MICROB-PREDICT

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

D3.2 Report on lipidomics and bile acids data

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1 Executive Summary

Analysis of bioactive lipid mediators has been completed in 308 samples from the MUCOSA-PREDICT cohort, corresponding to plasma collected at three different time-points from 100 patients with decompensated cirrhosis with and without ACLF. For this analysis, standard and up-to-date procedures have been performed (i.e LC-MS/MS). Solutions (stored at – 80 °C) were used to perform calibration curves and the recovery of all internal standards was calculated for each individual sample (see below, section 12). Recoveries > 80 % were considered valid. After quality control using internal standards, data have been obtained for all 308 samples. A complete and extensive analysis integrating the findings with other omics and with clinical data is currently undergoing.

A bile acid detection methodology that targets primary, secondary and tertiary bile acids was undertaken. Both plasma and faeces underwent characterization. 61 plasma bile acids and 52 faecal bile acids were recovered with quality control and quantification performed using internal standards similar to bioactive lipid mediators.

2 Background and rationale

Patients with acute decompensation of cirrhosis (AD) frequently develop acute-on-chronic liver failure (ACLF). ACLF is closely associated with multiple organ failures, recurrent infections and high short-term mortality and is mostly driven by innate immune system dysfunction, leading to exacerbated systemic inflammation, immune paralysis and tissue immunopathology. Indeed, plasma cytokines are unusually elevated in patients with ACLF and their levels directly correlate with ACLF severity. Moreover, a blood metabolite fingerprint specific for ACLF that is closely associated with the levels of inflammatory markers and the presence of organ failures has recently been identified.

Overall, these studies reinforce the concept that systemic inflammation and tissue/organ injury in ACLF is triggered by the concerted actions of cytokines/chemokines and amino acid derived factors that act as metabotoxins. Previous studies also provided evidence of the participation of bioactive lipid mediators in decompensated cirrhosis[1]. However, the exact contribution of lipid mediator species in the pathogenesis of systemic inflammation and development of organ failures in ACLF is not yet established.

Lipid mediators are signalling molecules with potent and diverse actions on blood and tissue homeostasis and responses to stress and injury[2].

FXR is a ligand-activated transcription factor belonging to the nuclear receptor superfamily and with bile being the receptors most potent natural ligand. Following binding of bile acids to FXR, the receptor translocates to the nucleus and through its DNA-binding domain directly influences the transcription of a large variety of tar. FXR sits at a key locus of metabolic regulation, inflammation, and driving key regulator functions.

Defective farnesoid-X-receptor signalling may be involved in hepatic inflammation, portal hypertension, and intestinal bacterial translocation, factors which are known to promote the development of ACLF.

Measurement of plasma and faecal bile acids may therefore give valuable clues to the gut derived source of these potent inflammatory mediators.

3 Samples

FCRB has received 308 plasma samples from the MUCOSA-PREDICT cohort, which have been drawn from 100 patients with decompensated cirrhosis with and without ACLF at three different time-points. A total of 308 samples were extracted using a modified Bligh and Dyer procedure and analyzed by LC-MS/MS using the Analyst 1.5.1 software package.

Kings College London and the National Phenome Centre received 308 plasma and faecal samples from the MUCOSA PREDICT cohort as per FCRB. The quantification of bile acids in these matrices is described below.

4 Experimental methods

4.1 Lipids

Two-hundred µl of plasma were spiked with an internal standard consisting of 15-HETE-d8, LTB_4-d4, PGE_2-d2 (Cayman Chemical, Ann Arbor, MI) (5 ng each) in 500 µL acetonitrile, 100 µL water and 5 µL 2,6-di-tert-butyl-4-methylphenol (BHT 10 mg/mL) and shaked vigorously. The samples were brought to pH 6 with 2 mL phosphate buffer. After centrifugation, supernatants were submitted to solid-phase extraction (SPE) in Bond Elute Certify II columns (Agilent Technologies, Santa Clara, CA) preconditioned with 3 mL methanol, followed by 3 mL of 0.1 mol/L phosphate buffer containing 5% methanol (pH 6) in a SPE Vacuum Manifold. The SPE-columns were then washed with 3 mL methanol/H_2O (50/50, vol/vol) and eluted with 2 mL of n-hexane/ethyl acetate (25:75, vol/vol) with 1% acetic acid. The eluate was evaporated on a heating block at 40°C under a stream of nitrogen. The solid residues were dissolved in 70 µL acetonitrile/H2O (50/50, vol/vol) and analyzed using an Agilent 1290 HPLC system with binary pump, multisampler and column thermostat with a Zorbax Eclipse plus C-18 column (2.1 x 150 mm, 1.8 µm) using a solvent system of aqueous acetic acid (0.05%) and acetonitrile/methanol (50:50, vol/vol). The elution gradient was started with 5% organic phase, which was increased within 0.5 min to 56%. The flow rate was set at 0.3 mL/min and the injection volume was 15 µL in a HPLC coupled with an Agilent 6495 Triplequad mass spectrometer (Agilent Technologies) with ESI source. Analysis was performed with multiple reaction monitoring in negative mode with the following source parameters (drying gas: 115 °C/16 L/min, sheath gas: 390°C/12 L/min, capillary voltage: 4300 V, nozzle voltage: 1950 V and nebulizer pressure: 35 psi). The LC-MS/MS conditions used to profile arachidonic acid derived metabolites are described in Table 1.

