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

# D3.3 Report on histological data of gut barrier

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## Abbreviations

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| **ACLF** | Acute-on-chronic liver failure |
| **AD** | Acute decompensation |
| **BT** | Bacterial translocation |
| Confidential information | |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **GI** | Gastrointestinal tract |
| **HCONT** | Healthy control |
| Confidential information | |
| **IHC** | Immunohistochemistry |
| **IIT** | Indirect immunofluorescent techniques |
| **INR** | International normalized ratio |
| **MELD** | Model For End-Stage Liver Disease |
| **ROC** | Receiver operating characteristic |
| Confidential information | |

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## 1. Executive Summary

Significantly increased Confidential information of the gut mucosa were hypothesized according to disease severity and unfavorable outcomes due to enhanced bacterial translocation (BT). Furthermore, serologic markers of Confidential information reliably reflect these pathological processes in the gut mucosa**. Methods:** Accordingly, formalin fixed and paraffin embedded tissue sections of duodenum biopsy (n=72) obtained by upper gastrointestinal endoscopy were stained for haematoxylin and eosin and predefined morphological parameters Confidential information were evaluated. Confidential information Confidential information Additional immunohistochemistry staining (IHC) was performed for Confidential information Confidential information Confidential information. Automated image analysis evaluation of the entire biopsy specimen area (i.e., the lamina propria) was introduced with the expectation to identify IHC labeled Confidential information more reliably in the samples and quantify them as numeric parameters, eliminating the subjective aspects of classical histopathological evaluation. ELISA and indirect immunofluorescent techniques (IIT) were applied to determine

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## 2. Deliverable report

### a. Background/Introduction

The crosstalk between the gut and liver is increasingly recognized in recent years. The intestinal mucosa serves as a double function barrier. On the one hand it limits the systemic dissemination of microbes and their toxic components while on the other hand it allows nutrients to access the circulation. Cirrhosis is associated with alterations in the gut microbiota composition and distribution of intestinal barrier. Small intestinal bacterial overgrowth and the disruption of both the mucosal immunity and the gut barrier integrity lead to sustained leakage of antigens, including bacteria (mainly Gram-negative ones), and bacterial products from the gut lumen (i.e., bacterial translocation, BT) to the portal circulation. Enhanced BT results in a more profound activation of proinflammatory signaling cascade and consequently increases tissue damage of the liver accelerating the progression of chronic liver diseases. Confidential information Confidential information play a pivotal role in intestinal immunity. In the hepatobiliary system and gut mucosa, the vast majority of these cells Confidential information Therefore, in

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Confidential information We hypothesized that there are significantly increased total

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## b. Material and methods

**Patients and Samples**

The prerequisites of this deliverable were completed as part of the activities of Work Package 1 (WP1), which actions are summarized in D1.2. The **patients** of MUCOSA-PREDICT cohort were selected from the concluded PREDICT study and carefully assembled as a mono-center cohort to fully control the possible geographic confounders of gut barrier failure. It comprises 93 patients with a total of 322 visits, including scheduled visits like the baseline visit, 4-8-12 week follow-up visits and the dedicated biopsy visit; in addition, unscheduled visits were performed during the course of the study and have been included as well, namely the visit at the time of ACLF development and its 1-week follow-up visit and hospital readmission visits. The clinical data of the patients were harmonized in the master template containing 400 clinical and biological variables.

Depending on patients' clinical status, the gut tissue biopsies were often not collected on the same day as the visits, so for the analysis we matched the biopsies to the visit closest in time. In order to obtain the most accurate data about the clinical status at the time of the endoscopy, dedicated biopsy visits were performed in selected cases. The average time difference between the gut biopsy sampling and clinical data collection is 3 days, median 1 day, min 0 day, max 41 days.

**Table 1** summarizes how the endoscopy and histology sampling were organized for MUCOSA PREDICT patients at the University of Debrecen. There were 8 pre-defined consecutive locations for the sampling (in the stomach, duodenum, terminal ileum, colon and rectum). The number of histology tissue samples are less than the fresh frozen samples for omics characterization due to some technical issues or patient concerns (e.g., extra biopsies for histology was not agreed or neglected for safety concerns due to the condition of patients). There is a striking difference in the sample number of one location, which is the terminal ileum.

**Methods of histology evaluation**

Morphological analysis of the gut tissue biopsies stained by haematoxylin and eosin was performed for the Confidential information by a senior pathologist as a part of the routine evaluation procedure. We also performed additional immunohistochemistry (IHC) and Confidential information (II) was evaluated. For the present deliverable we report the evaluation of 72 duodenum biopsy sample.

***Morphological analysis of the*** Confidential information

The formalin fixed and paraffin embedded tissue sections were stained for haematoxylin and eosin. A senior gastrointestinal pathologist assessed the epithelial layer according to pre-defined parameters that were the followings:

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***Morphological analysis of the*** Confidential information

Beside the morphological features immunohistochemistry (IHC) evaluation was used for identification of Confidential information was also evaluated. IHC staining method of the Confidential information is summarized in **Figure 1**.

