

## Modulation of Metabolic Zonation of Glutamine Synthetase and Cytochrome P-4502E1 in Hepatic Fibrosis of Aged Cadavers

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### ABSTRACT

**AIMS:** Expression of glutamine synthetase (GS) and cytochrome P-4502E1 (CYP2E1) are notable physiological processes characterizing hepatic metabolic zonation. It is unknown whether liver fibrogenesis modulates GS zonation. CYP2E1 is upregulated in liver fibrogenesis. The present study evaluated metabolic zonation of GS and CYP2E1 in progressive stages of liver fibrosis and whether their expressions were correlated.

**METHODS:** 32 cadaveric livers (mean age, 84.8 ± 1.9) with minimal fibrosis, septal fibrosis, bridging fibrosis, incomplete cirrhosis and cirrhosis were studied. Consecutive paraffin sections were immunostained for GS or CYP2E1. GS-positive hepatocytes in perivenous and non-perivenous areas were presented as numbers per central vein and staining scores. Zonal distribution of CYP2E1 was scored.

**RESULTS:** GS-positive hepatocytes increased as fibrosis progressed cumulating in a significantly higher count at the bridging fibrosis stage. The increase was primarily attributed to a greater perivenous hepatocyte GS expression with minor contribution from non-perivenous cells. There was a significantly higher incidence of GS immunoreactivity in the non-perivenous area at the bridging fibrosis stage. GS expression was variable in incomplete cirrhosis and negligible in cirrhosis. GS-positive cell numbers correlated with their staining scores. CYP2E1 zonation was centrilobular and midlobular and occasionally periportal. CYP2E1 immunoreactivity was diffuse in nodular hepatocytes of cirrhosis. Staining scores of GS and CYP2E1 were not correlated.

**CONCLUSIONS:** Increased GS metabolic zonation may reflect a regenerative cellular response to liver injury caused by fibrosis and/or aging-related pathologies. While CYP2E1 expression shows little changes in fibrosis progression, it may have a role in effectuating aging-related hepatic pathologies.

**Key words:** Glutamine synthetase; Cytochrome P-4502E1; Metabolic zonation; Hepatic central vein; Hepatic fibrosis; Elderly cadavers

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### INTRODUCTION

Hepatic metabolic zonation characterizes diverse metabolic functions of hepatocytes along the centro-portal axis of the liver lobule<sup>[1,2,3]</sup>. Physiologically, glutamine synthetase (GS) detoxifies the ammonia entering the liver from the intestines, maintains nitrogen balance and regulates pH in the liver<sup>[4,5]</sup>. Biologically, GS serves as an immunohistological marker for perivenous hepatocytes that make up the first three cell layers around the central vein. The localization of GS to perivenous hepatocytes has been described in various mammalian species including humans<sup>[6]</sup>. It is unknown whether fibrogenesis modulates the zonation of GS in either experimental or human hepatic fibrosis.

Cytochrome P-4502E1 (CYP2E1) is a principal family member of the cytochrome P-450 microsomal monooxygenase enzyme<sup>[7]</sup>. It is constitutively expressed in the liver and other extrahepatic organs. Hepatic CYP2E1 metabolizes ethanol, drugs, fatty acids, anesthetics, mutagens, and procarcinogens. CYP2E1 catalytic activity generates potent oxidative radicals that cause tissue damage. Further, alcohol consumption upregulates CYP2E1 expression in the liver. In human liver, CYP2E1 immunoreactivity has a broader lobular distribution than that of GS, occurring predominantly in centrilobules and sometimes extending to midlobules<sup>[8,9]</sup>. Unlike GS, CYP2E1 up-regulation is known to be associated with liver fibrogenesis in human and animal models<sup>[10,11,12,13]</sup>. Nonetheless, whether there is any link between GS expression and CYP2E1 expression in hepatic fibrosis is not known.

Hepatic fibrosis is prevalent in elderly cadavers with diverse causes of death<sup>[14]</sup>. The fibrosis includes central vein fibrosis, perisinusoidal fibrosis, portal tract fibrosis, septal fibrosis with septa formation, bridging fibrosis with linking septa, incomplete cirrhosis (or pre-cirrhosis) and cirrhosis. Variable degrees of parenchymal inflammation and steatosis accompany these fibrotic changes. Accordingly, the liver of cadavers offers an opportunity to investigate into the histogenesis of metabolic zonation of GS and CYP2E1 in hepatic fibrosis of human liver. Therefore, we analyzed expression of GS and CYP2E1 in cadaveric livers with progressive stages of hepatic fibrosis. Specifically, the present study evaluated: (1) expression of GS and CYP2E1 by immunohistochemistry, (2) the number of GS immunoreactive hepatocytes in the perivenous and non-perivenous areas to determine the extent of GS metabolic zonation, (3) zonal expression of CYP2E1, and (4) any correlation between GS and CYP2E1 expression.

## MATERIALS AND METHODS

### Liver Samples

Liver tissue of embalmed cadavers from our previous studies provided the source of specimens in the present investigation<sup>[14,15]</sup>. These liver samples were obtained from cadavers at the end of the Gross Anatomy course at the Icahn School of Medicine at Mount Sinai when the organs were no longer needed. The tissue (~ 1 x 1 x 0.5 cm in size) was excised from the right lobe, put in 10% formalin and processed for paraffin embedment. These cadaveric livers presented variable tissue preservation (good, fair or poor), evaluated by hematoxylin and eosin staining<sup>[14]</sup>. Based on combined tissue preservation (good and at least fair) and fibrotic changes, 32 liver samples were selected for the present investigation with their demographics summarized in the RESULT. These included 8 livers with minimal fibrosis, 7 with septal fibrosis, 7 with bridging fibrosis, and 6 cases of incomplete cirrhosis, and 4 cases of cirrhosis. The criteria for fibrosis staging that were based on Sirius red staining of collagens in liver sections are presented below. The size of liver sections (~ 1 x 1 cm) was sufficiently large to present in general 10 well-defined liver lobules that were demarcated by a central vein in the center with four or more portal tracts at the periphery.

