ROLE OF GLUTAMINASE AND THE LENGTH OF GLS MICROSATELLITE IN BIOPSY-PROVEN NAFLD PATIENTS

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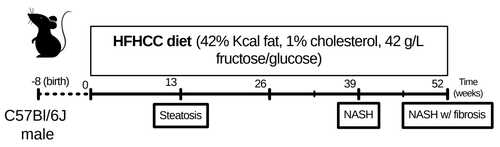
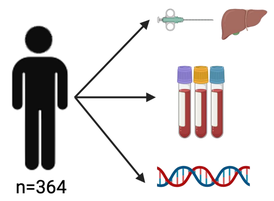
Objective

Glutaminolysis might play an essential role in NAFLD. The aim of this project was to study the role of GLS, beyond ammonia production, in an animal model of NAFLD and in biopsy-proven cohort of patients

Methods

Thirty one male C57BL/6J mice were fed a HFHCC diet (n=25) or chow (n=5) for 52 weeks. *Gls* gene expression, Gls activity and protein expression were assessed by qPCR, fluorometric and IHC assays. Ammonia levels were determined in blood using Arkray's ammoniameter.





Within a cohort of 364 biopsy-proven NAFLD patients, we determined *GLS* expression, serum glutamine and glutamate levels and genotypes for *PNPLA3*. The length of a microsatellite in the promoter region of *GLS* was assessed by capillary electrophoresis (long allele > 14 GCA repeats).

Results

Hepatic *Gls* gene and protein expression were found to increase along with the progression of NAFLD (Fig1a-b), being associated with ballooning (p=0.0006) and significant fibrosis (p=0.019). Functionally, Gls mitochondrial activity was found increased in NASH compared to control liver tissue (Fig.1c), also correlating with protein expression by IHC (p<0.05). Besides, blood ammonia levels were higher in NASH mice (Fig. 1d), altogether suggesting greater glutaminase activity.