4.2 Bile acids

Bile acids were quantified from thawed plasma and faecal samples using the protocols previously described[3].

Briefly, BA analysis was performed by ACQUITY ultraperformance liquid-chromatography (UPLC) (Waters Ltd., Elstree, UK) coupled to either a Xevo G2-S Q-ToF mass spectrometer (for profiling application) or a Xevo TQ-S mass spectrometer (for targeted detection application) (Waters, Manchester, UK). Electrospray ionization source operating in negative ion mode (ESI^-) was used.

The reversed-phase chromatographic method consisted of a mobile phase system, paired with a shorter alkyl chain stationary phase (C8) to facilitate both the separation of BA species and the elution of lipidic matrix content. An ACQUITY BEH C8 column (1.7 µm, 100 mm × 2.1 mm) was selected and used at an operating temperature of 60 °C. The mobile phase solvent A consisted of a volumetric preparation of 100 mL of acetonitrile added to 1 L of ultrapure water, with a final additive concentration of 1 mM ammonium acetate and pH adjusted to 4.15 with acetic acid. Mobile phase solvent B consisted of a volumetric preparation of acetonitrile and 2-propanol in a 1:1 mixture. The injection volume of all samples was 5 µL. To minimize injector carry-over, 3 wash cycles of weak (H_2O/2-propanol, 90:10) and strong (2-propanol) solvent preparations were performed simultaneously with sample analysis.

Mass spectrometry parameters were as follows: capillary voltage was set at 1.5 kV, cone voltage at 60 V, source temperature at 150 °C, desolvation temperature at 600 °C, desolvation gas flow at 1000 L/h, and cone gas flow at 150 L/h. Bile acid species yielding characteristic fragments when subjected to collision-induced dissociation were assayed using multiple reaction monitoring (MRM), while those that did not fragment were assayed by selected ion monitoring (SIM).

5 Data analysis

All peak areas obtained from HPLC-MS/MS were integrated and quantified to obtain a final concentration of targeted lipid mediators. Data were assessed for preliminary analysis before submission to the data-hub for extensive characterization.

RAW data files were processed and integrated with TargetLynx v4.2[4]. Calibration curves were obtained by fitting a 2-parameter (intercept, slope) linear response curve to the calibration injection series, with a weighting factor of 1/x. Points at lower and upper ends with a relative residual deviation larger than 25% or 15%, respectively, were excluded, and only calibration curves with r2 > 0.99 were accepted. The lower and upper limits of quantification (LLOQ and ULOQ) were defined as the lowest and highest points in the final calibration curve. For those compounds where no acceptable calibration curves could be obtained, marked as “monitored”, relative abundances were reported instead of absolute concentrations. Since LLOQ, and ULOQ values were determined per analytical batch, they were further unified across the whole data acquisition by taking the highest (LLOQ) and lower (ULOQ) values achieved. The measurements were combined into a data matrix using the nPYc-Toolbox[1], and the precision and accuracy estimated for the multiple concentrations in analytical quality control samples.