### Hepatic Fibrosis Staging

**Minimal fibrosis:** with or without central vein fibrosis; variable perisinusoidal/pericellular fibrosis; no evidence of portal tract fibrosis.

**Septal fibrosis:** portal tract fibrosis with developing septa emerging from the portal tract; or central vein fibrosis with developing septa extending from the vein; variable perisinusoidal/

pericellular fibrosis.

**Bridging fibrosis:** portal tract fibrosis with portal-portal linking septum, portal-central linking septum or central-central linking septum; variable perisinusoidal/pericellular fibrosis; no evidence of nodule-like formation.

**Incomplete cirrhosis:** an advanced stage of bridging fibrosis showing incomplete nodules or vague nodularity marked by broad fibrous septa in the parenchyma. This stage is also called pre-cirrhosis.

**Cirrhosis:** complete parenchymal nodule formation with thickened connective septa demarcating the nodules of either micro-nodules or macro-nodules.

In all these stages, there were variable amounts of parenchymal inflammation and portal tract inflammation. Likewise, steatosis is variably present.

### Immunohistochemistry and Antigen Retrieval

For antigen retrieval of GS immunoreactivity, paraffin-embedded liver sections were simultaneously deparaffinized, rehydrated and antigen unmasked with Trilogy™ solution (Cell Marque, Rocklin, CA), which is an EDTA-based solution. The solution was heated to a sub-boiling temperature for 30 min in a kitchen-type steamer, followed by rinsing in another hot bath of Trilogy for an additional 30 min<sup>[16]</sup>. After cooling, the slides were washed three times in distilled water and then phosphate-buffered saline (PBS). Liver sections were sequentially incubated with peroxide block (10 min), nonspecific protein block (10 min) and a rabbit polyclonal GS antibody (GeneTex, Irvine, CA) at a 1:500 dilution in 1% bovine serum albumin/PBS for 90 min at room temperature. Consecutive sections were stained for CYP2E1 with a polyclonal antibody at a dilution of 1:200 to 500 (Boster, Pleasanton, CA). For antigen retrieval of CYP2E1 immunoreactivity, sections were heated in a bath of sodium citrate (0.01 M at pH 6.0) at a sub-boiling temperature for 30 min prior to application of the antibody.

The immunoreaction was detected with rabbit polymer-HRP (Dako Envision+System, Carpinteria, CA) or ImmPress™ HRP Reagent kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine tetrahydrochloride was used as a chromogen to yield a brown color reaction product. Sections were counterstained with Harris hematoxylin for nuclei or, in addition, post-stained with Sirius red for collagens.

### Hepatic Central Veins

The central vein was defined as a vein located in the central position of the classic liver lobule and revealed a cross-sectional diameter of less than 150 μm<sup>[14]</sup>. The diameters of central veins were determined using a Nikon Eclipse 50i microscope (Nikon Instruments, Melville, NY). Central veins were selected for analysis if they fulfilled the following criteria on transverse or oblique sections: the largest diameter did not exceed 150 μm and the ratio of two perpendicular diameters was at least 0.5. Thus, longitudinally sectioned central veins were not taken for the analysis.

### Perivenous Hepatocytes and Non-perivenous Hepatocytes

Perivenous hepatocytes were defined as cells occupied cell layers 1-3 around a hepatic central vein. Non-perivenous hepatocytes were cells resided beyond (distal to) the third cell layers. In each liver, five to six central veins were selected for analyzing the distribution of hepatocytes that were positive for GS immunostaining in the centrilobular areas. Cell counts were performed under the Nikon microscope at a magnification of 400-600x. Counts of perivenous GS-

positive hepatocytes or non-perivenous GS-positive hepatocytes were presented as number per central vein. Perivenous plus non-perivenous GS-positive hepatocytes represented total cell number per central vein.

### Staining Scores for GS-Positive Hepatocytes

The distribution of GS-positive hepatocytes around a central vein was also expressed in terms of staining scores on a scale of 1-4: 1 indicated the presence of GS-positive cells in cell layer 1; 2 indicated the presence of GS-positive cells in layers 1 and 2; 3 indicated the presence of GS-positive cells in layers 1-3; and 4 indicated the presence of GS-positive cells beyond (distal to) layer 3 and these hepatocytes cells were considered as non-perivenous hepatocytes. Thus a staining score of 4 indicated that GS expression extending to the non-perivenous area of the centrilobular zone. Also, a correlation between staining scores and total GS-positive cells was analyzed.

### CYP2E1 Staining Scores

Expression of CYP2E1 was evaluated based on the distribution of its immunoreactivity in the liver lobules. The value was expressed in terms of lobule zonal staining scores: 1 for centrilobular, 2 for midlobular, and 3 for panlobular. In each liver, 5-6 well-oriented lobules were analyzed for CYP2E1 zonal distribution. Staining scores for CYP2E1 and GS were used to determine any correlation between these two parameters.

### Statistics

Data were reported as means  $\pm$  SEM. Statistical significance was determined by Student's t-tests (unpaired, two-tailed) or the chi-square test. The correlation between two parameters was evaluated by the Pearson coefficient correlations.  $P < 0.05$  was considered statistically significant. Statistics were performed using Microsoft<sup>TM</sup> Excel<sup>TM</sup> for Mac 2011, version 14.50.

