatures of ACLF can be drawn from serum, fecal and urinary samples of the MUCOSA-PREDICT cohort.

Matrice	model	AUC	AUC_CI
sera	svmLinear	0.944	0.93-0.96
sera	pls	0.942	0.93-0.95
feces and sera	rf	0.942	0.93-0.95
feces and sera and urines	rf	0.936	0.93-0.95
sera and urines	pls	0.934	0.93-0.94
sera and urines	rf	0.931	0.92-0.94
sera	rf	0.929	0.92-0.94
sera	knn	0.926	0.92-0.94
sera and urines	svmLinear	0.926	0.91-0.94
feces and sera and urines	pls	0.925	0.91-0.94
feces and sera	svmLinear	0.924	0.91-0.94
feces and sera and urines	svmLinear	0.924	0.90-0.94
feces and sera	pls	0.915	0.90-0.93
urines	pls	0.904	0.89-0.92
feces and urines	rf	0.898	0.89-0.91
feces and urines	pls	0.883	0.87-0.90
feces and urines	svmLinear	0.869	0.84-0.90
urines	svmLinear	0.868	0.84-0.89
urines	rf	0.867	0.85-0.88
sera and urines	knn	0.861	0.85-0.88
feces and sera	knn	0.860	0.85-0.87
feces and sera and urines	knn	0.859	0.84-0.87
feces	rf	0.809	0.79-0.83
urines	knn	0.787	0.77-0.80
feces and urines	knn	0.785	0.77-0.80
feces	pls	0.697	0.68-0.72
feces	svmLinear	0.660	0.62-0.70
feces	knn	0.579	0.56-0.60

Figure 3: ROC curves for the ACLF/AD metabolomic signatures

Regarding serum samples, 75 metabolites out of 222 exhibited increased concentrations in ACLF patients compared to AD ones, whereas 18 metabolites had decreased levels. Of note, 25 of the metabolites of the 38-metabolite blood fingerprint specific for ACLF and previously highlighted by Moreau et al.[1] were detected and had similar concentration trends. Regarding fecal samples, 43 metabolites exhibited increased levels (including 16 common with serum samples) in ACLF patients compared to AD ones, whereas 30 had decreased levels (including 3 in common with serum samples). Regarding urines, the concentrations of 46 metabolites were decreased in ACLF samples, whereas 19 metabolites had increased levels, including 9 in common with serum samples (i.e., mainly amino-acid and sugar derivatives and acylcarnitines).

To summarize, the greatest number of metabolites whose concentrations are impacted

by ACLF is observed in serum samples. Interestingly, some urinary and fecal metabolites not detected in serum also have their concentrations impacted by ACLF. This is for example the

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5.2.b. A metabolomics signature enabling to discriminate between stable (SDC) and unstable (UDC) acute decompensated patients can be drawn from serum samples of the MUCOSA-PREDICT cohort.

This signature mainly corresponds to serum metabolites whose concentrations are increased in UDC patients. It includes 20 metabolites, which are already part of the ACLF/AD signature, but which exhibit moderate (*i.e.*, corrected p values between 0.05 and 0.01) to intermediate (*i.e.*, corrected p values between 0.01 and 0.001) statistical significance. There are also 4 fecal metabolites, but they have moderate statistical significance. The most significant metabolites

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5.2.c. A prognostic metabolomics signature of ACLF development in AD patient can be drawn from serum samples of the MUCOSA-PREDICT cohort.

The comparison of the metabolomic data obtained from AD patients that will develop ACLF and from those who will not, enabled to draw a prognostic signature of ACLF development. It is composed of 50 and 3 metabolites whose concentrations are increased and decreased in preACLF patients, respectively. These 50 metabolites are already part of the ACLF/AD signature (cf section 5.2.a).

