Development of hepatocellular carcinoma in the extended GAN diet-induced obese mouse model of NASH with advanced fibrosis

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BACKGROUND & AIM

Non-alcoholic steatohepatitis (NASH) predispose to the development of severe fibrosis and hepatocellular carcinoma (HCC). Preclinical animal models resembling NASH driven HCC development are important tools for exploring novel pharmacological interventions for HCC. The present longitudinal study aimed to characterize disease progression in the extended GAN (Gubra-Amylin NASH) diet-induced obese (DIO) mouse model of NASH.

1 Study outline

Group	Animal	Group name	Number of animals
1	LEAN-CHOW	Chow	10
2	DIO-NASH	28w	16
3	DIO-NASH	38w	16
4	DIO-NASH	48w	16
5	DIO-NASH-HCC	58w	16
6	DIO-NASH-HCC	68w	16
7	DIO-NASH-HCC	78w	16
8	DIO-NASH-HCC	88w	16

Macroscopic liver tumor evaluation:
+ Number and size of tumors

Histopathological liver tumor evaluation:
+ Reticulin staining
+ HE staining

Liver histology:
+ NAFLD Activity Score (HE)
+ Fibrosis Stage (PSR)
+ Morphometric image analysis:
- Hepatocytes with lipids (HE)
- Inflammatory foci (HE)
- Inflammation (Gal-3) (IHC)
- Fibrosis (PSR)
- Collagen (Col1a1) (IHC)
- Stellate cell activation (α-SMA) (IHC)
- Proliferation (KI67) (IHC)
- Biliary/progenitor cells (CK7/19 (IHC)

Liver RNAseq and bioinformatic analysis

2 Metabolic and biochemical parameters

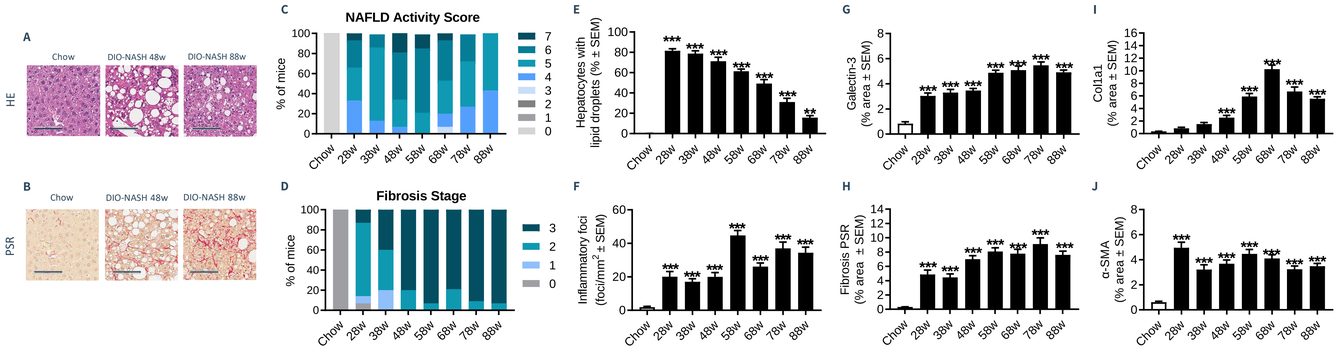

Figure 2. Disease progression in the DIO-NASH mouse model. Representative images for **(A)** HE and **(B)** PSR staining (scale bar, 100 µm). Histopathological scores were determined by Gubra Histopathological Objective Scoring Technique (GHOST) deep learning-based image analysis for **(C)** NAFLD Activity Score. **(D)** Fibrosis stage. **(E)** Hepatocytes with lipid droplets (%). **(F)** Inflammatory foci (foci/mm2). IHC image analysis of **(G)** Liver galectin-3 (% area). **(H)** Liver fibrosis PSR (% area). **(I)** Liver Collagen 1a1 (% area). **(J)** Liver α-SMA (% area). Mean ± SEM. **p<0.01, ***p<0.001 compared to chow group (Dunnett's test one-factor linear model).

3 Evaluation of HCC tumor burden

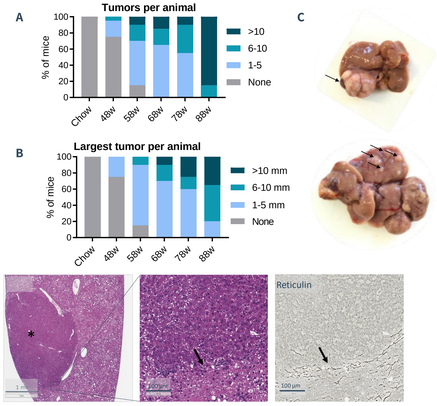

Figure 3. GAN DIO-NASH mice show progressive HCC tumor burden.
Macroscopic tumor assessment for **(A)** Number of tumors per animal, and **(B)** Largest tumor per animal (diameter, mm). **(C)** Representative livers after 68 weeks on GAN diet. Arrows indicate macroscopic hepatic neoplasms. **(D)** Representative photomicrophraphs of HE- and reticulin-stained tumor sections. High resolution images demonstrates increased hepatocyte nuclear/cytoplasmic ratio (condensed cytoplasm with normal or enlarged nuclei) and absent reticulin trabecular framework. Asterisk marks a large tumor and arrows indicate the compression zone between the neoplastic and normal liver parenchyma.