## RESULTS

The demographics of the 32 cadavers are summarized as follows: The mean age was  $84.8 \pm 1.9$  years (range 57-100 years;  $N = 32$ ). Sixteen were male and 16 were female. Of the males, 9 were younger than 84 years and 7 were older than 84 years. Of the females, 9 were younger than 80 years and 7 were older than 80 years. Causes of death were available in 18 people: 11 were pulmonary, cardiac and vascular-related with or without additional diseases, 2 were cancer-related, 1 had cerebellum hemorrhage, 2 had Parkinson's disease, and 2 were liver cirrhosis-related. Although liver disease was not indicated as the cause of death in 16 of the 18 cadavers, it did not exclude that they might have ongoing liver disease. Moreover, it is not known if liver disease was indicated among the 14 donors with no available causes of death.

### Survey of GS Expression in Centrilobular Parenchyma

Figures 1 (a-g) highlighted GS expression in the cadaveric livers. The immunoreactivity of GS was generally associated with perivenous hepatocytes that were disposed in 1-3 cell layers wide around hepatic central veins. In many occasions, GS-positive hepatocytes were observed extending from the perivenous area for variable distances to non-perivenous area of the centrilobular parenchyma. Remarkably, no GS staining of hepatocytes was present in the midlobular and periportal zones.

There were GS-positive hepatocytes disposed in a single layer along the wall of sublobular veins and collecting veins (Figure 2). Hepatocytes juxtaposed the larger hepatic veins lacked GS staining. No GS immunoreactivity was seen in any of the structures associated

with the portal tracts—bile ductules and canals Hering. Likewise, the endothelial lining of hepatic central veins and sinusoids were negative for GS. Kupffer cells resided in the sinusoidal lumen constantly stained for GS.

The anatomical distribution of GS expression in the liver lobules was generally applied to cases with minimal fibrosis, septal fibrosis and bridging fibrosis, and in the first glance, the number of GS-positive hepatocytes appeared to be higher in the livers with septal fibrosis and bridging fibrosis than that with minimal fibrosis. In the more advanced fibrotic stage of incomplete cirrhosis, GS immunostaining in the livers became more variable, presenting prominent, minimal or negative staining. In end-stage cirrhosis, nodular hepatocytes were not stained for GS (Figure 3). In rare instances, sporadic hepatocytes nearby the fibrous septa showed a trace of GS staining.

### Quantification of GS Expressing Hepatocytes in Progressive Hepatic Fibrosis

Having observed the distribution of GS-positive hepatocytes in the liver lobules, their numbers were counted according to the stages of fibrosis (Figure 4a). Expressed as the number of GS-positive cells per central vein, there was an obvious trend for increase as fibrosis progresses from minimal fibrosis to septal fibrosis and to bridging fibrosis, reaching a significant higher count at the latter stage (40.5 % higher vs. minimal fibrosis,  $p = 0.029$ ). Counts of GS-positive hepatocytes were highly variable in incomplete cirrhosis. In cirrhosis no GS-positive cells were scored. To assess the contribution of perivenous hepatocytes vs. non-perivenous cells to the total GS-positive cell population, the counts were presented according to their perivenous location and non-perivenous location (Figure 4b). Both perivenous cell counts and non-perivenous cell counts showed trends for increase in septal fibrosis and bridging fibrosis with a significantly higher perivenous hepatocyte count at the bridging fibrosis stage (28.7 % higher vs. minimal fibrosis,  $p = 0.019$ ). These data reflect an enlargement of perivenous GS metabolic zonation accompanied by an extension to the non-perivenous parenchyma in fibrosis progression.

### GS Staining Scores

GS expression was also expressed in terms of staining scores (Table 1). The scores showed a trend for increase from  $2.42 \pm 0.33$  in livers with minimal fibrosis to  $2.93 \pm 0.25$  with septal fibrosis and to  $3.37 \pm 0.20$  with bridging fibrosis; the latter score was significantly higher compared to that of minimal fibrosis ( $p = 0.029$ ). These staining scores were significantly correlated with total perivenous and non-perivenous GS-positive cell counts (Figure 5)—providing an alternate semi-quantitative means for assessing GS expression.