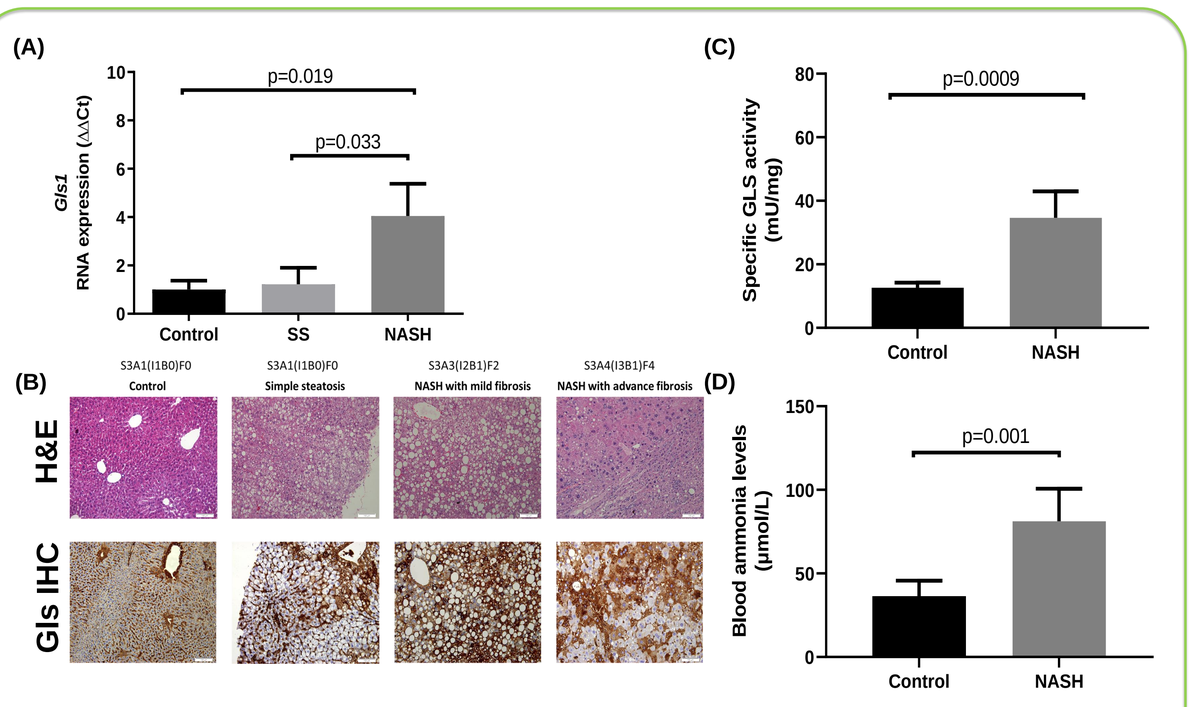


Figure 1. A) Hepatic *Gls* expression. **B)** Immunohistochemical staining with GLS in mouse tissue at different stages of the disease (Control, simple steatosis, NASH with mild fibrosis and NASH with advanced fibrosis). **C)** Mitochondrial GLS activity in Control vs NASH **D)** Blood ammonia levels in Control vs NASH.

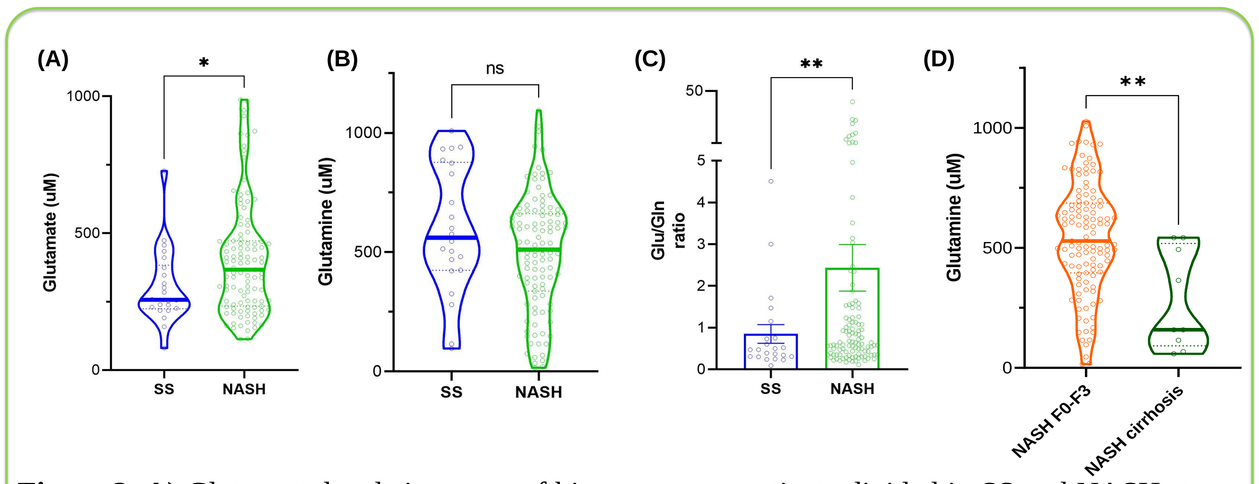


Figure 3. A) Glutamate levels in serum of biopsy-proven patients divided in SS and NASH stages of the disease. **B)** Glutamine levels in serum of biopsy-proven patients divided in SS and NASH stages of the disease **C)** Ratio glutamate/glutamine of biopsy-proven patients divided in SS and NASH stages of the disease. **D)** Glutamine levels in cirrhosis vs non-cirrhosis NASH patients

Also, glutamine levels decreased with fibrosis progression; higher differences were observed in cirrhosis vs non-cirrhosis (278±206 vs 538±231; p=0.001) (Fig. 3d).

In a cohort of 364 biopsy-proven patients with NAFLD, 261 had NASH according to SAF score. Multivariate analysis demonstrated that the length of the microsatellite in GLS was associated with NAFLD phenotype independently of other clinical or genetic (*PNPLA3* genotype) factors (Table 1). The long-long form of the microsatellite was more frequent in patients with simple steatosis, which could suggest that the genotype, by lowering *GLS* expression, may protect from progression to NASH.

Pharmacological inhibition of GLS *in vitro* decreased lipid droplet accumulation upon fatty acids overload in HepG2 cells (Fig. 2a). Besides, either CB839 administration or GLS silencing by siRNA led to lower activation of hepatic stellate cells (Fig. 2b-c). This suggests a role for GLS in steatosis and fibrogenesis.