6 Quality control

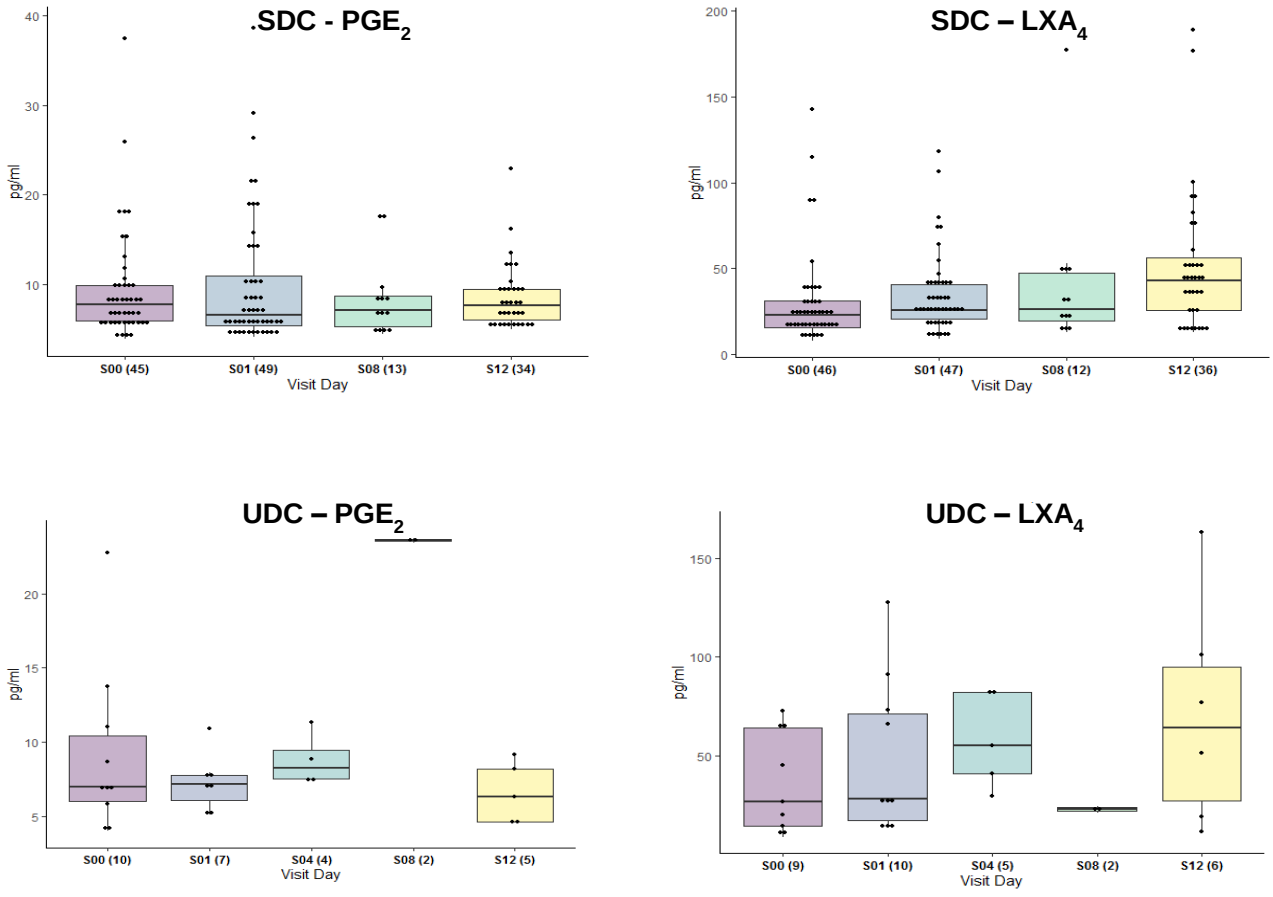
Internal standard solutions (stored at – 80 °C) were used to perform calibration curves and the recovery of all internal standards was calculated for each individual sample (see below, section 12). Recoveries > 80 % were considered valid. After quality control, data was obtained for 308 samples. All samples were successfully analyzed for both lipid and bile acid assays in terms of recoveries although quantification accuracy differed with bile acid data as noted in Supplementary Table 2 and 3.

7 Statistical analysis

Preliminary statistical analysis of data (mean and standard error of mean) has been conducted (see below section 8). Extensive analysis integrating all clinical data will be performed by WP4 (not part of this deliverable).

8 Results [organized according to the objectives]

We compared the levels of two representative lipid mediators, namely prostaglandin (PG) E_2, which is an arachidonic acid-derived lipid mediator with potent inflammatory and immunosuppressive properties, and lipoxin (LX) A_4, which is another arachidonic acid-derived lipid mediator, but with potent anti-inflammatory and proresolution properties in patients with stable decompensated cirrhosis (SDC) and unstable decompensated cirrhosis (UDC). In patients with SDC, plasma levels of both PGE_2 and LXA_4 remained invariable, whereas in patients with UDC, levels followed a fluctuating profile along the different periods of assessment.