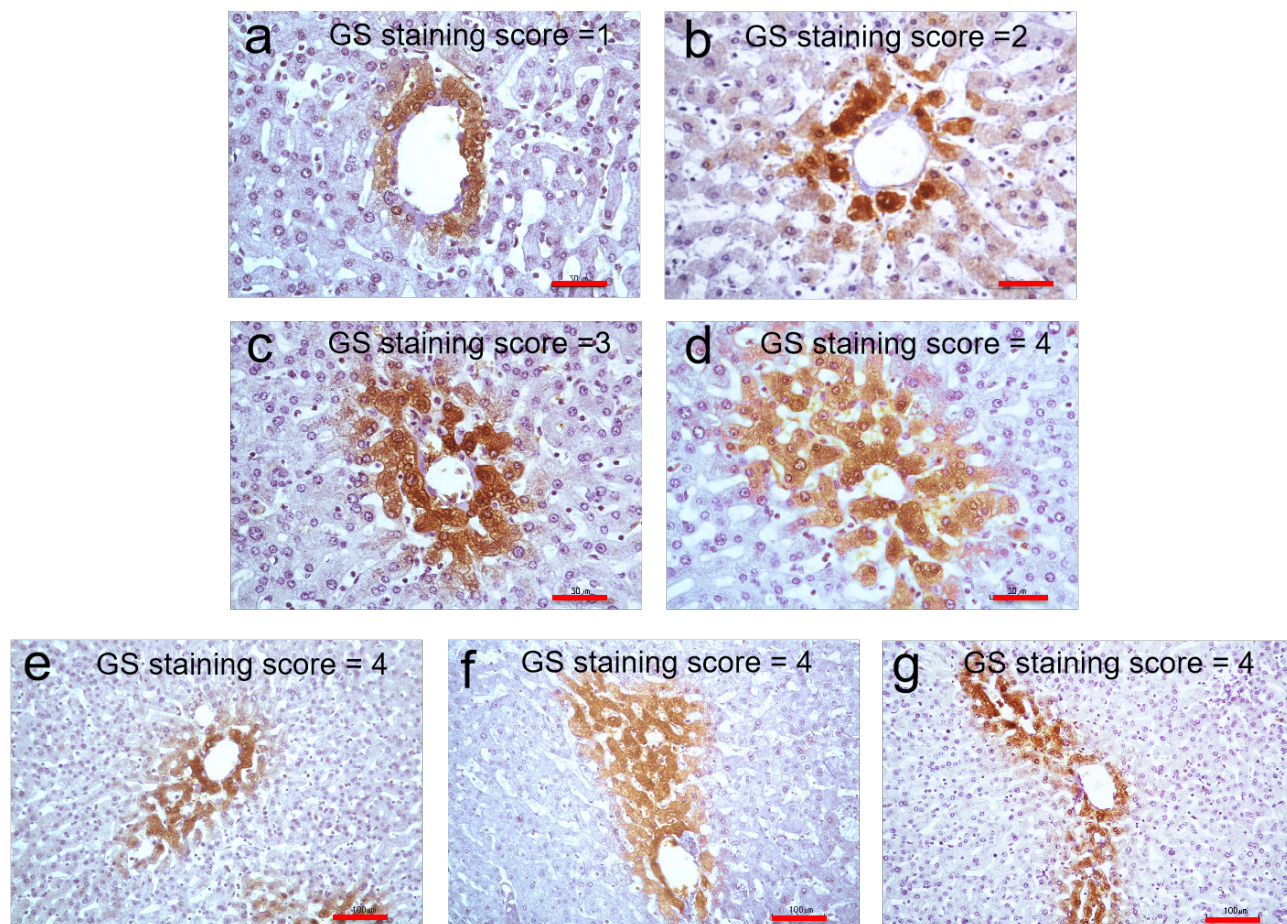
### Incidence of GS expression in Non-perivenous Area

Cell counts (Figure 4b) disclosed distribution of GS-positive hepatocytes in the non-perivenous area, the presence of which was considered as an expansion of GS zonation from the perivenous compartment distally to the centrilobular parenchyma. To substantiate this, the incidence of non-perivenous GS distribution was calculated and was expressed in terms of percent of central veins. As shown in Table 2, in the liver with minimal fibrosis, there was 28.2% of the central veins had non-perivenous GS-positive hepatocytes. In the liver with septal fibrosis, the incidence 48.7% of the veins, and in the liver with bridging fibrosis, the incidence was 63.6% of the veins ( $P = 0.037$  vs minimal fibrosis). In incomplete cirrhosis, GS expression was too variable to include for the analysis. No central vein-associated GS staining was observed in cirrhosis.

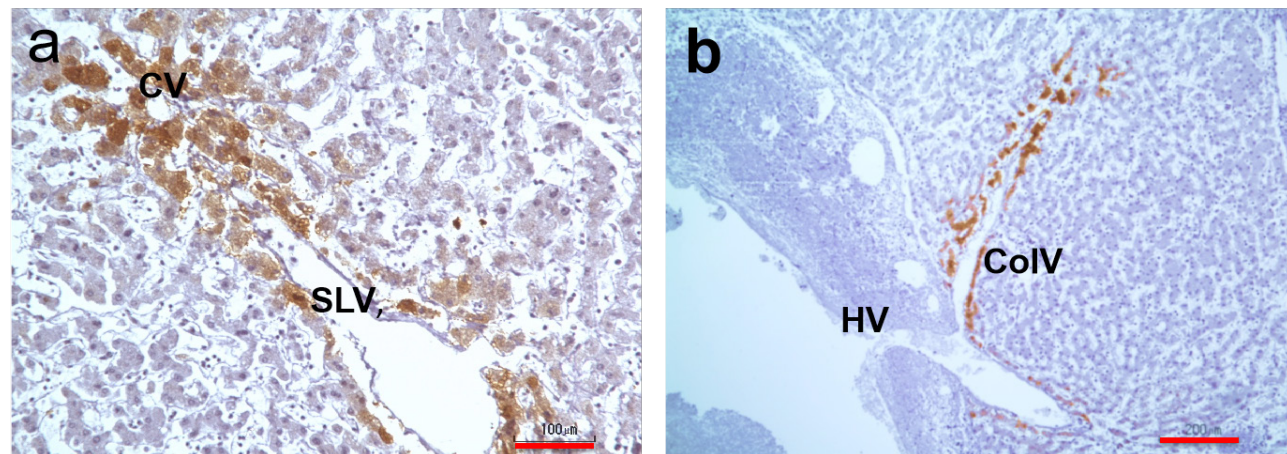
**CYP2E1 Expression in Liver**

Figure 6 demonstrates the zonal distribution of CYP2E1 expression in the liver lobules by immunohistochemistry. CYP2E1 immunoreactivity was also presented in terms of zonal

staining scores (Table 1). The scores were  $1.96 \pm 0.17$ ,  $1.80 \pm 0.14$  and  $1.88 \pm 0.22$  for minimal fibrosis, septal fibrosis and bridging fibrosis, respectively. The scores reflected centrilobular and midlobular expression of CYP2E1 and, in some instances,