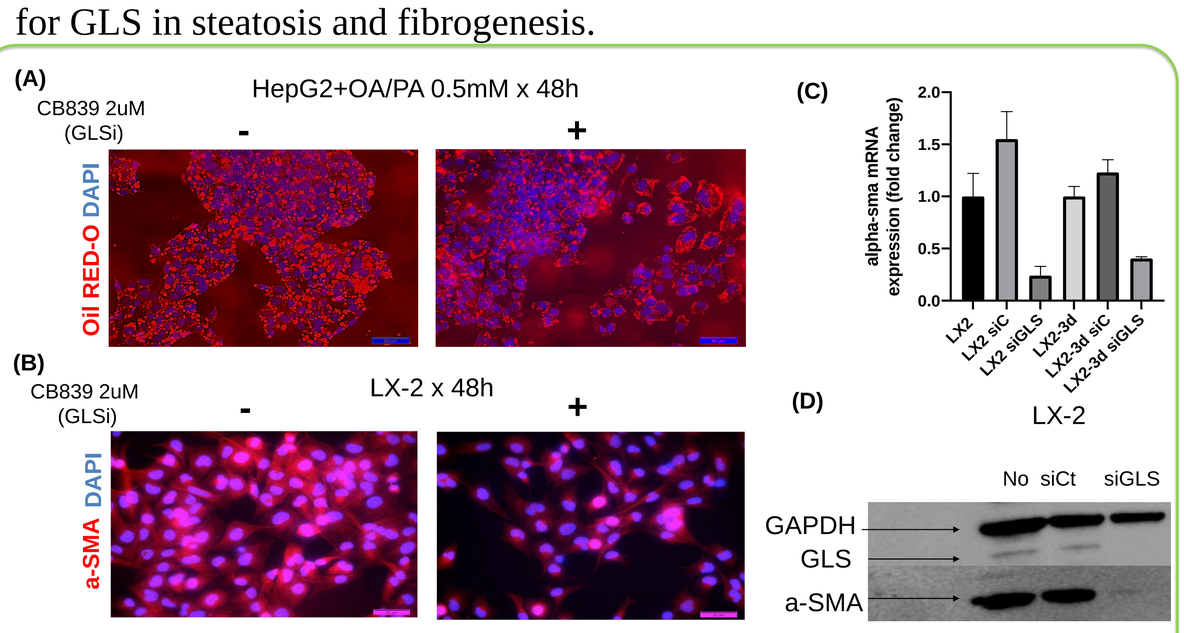


Figure 2. A) GLSi CB839 decreases lipid droplet accumulation in HepG2 cells. **B)** GLS inhibitor decreased a-SMA immunofluorescence in LX-2 cells. **C)** and **D)** Gene expression levels and WB of a-SMA upon GLS silencing by siRNA in LX-2 cells.

In humans, we confirmed higher GLS expression along with the progression of the disease; NASH patients with advanced vs mild fibrosis had 5.2±4.4 times higher expression (p=0.09; n=6/group). Also, we found that serum glutamate concentration (324±94 vs 438±126 uM; p=0.009) were higher in patients with NASH than in patients with SS (Fig. 3a). Although glutamine levels did not reach statistical significance, the glutamate/glutamine ratio (0.9±1.0 vs 4.1±8.4; p=0.035) (Fig. 3c) were increased in NASH patients, reflecting greater glutaminase activity.

	Simple steatosis (103)	NASH (261)	Univariate	Multivariate
Age (years)	46.6±13.9	52.0±13.8	ns	ns
Sex (female, %)	55.3% (57)	47,1% (123)	ns	0.04
BMI	34.95±9.7	35.9±8.7	0.009	0.036
T2DM (yes)	9.7% (10)	41.0% (107)	ns	0.001
AST	33.26±29.05	42.36±24.84	0.004	0.009
ALT	50.51±46.08	60.55±40.02	0.042	ns
PNPLA3-GG	15,5% (16)	21.8% (57)	0.112	ns
msGLS (long-long)	12.6% (13)	3.1% (8)	0.001	0.002

Table 1. Clinical characteristics of the patients based on the stage of the disease. Data is expressed as mean ± SD for the quantitative variables and as %(n) for the qualitative variables.

Conclusions

- Glutaminase expression and activity are increased in murine and human NASH.
- *In vitro* studies support its role in the pathogenesis of NAFLD.
- The length of the microsatellite was found to be a diagnostic factor of NASH, independently of previously known clinical/genetic factors. Further understanding of the regulation of GLS expression mediated by microsatellite length in NAFLD field is warranted.

Acknowledgments

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