4 Histological markers for proliferation, biliary and progenitor cells

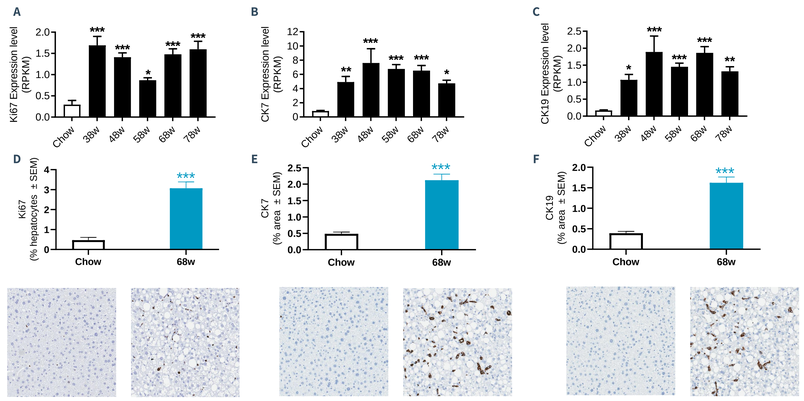

Figure 4. GAN DIO-NASH mice show increased markers for hepatocellular proliferation and activated biliary/progenitor cells.
Liver RNA expression levels for **(A)** Ki67, **(B)** Cytokeratin 7 (CK7) and **(C)** Cytokeratin 19 (CK19) during extended GAN diet exposure. IHC image analysis and representative images (panels D-F). (**D**) % of hepatocytes ki67 positive. **(E)** % area of CK7. **(F)** % area of CK19. Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 to chow group (Dunnett's test one-factor linear model). Scale bar, 100 µm.

5 Hepatic transcriptomic profile

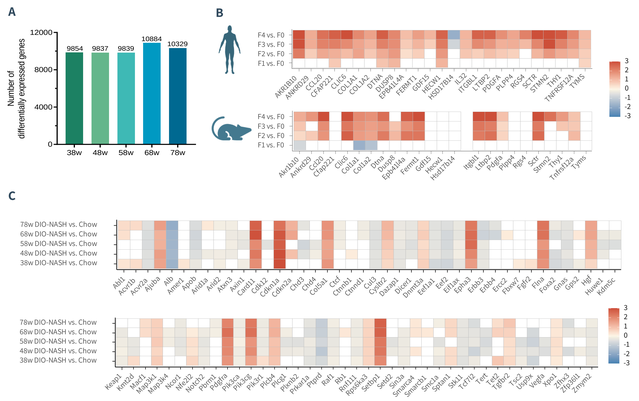

Figure 5. GAN DIO-NASH mice show clinically relevant core gene expression signatures of progressive fibrosis and HCC
(A) Total number of differentially expressed hepatic genes compared to Chow. **(B)** Core gene signatures of fibrosis progression in NASH patients (Govaere *et al.* Sci Transl Med 12, eaba4448, 2020) vs. DIO-NASH mice (Møllerhøj *et al.* Clin Transl Sci 15:1167-1186, 2022). **(C)** Candidate driver genes in human NASH-HCC (adapted from Pinyol *et al.*, J Hepatol. 75:865-878, 2021) significantly regulated in GAN DIO-NASH mice. Blue and red colour gradients indicate significantly (p<0.05) down-regulated and up-regulated gene expression, respectively. White boxes indicate genes not significantly regulated (p>0.05) compared to Chow.

CONCLUSION

+ DIO-NASH mice demonstrated advanced NAFLD Activity Score (≥4) after ≥28 weeks on GAN diet.
+ DIO-NASH mice progressed to bridging fibrosis (stage F3) after ≥48 weeks on GAN diet.
+ DIO-NASH mice consistently developed liver tumors after ≥58 weeks on GAN diet.
+ DIO-NASH mice histopathological tumor evaluation demonstrated HCC.
+ DIO-NASH mice demonstrated increased quantitative histological markers for proliferation, biliary and progenitor hepatic cells.
+ DIO-NASH mice demonstrated clinically relevant core gene expression signatures of progressive fibrosis and HCC.
+ The GAN DIO-NASH-HCC mouse model is suitable for profiling novel drug therapies for advanced fibrosing NASH-driven HCC.

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