**Figure 1** Immunoperoxidase staining for GS in aged cadaveric livers. Representative images showing distribution of GS immunoreactive hepatocytes surrounding hepatic central veins. GS-positive hepatocytes are discernible: (a) in the first cell layer adjacent to the rim of the central vein; (b) in cell layer 2; (c) in cell layer 3; and (d) beyond or distal to cell layers 1-3. Hepatocytes in cell layers 1-3 are considered as perivenous hepatocytes and their distributions are given a GS staining score of 1, 2 and 3, respectively. Hepatocytes beyond cell layers 1-3 are considered as non-perivenous hepatocytes and the distribution is given a GS staining score of 4. (e-g) are images demonstrating various modes of GS immunostaining extension from perivenous areas to non-perivenous areas: (e) Slight extension of GS-positive hepatocytes from one pole of the central vein to the non-perivenous area; (f) Marked extension of GS-positive hepatocytes from one pole of the central vein to the non-perivenous area; and (g) GS-positive hepatocytes extending from opposite poles of the central vein to non-perivenous areas. The distribution of GS immunoreactive hepatocytes described in (e-g) is given a staining score of 4. No extension of GS staining beyond the centrilobular zone was observed. Note fibrous tissue of the venular wall is stained blue with Harris hematoxylin counterstaining. Scale bars: (a-d) 50 µm; (e-g) 100 µm.



**Figure 2** (a) GS immunoreactivity in transition of the hepatic central vein to sublobular vein. GS-positive multi-layered hepatocytes around the central vein become a single-layered in the wall of the sublobular vein. (b) Junction of the collecting vein and hepatic vein. GS immunoreactivity associated with the wall of the collecting vein abruptly disappears in transition to hepatic vein. Hematoxylin counterstained. CV, central vein; SLV, sublobular vein; collecting vein; HV, hepatic vein. Scale bars: (a) 100 µm; (b) 200 µm.

periportal expression, but the values did not differ between the fibrosis stages. Of the 6 livers with incomplete cirrhosis, only 2 were found to stain positively for CYP2E1, showing midlobular to periportal distribution. In cirrhosis, 3 of the 4 livers showed staining of hepatocytes for CYP2E1 in the nodular parenchyma, which was particularly apparent in hepatocytes with fatty changes (Figure 6e).

**Table 1** Staining scores for GS and CYP2E1 in hepatic fibrosis.

Staining scores	Fibrosis stage		
	Minimal (8 livers)	Septal (7 livers)	Bridging (7 livers)
GS	2.42 ± 0.33	2.93 ± 0.25	3.37 ± 0.20*
CYP2E1	1.96 ± 0.48	1.80 ± 0.14	1.82 ± 0.22

GS, Glutamine synthetase; CYP2E1, Cytochrome P-4502E1. Staining scores are means ± SEM. \*GS staining score for bridging fibrosis is significantly higher than that of minimal fibrosis,  $P = 0.0296$ . CYP2E1 zonal staining scores did not differ between fibrosis stages.

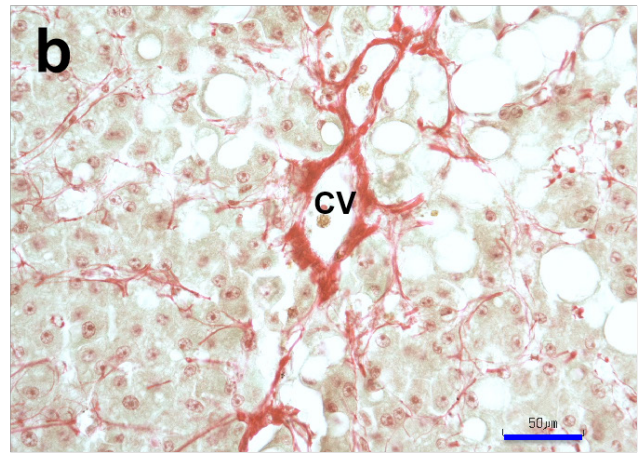
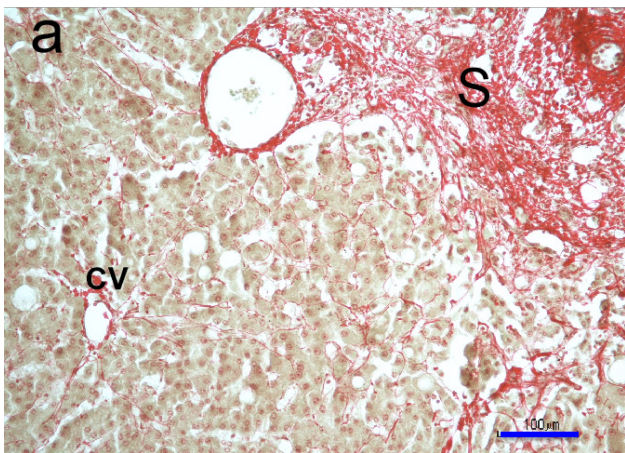
**Lack of Correlation between GS Expression and 2E1 Expression**

To evaluate whether any correlation between GS expression and CYP2E1 expression, the staining scores for GS were plotted against the zonal staining scores for CYP2E1 from livers minimal fibrosis, septal fibrosis and bridging fibrosis (from data in Table 1).