The generated data has been transferred to the data hub and WP4 for further analyses for both lipids and bile acids.

9 Discussion

Fluctuations in the plasma levels of bioactive lipid mediators are observed in patients with UDC, who have a worse prognosis than patients with SDC. Regarding bile acids, data demonstrate that extensive primary, secondary and tertiary bile acids can be retrieved and quantified from plasma and faeces in patients with acute decompensation of cirrhosis. Performance of this assay was high with full quantification available for >50% of bile acids. BAs not passing FDA quality control requirements are suitable for analysis, but absolute concentrations cannot be attributed at this stage. However, the extensive coverage will give important additional information into the WP4 data integration.

10 Outlook

Publication will be considered after extensive analysis integrating the findings with clinical data. The aim of this WP was to accurately quantify lipids and bile acids in several matrices from samples of patients from the PREDICT cohort.

11 Conclusion

The work described in this report fulfils the requirements for the deliverable for both lipids and bile acids. Please refer to WP4 for analysis with respect to clinical metadata.

12 Tables and other supporting documents

Table 1: Measured lipid mediators

Compound name	Precursor ion	Product ion	Collision energy	Retention time* (min)
TXB_2	367.2	169.1	16	4.109
TXB_2	367.2	195.1	10	4.109
PGE_2-D4*	355.2	275.2	18	5.289
$PGF_{2\alpha}$	353.2	193.1	26	5.389
$PGF_{2\alpha}$	353.2	291.2	21	5.389
PGE_2	351.2	315.2	10	5.459
LTB_4-D4*	339.2	197.1	16	10.346
LTB_4	335.2	129.0	20	10.476
LTB4	335.2	195.1	16	10.476
15-HETE-D8*	327.2	226.2	11	15.26
5-HETE	319.2	115.1	14	16.74
5-HETE	319.2	191.2	14	16.74

*Used as internal standards.

Detected bile acids in the MICROB-PREDICT cohort are given below.

Table 2: 52 quantified faecal bile acids in the MICROB-PREDICT cohort. If no Unit is given the molecule is monitored and not fully quantified.

Bile Acid	Concentration
5-beta-Cholenic Acid-7-alpha-ol-3-one	noUnit

Bile Acid	Concentration
3-alpha-Hydroxy-7 Ketolithocholic Acid	noUnit
3-alpha-Hydroxy-7,12-Diketocholanic Acid	nM
5-beta-Cholanic Acid-3-alpha, 6-alpha-diol-7-one	nM
3-Dehydrocholic Acid	nM
omega-Muricholic Acid	nM
Glycodeoxycholic Acid	nM
Glycocholic Acid	nM
Taurolithocholic Acid	nM
Tauroursodeoxycholic Acid	nM
Taurodeoxycholic Acid	nM
Taurohyocholic Acid	nM
Tauro-omega-Muricholic Acid	nM
Taurolithocholic Acid 3-Sulfate	nM
Taurochenodeoxycholic Acid 3-Sulfate	nM
Glycocholic Acid 3-Sulfate	nM
Glycolithocholic Acid 3-Sulfate	nM
Taurodeoxycholic Acid 3-Sulfate	nM
Tauroursodeoxycholic Acid 3-Sulfate	nM
Taurocholic Acid 3-Sulfate	nM
5-Cholenic Acid-3-beta-ol	noUnit
3-Ketocholanic Acid	noUnit
Lithocholic Acid	noUnit
Isolithocholic Acid	noUnit
Allolithocholic Acid	noUnit
3,7-Diketocholanic Acid	noUnit
3,6-Diketocholanic Acid / BA13 3,12-Diketocholanic Acid	noUnit
3-alpha-Hydroxy-12 Ketolithocholic Acid	noUnit
Murocholic Acid	noUnit
5-beta-Cholanic Acid-3-beta, 12-alpha-diol	noUnit
Deoxycholic Acid	noUnit
Chenodeoxycholic Acid	noUnit
Hyodeoxycholic Acid	noUnit
Ursodeoxycholic Acid	noUnit
beta-Muricholic Acid	noUnit
Cholic Acid	noUnit
Hyocholic Acid	noUnit
alpha-Muricholic Acid	noUnit
Glycolithocholic Acid	noUnit
Glycochenodeoxycholic Acid	noUnit
Glycoursodeoxycholic Acid	nM
Glycohyocholic Acid	noUnit
Taurochenodeoxycholic Acid	noUnit
Taurocholic Acid	noUnit

