Histological and molecular characterization of the GAN diet-induced obese mouse model of advanced MASH with progression to advanced fibrosis and HCC

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**Background & Aim**
Metabolic dysfunction-associated steatohepatitis (MASH) is the molecular alterations leading to onset of MASH-HCC, however, the molecular alterations leading to onset of MASH-HCC are unclear. The present study aimed to evaluate disease progression in the translational GAN diet-induced obese (DIO) mouse model of advanced fibrosis MASH-HCC (GAN DIO-MASH-HCC mice).

**Methods**
Make C57BL/6j mice were fed the GAN diet high in saturated fat, fructose and cholesterol for 38-72 weeks (n=15 per group). Mice fed chow for 48-48 weeks (n=15) served as healthy controls. Terminal endpoints included AI-assisted Gubra Histopathological Objective Scoring Technique (GHOST); for NAFLD Activity Score and Fibrosis Stage; and histomorphometrics, flow cytometry, tumor classification by an expert clinical histopathologist. Brown RNA sequencing analysis was performed applying single gene set enrichment analysis (ssGSEA) and digital cytometry (xCell, EC Typex). Transcriptional profiles of murine MASH and MASH-HCC were compared to hepatic transcriptomes from human MASH-HCC patients (n=45, GSE193084).

**Conclusion**
- The GAN-DIO-MASH-HCC mouse spontaneously develops HCC on the background of progressive advanced fibrosis
- HCC molecular signature in GAN DIO-MASH-HCC mice recapitulates poor prognostic human MASH-HCC and HCC immune microenvironment
- The translational GAN DIO-MASH-HCC mouse model is highly applicable for profiling novel drug therapies targeting NASH-HCC, including first-line immune checkpoint inhibitor therapies

**Figure 1. Disease progression in GAN DIO-MASH-HCC mice.** GAN DIO-MASH-HCC mice were divided into 4 treatment groups: Chow, DIO, GAN diet feeding, and GAN diet feeding + 4 weeks of MASH. Body weight and feed consumption were monitored weekly. Histological scores were determined by image analysis (magnification range 250x). (A) Hepatic fibrosis was scored in liver sections (0–6), which reflects the degree of fibrosis. Fibrosis grade was determined based on fibrosis score and steatosis score, which was scored on a 4-point scale. The steatosis score reflects the degree of steatosis, with higher values indicating higher degrees of steatosis. Histological scores were calculated as the mean of 3 replicates. (B) Liver inflammation score was determined based on the lobular inflammation score (LIS) and the portal inflammation score (PIS). The LIS reflects the degree of inflammation in the lobules, while the PIS reflects the degree of inflammation in the portal tracts. (C) Lobular inflammation score was determined based on the presence of inflammatory cells in the lobules, with higher values indicating higher degrees of inflammation. (D) Portal inflammation score was determined based on the presence of inflammatory cells in the portal tracts, with higher values indicating higher degrees of inflammation. (E) Tumor occurrence was determined based on the presence of tumors in the liver, with higher values indicating higher degrees of tumor occurrence.

**Figure 2. Tumor occurrence in GAN DIO-MASH-HCC mice.** Tumor occurrence was determined based on the presence of tumors in the liver, with higher values indicating higher degrees of tumor occurrence. Tumor occurrence was assessed by histological analysis of liver sections, with tumors classified as either small (≤2 mm) or large (>2 mm). Tumor occurrence was determined in a blinded manner. Tumor occurrence was determined based on the presence of neoplastic lesions in the liver, with higher values indicating higher degrees of tumor occurrence. Tumor occurrence was assessed by histological analysis of liver sections, with tumors classified as either small (≤2 mm) or large (>2 mm). Tumor occurrence was determined in a blinded manner.

**Figure 3. Tumor histological classification.** Tumor histological classification was determined based on the presence of tumors in the liver, with higher values indicating higher degrees of tumor occurrence. Tumor histological classification was assessed by histological analysis of liver sections, with tumors classified as either small (≤2 mm) or large (>2 mm). Tumor histological classification was determined in a blinded manner. Tumor histological classification was determined based on the presence of neoplastic lesions in the liver, with higher values indicating higher degrees of tumor occurrence. Tumor histological classification was assessed by histological analysis of liver sections, with tumors classified as either small (≤2 mm) or large (>2 mm). Tumor histological classification was determined in a blinded manner.

**Figure 4. Mitotic-related hepatic gene expression signatures in GAN DIO-MASH-HCC mice.** Principal component analysis (PCA) of all samples based on top 100 most variable gene expression levels. (A) Tumor burden, (B) myeloid cell population, (C) lymphoid cell population, (D) fibrosis score, (E) steatosis score. The PCA was performed using the R package FactoMineR. (A) Tumor burden was determined based on the presence of tumors in the liver, with higher values indicating higher degrees of tumor occurrence. Tumor burden was assessed by histological analysis of liver sections, with tumors classified as either small (≤2 mm) or large (>2 mm). Tumor burden was determined in a blinded manner. Tumor burden was determined based on the presence of neoplastic lesions in the liver, with higher values indicating higher degrees of tumor occurrence. Tumor burden was assessed by histological analysis of liver sections, with tumors classified as either small (≤2 mm) or large (>2 mm). Tumor burden was determined in a blinded manner.

**Figure 5. Hepatic immune cell composition in GAN DIO-MASH-HCC mice.** Hepatic immune cell composition was determined based on the presence of immune cells in the liver, with higher values indicating higher degrees of immune cell composition. Hepatic immune cell composition was assessed by histological analysis of liver sections, with immune cells classified as either T-cells, B-cells, NK cells, or NKT cells. Hepatic immune cell composition was determined in a blinded manner. Hepatic immune cell composition was determined based on the presence of immune cells in the liver, with higher values indicating higher degrees of immune cell composition. Hepatic immune cell composition was assessed by histological analysis of liver sections, with immune cells classified as either T-cells, B-cells, NK cells, or NKT cells. Hepatic immune cell composition was determined in a blinded manner.

**Figure 6. Digital cytometry of tumor: Human MASH-HCC xenografts.** Digital cytometry was performed using the ImageJ software. (A) Tumor: Human MASH-HCC xenografts. Tumor: Human MASH-HCC xenografts were analyzed by digital cytometry. Tumor: Human MASH-HCC xenografts were stained with antibodies specific to human-specific markers (CD4, CD8, CD11b, and CD19) and human-specific nuclear marker (H129). Tumor: Human MASH-HCC xenografts were analyzed in a blinded manner. Tumor: Human MASH-HCC xenografts were analyzed by digital cytometry. Tumor: Human MASH-HCC xenografts were stained with antibodies specific to human-specific markers (CD4, CD8, CD11b, and CD19) and human-specific nuclear marker (H129). Tumor: Human MASH-HCC xenografts were analyzed in a blinded manner.