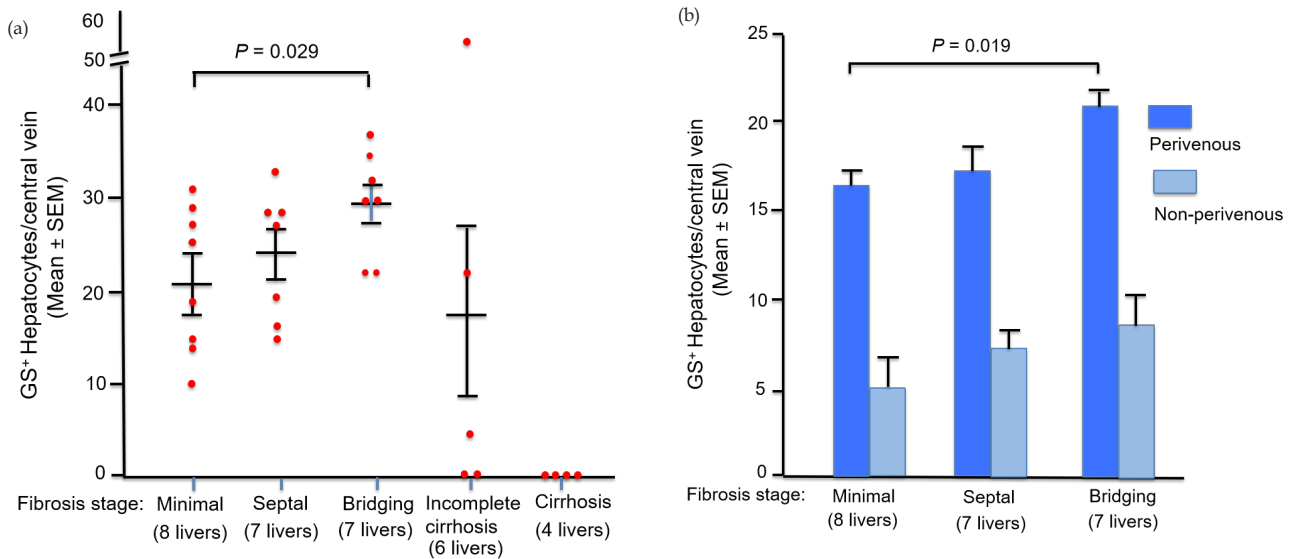
**Table 2** Incidence of GS expression in non-perivenous area in hepatic fibrosis.

	Fibrosis stage		
	Minimal (46 CV)	Septal (41 CV)	Bridging (41 CV)
CV with no extension, %	71.7	51.2	36.6*
CV with extension, %	28.3	48.7	63.4*

CV, central vein. Number of veins analyzed at the fibrosis stage is indicated in the parenthesis. Extension of GS expression to non-perivenous area is indicated when the GS staining score associated with a CV is 4 (see Materials and Methods). Statistical significance was tested by chi-square test. \*Bridging fibrosis vs. minimal fibrosis,  $P = 0.03774$



**Figure 3** Cirrhosis. (a) Hepatocytes within the nodule—including those around the central vein—lack GS immunoreactivity. The fibrous tissue of the septum also lacks GS staining. (b) Higher magnification view of a fibrotic central vein and adjacent fatty parenchymal cells showing absence of GS immunoreactivity. CV, central vein; S, fibrous septum. Counterstained with hematoxylin followed by Sirius red stain for collagens. Scale bars: (a) 100 µm; (b) 50 µm.



**Figure 4** (a) GS expression in progressive stages of hepatic fibrosis of cadavers. GS-positive hepatocytes were counted in perivenous and non-perivenous areas surrounding a hepatic central vein and the counts are expressed as the total number of positive cells per central vein. Each point represents the mean cell counts from each liver according to the fibrosis stages. The number of GS-positive hepatocytes is highest at bridging fibrosis stage, which is significantly higher than that of minimal fibrosis stage. In incomplete cirrhosis, GS cell counts are high, intermediate or undetectable. GS cell counts are absent or negligible in cirrhosis. (b) Perivenous and non-perivenous GS expression according to three stages of liver fibrosis. Counts of GS-positive hepatocytes are presented according to their perivenous and non-perivenous locations with respect to the hepatic central vein. There is a trend for increase in fibrosis progression with a significantly higher perivenous cell count at bridging fibrosis stage vs. minimal fibrosis stage.

Correlation analysis revealed a lack of significance between these two parameters, suggesting that GS expression was unlikely associated with CYP2E1 expression.

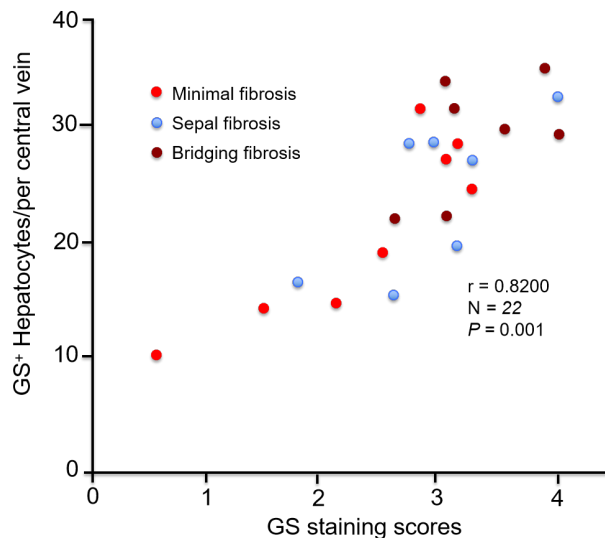
## DISCUSSION

The present study demonstrated that the metabolic zonation of GS undergoes modulation in hepatic fibrosis of aged cadavers. Increased GS expression by hepatocytes in the centrilobular zone reflects an enlargement of perivenous GS zonation along with an expansion GS to the non-perivenous parenchyma as fibrosis progresses from minimal fibrosis to septal and to bridging fibrosis. However, with further progression to the more advanced stage of incomplete cirrhosis, GS expression becomes variable leading to a nearly complete absence of GS expression in end-stage cirrhosis. On the other hand, CYP2E1 expression in aged liver is maintained in the centrilobular and midlobular zones with occasional expansion to the periportal area as fibrosis progresses to the bridging fibrosis stage. In cirrhosis, CYP2E1 immunoreactivity is particularly revealing in nodular hepatocytes with fatty changes. The staining scores for GS and CYP2E1 are not correlated, suggesting that expression of GS and CYP2E1 are likely not metabolically linked.