Bile Acid	Concentration
Lithocholic Acid 3-Sulfate	noUnit
Cholic Acid 3-Sulfate	noUnit
Deoxycholic Acid 3-Sulfate	noUnit
Chenodeoxycholic Acid 3-Sulfate	noUnit
Ursodeoxycholic Acid 3-Sulfate	noUnit
Glycochenodeoxycholic Acid 3-Sulfate	noUnit
Glycoursodeoxycholic Acid 3-Sulfate	noUnit
Glycodeoxycholic Acid 3-Sulfate	noUnit

Table 3: 61 quantified plasma bile acids in the MICROB PREDIC cohort. If no Unit is given the molecule is monitored and not fully quantified.

Bile Acid	Concentration
3-alpha,12-alpha, 23-Nordeoxycholic Acid	nM
9(11), (5-beta)-Cholenic Acid-3-alpha-ol-12-one	nM
5-alpha-Cholanic Acid-3, 6-dione |3,7-Diketocholanic Acid	nM
3,6-Diketocholanic Acid| 3,12-Diketocholanic Acid	nM
8(14),(5-beta)-Cholenic Acid-3-alpha,12-alpha-diol	nM
5-alpha-Cholanic Acid-3-alpha-ol-6-one	nM
3-alpha-Hydroxy-7 Ketolithocholic Acid	nM
3-alpha-Hydroxy-12 Ketolithocholic Acid	nM
Murocholic Acid	nM
3,7,12-Dehydrocholic Acid	nM
3-alpha-Hydroxy-7,12-Diketocholanic Acid	nM
3-alpha-Hydroxy-6,7-Diketocholanic Acid	nM
5-beta-Cholanic Acid-3-alpha, 6-alpha-diol-7-one	nM
3-Dehydrocholic Acid	nM
12-Dehydrocholic Acid	nM
Ursodeoxycholic Acid	nM
omega-Muricholic Acid	nM
beta-Muricholic Acid	nM
Cholic Acid	nM
Hyocholic Acid	nM
alpha-Muricholic Acid	nM
Glycohyodeoxycholic Acid	nM
Glycochenodeoxycholic Acid	nM
Glycodeoxycholic Acid	nM
Glycoursodeoxycholic Acid	nM
3,7,12-Glycodehydrocholic Acid	nM
Glycocholic Acid	nM
Taurolithocholic Acid	nM
Glycohyocholic Acid	nM
Tauroursodeoxycholic Acid	nM

Bile Acid	Concentration
Taurohyodeoxycholic Acid	nM
Taurochenodeoxycholic Acid	nM
Taurodeoxycholic Acid	nM
3,7,12-Taurodehydrocholic Acid	nM
Taurohyocholic Acid	nM
Tauro-beta-Muricholic Acid | Tauro-alpha-Muricholic Acid | Tauro-omega-Muricholic Acid	nM
Taurocholic Acid	nM
Taurolithocholic Acid 3-Sulfate	nM
Lithocholic Acid 3-Sulfate	nM
Cholic Acid 7-Sulfate	nM
Cholic Acid 3-Sulfate	nM
Deoxycholic Acid 3-Sulfate	nM
Chenodeoxycholic Acid 3-Sulfate	nM
Ursodeoxycholic Acid 3-Sulfate	nM
Glycochenodeoxycholic Acid 3-Sulfate	nM
Taurochenodeoxycholic Acid 3-Sulfate	nM
Glycocholic Acid 3-Sulfate	nM
Glycolithocholic Acid 3-Sulfate	nM
Taurodeoxycholic Acid 3-Sulfate	nM
Tauroursodeoxycholic Acid 3-Sulfate	nM
Glycoursodeoxycholic Acid 3-Sulfate	nM
Glycodeoxycholic Acid 3-Sulfate	nM
Taurocholic Acid 3-Sulfate	nM
5-Cholenic Acid-3-beta-ol	noUnit
Lithocholic Acid	noUnit
5-beta-Cholenic Acid-7-alpha-ol-3-one	noUnit
5-beta-Cholanic Acid-3-beta, 12-alpha-diol	noUnit
Deoxycholic Acid	noUnit
Chenodeoxycholic Acid	noUnit
Hyodeoxycholic Acid	noUnit
Glycolithocholic Acid	noUnit

13 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.

14 References

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