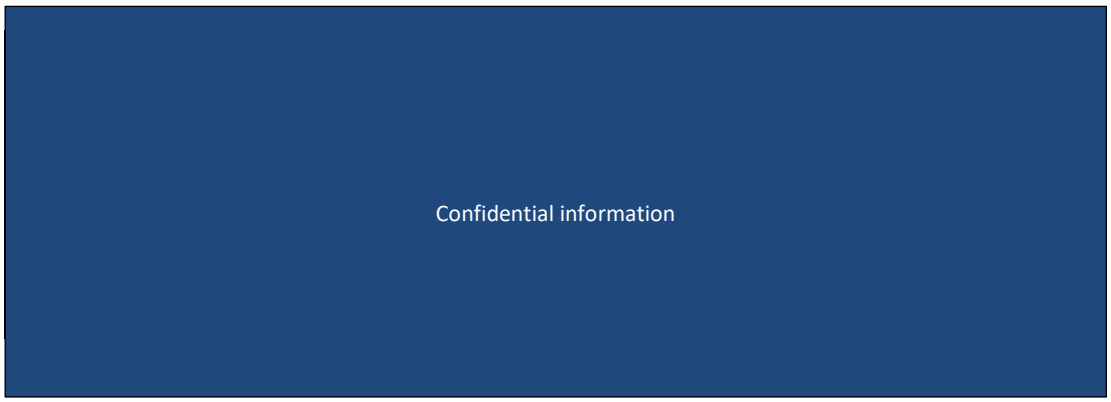
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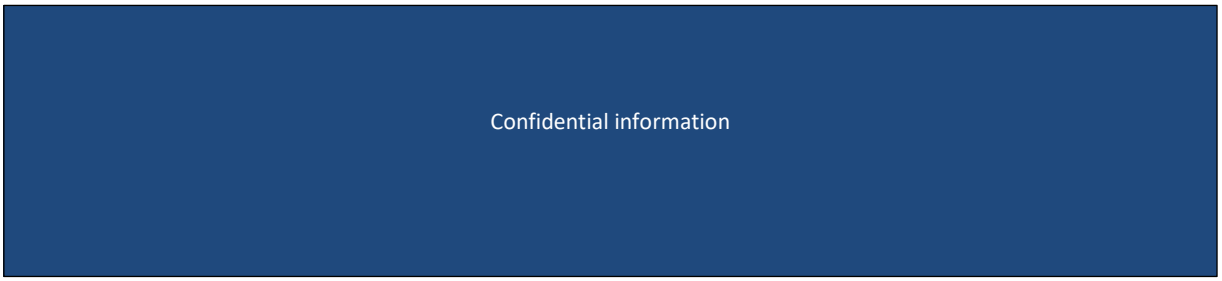
5.3. Searching for prognostic biomarker of albumin response in cirrhotic patients with acute decompensation.

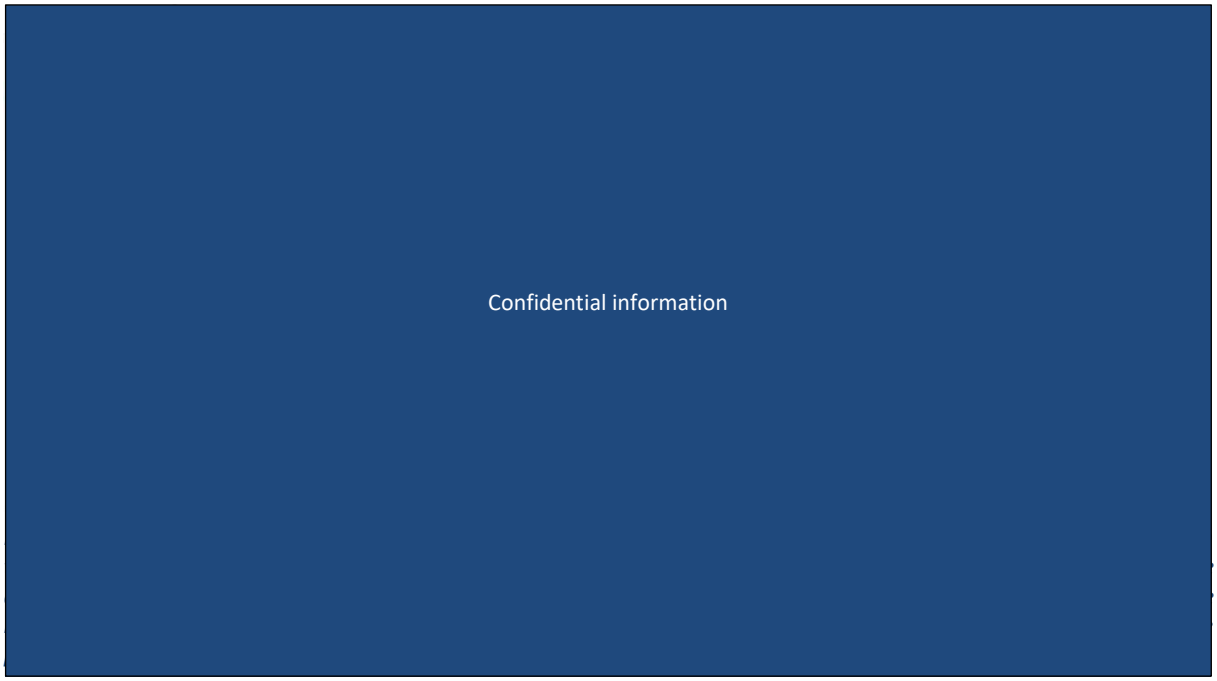
5.3.a. Analysis of samples of the MUCOSA-PREDICT cohort.