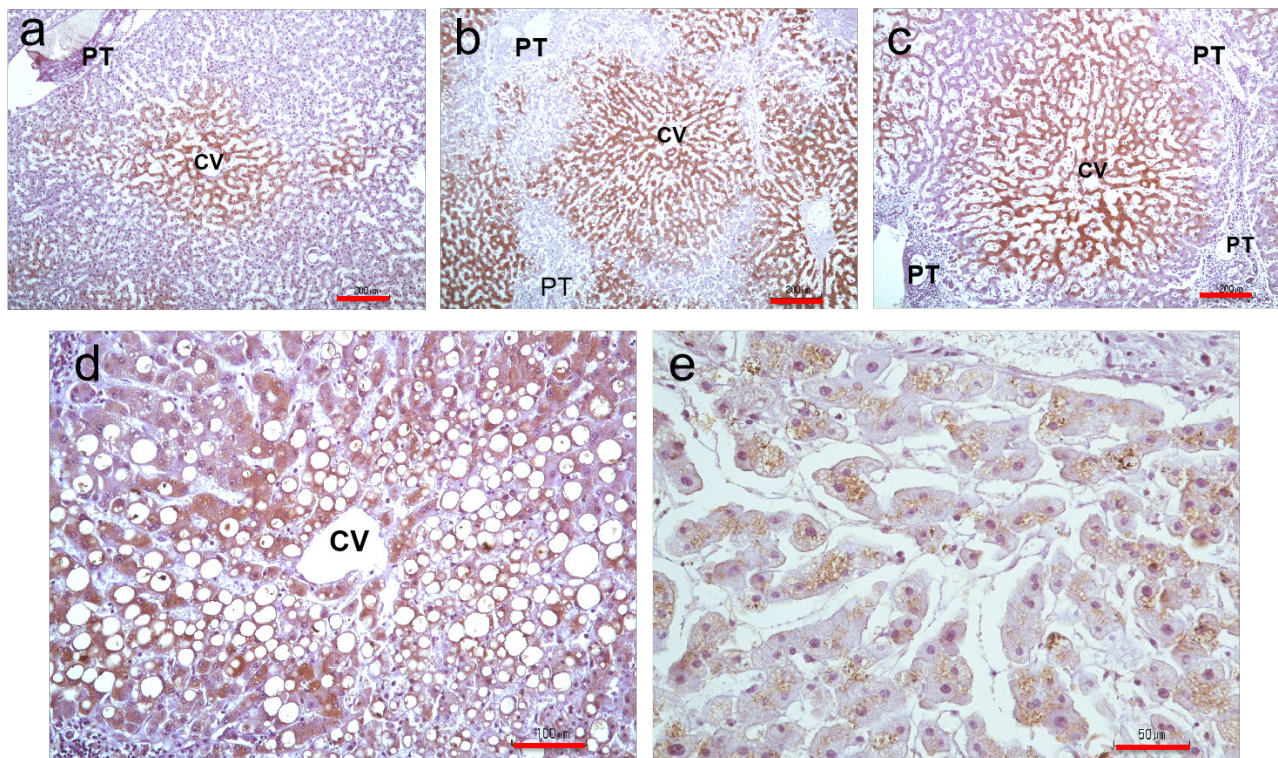
In mice, perivenous hepatocytes that form a single layer surrounding the central vein were found to selectively express Axin2 (axis inhibition protein2) mRNA<sup>[17,18,19,20]</sup>. Because no suitable antibody to detect Axin2 protein is available to date, GS has been used as a surrogate marker for Axin2 to label perivenous hepatocytes by immunohistochemistry. The present study confirmed that GS is an accurate and reliable immunohistological marker for perivenous hepatocytes of human liver, consistent with earlier studies in various mammalian species including humans<sup>[6]</sup>. Furthermore, the histological detection of hepatic GS immunoreactivity provides a tool

for evaluating lobular metabolic zonation of GS as it expands from the perivenous to non-perivenous area of the centrilobular zone.

The expansion of GS immunoreactivity from the perivenous hepatocytes distally to non-perivenous hepatocytes likely reflects an enlargement of the GS metabolic zonation in the aged liver with fibrosis, which may impact ammonia metabolism and hepatic metabolic homeostasis. The causal factors that promote the zonation change in elderly cadavers are not defined. Notwithstanding, old mice demonstrated a higher GS immunoreactivity in the pericentral



**Figure 5** Correlation between GS-positive hepatocytes and GS staining scores in hepatic fibrosis of cadavers. The numbers of GS-positive hepatocytes were plotted against the immunostaining scores from 8 livers with minimal fibrosis, 7 with septal fibrosis and 7 with bridging fibrosis. A significant correlation between these two parameters was found.



**Figure 6** Immunoperoxidase staining for CYP2E1 in hepatic fibrosis of cadavers. The images illustrate zonal distribution of CYP2E1 and zonal staining scores. (a) Centrilobular zone with score 1. (b) Midlobular zone with score 2. (c) Panlobular with score 3. (d) This image shows marked CYP2E1 immunostaining of hepatocytes containing macrovesicular fats. (e) In cirrhosis, CYP2E1 staining is apparent in nodular hepatocytes containing microvesicular fats. CV, central vein; PT, portal tract. Hematoxylin counterstained. Scale bars: (a-c) 200 µm; (d) 100 µm; (e) 50 µm.

area compared to young mice, the effect of which was potentiated by feeding with a high fat diet<sup>[21]</sup>. These old mice developed moderate to severe hepatic steatosis accompanied by pericentral inflammatory cell infiltrates and hepatocyte necrosis, reminiscent of non-alcoholic steatosis hepatitis (NASH). Whether the up-regulation of GS expression associated with fatty hepatocytes is a manifestation of regenerative response replacing injured hepatocytes is unclear from this study. Nevertheless, there is a possibility that the increased hepatic GS expression described in the present study with aged cadavers is a regenerative response to the impact of a combined aging-related change and increasing liver injury associated with fibrosis progression. This awaits additional study that includes histological normal liver samples of young people.