Automated image analysis evaluation of the entire biopsy specimen Confidential information Confidential information was introduced with the expectation to identify IHC labeled cellular and/or extracellular elements more reliably in the sample and quantify them as numeric parameters, eliminating the subjective aspects of classical histopathological evaluation. In order to obtain this the ROIs (covering the entire Confidential information were manually selected by three independent observers', which took approximately 1.5-3 hours per sample for each staining.

***Quantitative evaluation of IHC on whole slides***

The planned approach has been tested and optimized in preliminary experiments and aimed to take into consideration all the information that could be derived from the whole section for each case rather than restricting analysis to a limited number of fields of view. IHC has been done using standard pathodiagnostic procedures, except only haematoxylin counterstain has been applied together with the DAB reaction. Slides were scanned using a Pannoramic Confocal (3DHistech) digital pathology scanner in transmission RGB mode. The standard mrxs output format from the scanner was converted to OME.tiff for further processing in QuPath. Nuclear and DAB staining was linearly unmixed based on single stain fingerprints. Stromal areas were manually selected for analysis and the fused ROIs stored as a project. For assessing cellular --- Confidential information, cells were segmented using built in routines with optimized parameters and single cell data including cytoplasmic mean DAB OD (which was found the best parameter in preliminary optimization) were exported in list mode. This allowed comparison of raw histograms as well as setting categories of positivity. For Confidential information Confidential information histograms were created by using our custom written script for exploiting Fiji's analyze/histogram function. Histograms were normalized before comparison. When immunohistochemistry has been done in more than a single run, DAB reaction intensity was calibrated using QIFI beads processed identically as the sections. Histogram parameters or % distribution across positivity categories were analyzed against clinical data using chi-squared test for contingency tables and stepwise multiple regression for continuous variables.

*Quantification of* Confidential information

When all ROI was defined the developed automated algorithm determined the average DAB staining intensity in the cytoplasm and cell membrane of each segmented cell separately. In every sample, Confidential information of the cells showed a continuous - log-norm - distribution, the negative and positive (intensely stained) populations did not separate along a clear value. Therefore, we set up a series of intensity cut-off values starting from the lowest intensity and increased the threshold by 5% at each step. As the positivity threshold increased less and less cells were identified as positive. We correlated the obtained numbers with other clinical and

laboratory parameters to define the best cut-off for Confidential information, while we also took biological considerations into account. Based on the observed correlations, cells above the C positivity cut-off were defined as Confidential information

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*Quantification of* Confidential information

Confidential information

number based calculation we utilized a pixel based quantification method using our custom written script. The learning set included 7 AD and 9 ACLF samples. For all samples, pixel distribution histograms were extracted from the stromal ROIs.
Pixel numbers were between 50 – 180 million per sample (summarized total pixels 748 million and 815 million, respectively). We created histograms plotting the relative pixel frequencies corresponding to given pixel intensities containing all (stained and unstained) pixels of the sections (**Figure 2**). Next our goal was to define a cut-off for stained Confidential information pixels. Based on the histograms there was no unequivocal self-defining value. However, Confidential

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Designating this crossing point as a cut-off level, the fraction of pixels above this level is

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**Methods for evaluation of** Confidential information
ELISA and indirect immunofluorescent techniques were applied to determine various Confidential

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the specification of these ELISA methods are summarized in the **Table 2**. We also provide cut-off values according to the recommendation of the manufacturer or to 97.5% percentile of HCONT involved MUCOSA PREDICT.

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**Statistical analyses**
We investigated the association of histologic data with various demographic, clinical parameters, routine laboratory test results, liver and acute decompensation specific scores describing severity of the disease, and the disease outcomes (survivor, development of ACLF syndrome, spontaneous bacterial peritonitis and systemic bacterial infection). Finally, association of histologic data with Confidential information were also assessed. In our statistical analyses we used spearman correlation, nonparametric tests, ROC curves and Cox-regression.

### c. Results

***Association between*** Confidential information ***and clinical and laboratory variables***

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### d. Discussion

The gut-liver axis plays a pivotal role in the worsening of cirrhosis. Therefore, assessing the integrity and functional state of the intestinal barrier is of paramount importance to understand pathological processes more deeply and find novel treatment options. Since Confidential information Confidential information are essential for the function of the mucosal immunity of the gut, we hypothesized that Confidential information significantly increases in the intestinal Confidential information according to disease severity and unfavorable outcomes due to enhanced BT. We

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Whole sections were scanned and evaluated. We aimed to automatize the process by software to reduce the subjective factor. Additionally, a senior pathologist evaluated the Confidential information of the gut mucosa, according to standard criteria assessing Confidential information Confidential information

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### e. Outlook

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## 3. Tables and figures

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## 4. Acknowledgement and Disclaimer

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