We first of all focused on samples collected at inclusion and for patients that did not receive albumin at the sampling time in order to obtain prognostic biomarkers for albumin response. We failed to obtain statistically significant biomarkers by this mean.
We then decided to work on a diagnostic model by analyzing data corresponding to patients treated by albumin and being either responders (R) or non-responders (NR). By working on the 222 annotated serum metabolites, we obtained a signature of 9 metabolites able to

discriminate between R and NR patients (urine and fecal samples did not return any statistically relevant metabolites). This signature is displayed in the table 1.







5.3.b. Analysis of samples of the ANSWER cohort.

ANSWER is a multicentre, randomised, parallel, and open-label trial aimed at evaluating the benefit of long-term albumin administration for patients with a decompensated cirrhosis[2]. We analyzed 126 plasma samples collected from 63 patients at two time-points (i.e., at inclusion before albumin administration, and at the end of the protocol), with the same experimental conditions than those previously described in this report.

Of the 222 metabolites detected in serum samples of Mucosa-Predict, 166 were found in the plasma samples of the ANSWER cohort. Of note, 3 of the top 9 biomarkers identified in the MUCOSA-PREDICT cohort were not detected in the samples of the ANSWER cohort.

We first of all focused on samples corresponding to inclusion visits and on patients that did not receive albumin at the sampling time. We failed to obtain statistically significant biomarkers under these conditions. Then, we focused on samples corresponding to follow-up visits in order to find diagnostic biomarkers. Again, we failed to obtain statistically significant biomarkers. However, similar concentration trends as those observed in the MUCOSA-PREDICT cohort were observed for 2 metabolites Confidential information

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6. Conclusion

Our data showed that metabolomics analyses enable the stratification of cirrhotic patients with acute decompensation. Blood, but also urine and fecal metabolomic signatures can discriminate between AD patients having or not ACLF. However, the blood compartment appeared as the most informative, while urine and feces compartments did not bring any benefit in terms of classification. Furthermore, a serum metabolic signature could predict the development of ACLF in patients with decompensated cirrhosis. The peak tables related to our data have been shared with other WPs, especially WP4.

Regarding albumin response, we failed to obtain prognostic biomarkers using the samples of the MUCOSA PREDICT cohort, probably due to the small size of the cohort, which was not initially designed for this aim. However, 2 interesting candidates Confidential information Confidential information were highlighted. Unfortunately, by analysing the samples of the ANSWER cohort, similar concentration trends to those obtained in samples of MUCOSA-PREDICT were observed, although without no statistical significance.

7. Acknowledgement and Disclaimer

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This report reflects only the author's view and the Commission is not responsible for any use that may be made of the information it contains.

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