In normal human liver, GS is precisely expressed by perivenous hepatocytes around the central vein. However, modulation of GS expression occurs in the liver with various pathologies. Indeed, extension of GS immunoreactivity from the perivenous area towards the portal region was observed in adenomatous polyposis coli (APC) gene knockout mice with aberrant activation of  $\beta$ -catenin<sup>[22]</sup>. Conversely, overexpression of Wnt antagonist Dickkopf-1 (Dkk1) in mice, which blocked Wnt/ $\beta$ -catenin signaling, led to a loss of perivenous GS expression and subsequently converted the entire liver lobular cells to a periportal phenotype. Liver-specific deletion of  $\beta$ -catenin in knockout mice resulted in a loss of GS expression from perivenous hepatocytes and pericentral liver zonation<sup>[23,24,25]</sup>. Ectopic expression of the angiocrine Respondin1 (RSPO1) in adult mice expanded GS expression into the midlobular area<sup>[26]</sup>. Other studies found marked expression of GS in non-perivenous hepatocytes in liver disorders of regenerative states such as chronic HVB, HVC, focal nodular hyperplasia peritumorous hyperplasia, and in some hepatocellular neoplasms<sup>[27,28,29,30]</sup>. In alcoholic cirrhosis and NASH cirrhosis, hepatic GS expression was diminished or even lost from the nodular parenchyma<sup>[30,31]</sup>. The findings of diminished and variable GS expression in pre-cirrhosis and loss of GS in cirrhosis of aged liver appear to be in accord with this phenomenon. At variance, cirrhotic nodules of HVC patients were strongly stained for GS<sup>[29]</sup>. The loss of perivenous GS expression in cirrhosis is thought to reflect a disruption of perivenous hepatocytes-hepatic central vein contact as endothelial cells of the central vein serve as anatomical-molecular link to orchestrate perivenous biology, physiology and pathology<sup>[32]</sup> by providing a source of angiocrines Wnt2, Wnt 9b and RSPO3 [see 33 Mak and Shin, 2021 for review].

CYP2E1 expression is maintained as fibrosis progresses from minimum to septal and to bridging fibrosis of elderly cadavers. The CYP2E1 immunoreactivity is more extensively distributed in centrilobules, midlobules and occasionally in periportal area than that of normal human liver without fibrosis, which is predominantly centrilobular. CYP2E1 is also detectable in cirrhosis of the aged livers. The immunohistological findings point to a role for CYP2E1 in fibrogenesis of aged cadavers. In fact, increased CYP2E1 expression is associated with fibrosis in nonalcoholic fatty liver disease/NASH and alcohol-induced liver fibrosis of human and animal models<sup>[10,11,12,13]</sup>. However, the involvement of CYP2E1 in mediating liver physiology and pathology during the aging process is not well understood. Indeed, several studies reported that the levels and activities of CYP2E1 are either decreased or unchanged during the aging process of humans, rats and mice<sup>[34,35,36,37,38]</sup>. Accordingly, the effect of CYP2E1 in aging was examined in old mice using aged female Cyp2e1-null mice and wild type mice in conjunction with corresponding young mice<sup>[38]</sup>. The data implicated that CYP2E1 plays a prominent role in effectuating aging-related hepatic steatosis,

fibrosis and apoptosis possibly through increasing production of oxidative radicals. These changes are in line with the incidence of steatosis, various formations of parenchymal fibrosis, cirrhosis and cancer that were previously described in the elderly cadavers<sup>[14]</sup>.

GS and CYP2E1 are biochemical indicators of the activity of Wnt/ $\beta$ -catenin signaling. Their expression levels have been used to assess the capacity for liver regeneration in response to liver injury induced by the analgesic acetaminophen (APAP) and the hepatotoxin carbon tetrachloride, or following partial hepatectomy<sup>[reviewed in 32]</sup>. In people with HCV infection, GS and CYP2E1 immunostains were found to be useful indicators of hepatic zonation<sup>[29]</sup>. Unlike GS, the immunoreactivity of CYP2E1 is not entirely lost from cirrhosis of aged cadaveric liver. CYP2E1 immunostaining is observed in nodular hepatocytes of cirrhosis, particularly prevalent in hepatocytes showing fatty changes. Also, GS expression lacks a correlation with CYP2E1 expression, suggesting that the zonation of GS is likely metabolic independent of CYP2E1 zonation. This is unexpected given that both the GS and CYP2E1 gene and protein expressions are mediated by the Wnt/ $\beta$ -catenin signaling pathway<sup>[23,24,25,39]</sup>, the latter is known as the master regulator of hepatic zonation<sup>[2,3,32,40]</sup>.

In conclusion, GS serves as an accurate and reliable immunohistological marker for perivenous hepatocytes. Expansion of metabolic zonation of GS from the perivenous area to non-perivenous area may reflect a regenerative cellular response to liver injury caused by fibrosis and/or aging-related pathologies. In that case, the prevalence of GS immunoreactivity could serve as an immunohistological indicator of liver parenchymal cell regeneration. Loss of perivenous GS expression in cirrhosis may reflect a disruption of contact between the central vein endothelium and perivenous hepatocytes. Understanding the histogenesis of hepatic GS expression may help in therapeutic planning for enhancing hepatic regeneration in liver fibrosis disease. While CYP2E1 expression revealed little changes in the fibrosis progression of aged people, it nevertheless may have a role in effectuating aging-related hepatic pathologies.

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