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ORIGINAL ARTICLE

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Glycogenosis is common in nonalcoholic fatty liver disease and is independently associated with ballooning, but lower steatosis and lower fibrosis

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Abstract

Background/Aims: Glycogen synthesis and storage are normal hepatocyte functions. However, glycogenosis, defined as excess hepatocyte glycogen visible by routine H&E light microscopy, has not been well characterized in nonalcoholic fatty liver disease (NAFLD).

Methods: Glycogenosis in NAFLD liver biopsies was graded as "none", "focal" (in <50% of hepatocytes), or "diffuse" (in ≥50% of hepatocytes). Clinical and pathological variables associated with glycogenosis were assessed. 2047 liver biopsies were prospectively analysed.

Results: In adults and children, any glycogenosis was present in 54% of cases; diffuse glycogenosis was noted in approximately 1/3 of cases. On multiple logistic regression analysis, adults with glycogenosis tended to be older ($P = .003$), female ($P = .04$), have higher serum glucose ($P = .01$), and use insulin ($P = .02$). Adults tended to have lower steatosis scores ($P = .006$) and lower fibrosis stages ($P = .005$); however, unexpectedly, they also tended to have more hepatocyte injury including ballooning ($P = .003$). On multiple logistic regression analysis, paediatric patients with glycogenosis were more likely to be Hispanic ($P = .03$), have lower body weight ($P = .002$), elevated triglycerides ($P = .001$), and a higher fasting glucose ($P = .007$). Paediatric patients with glycogenosis also had less steatosis ($P < .001$) than those without.

Conclusions: Glycogenosis is common in adult and paediatric NAFLD, and is associated with clinical features of insulin resistance. Glycogenosis is important to recognize histologically because it may be misinterpreted as ballooning, and when

Abbreviations: AIC, akaike's information criteria; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index (weight in kg/height in metes^2); H&E, haematoxylin and eosin; HbA1c, haemoglobin A1c; HDL, high density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; IQR, inter-quartile range; NAFL, nonalcoholic fatty liver ("isolated steatosis"); NAFLD, nonalcoholic fatty liver disease; NAS, NAFLD activity score; NASH CRN, nonalcoholic steatohepatitis clinical research network; NASH, nonalcoholic steatohepatitis; OR, odds ratio; PAS, periodic acid-Schiff; PASD, periodic acid-Schiff with diastase; PPP1R3B, protein phosphatase 1 regulatory subunit 3B.

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diffuse, confusion with glycogen storage disorders or glycogenic hepatopathy must be avoided. The newly observed dichotomous relationship between glycogenosis and increased liver cell injury but decreased steatosis and fibrosis requires further study.

KEYWORDS
adults, children, hepatic glycogen, pathology, steatohepatitis

1 | INTRODUCTION

The liver is central to lipid and carbohydrate metabolism in the healthy state, but these become dysregulated in the setting of obesity and metabolic syndrome, and contribute to the pathogenesis of nonalcoholic fatty liver disease (NAFLD).[1] Steatosis, hepatocellular lipid accumulation, is a hallmark histopathologic lesion in NAFLD,[2] and the histological, laboratory, radiographic and clinical associations of steatosis have been widely studied.[3,4] However, relatively little is known about hepatocellular glycogen processing in NAFLD.

Hepatocytes normally contain glycogen in the unfasted state, but the hepatocellular glycogen is not distinctly visible by light microscopy using routine haematoxylin & eosin (H&E). In contrast, glycogen is distinctly visible by routine methods in pathological conditions such as glycogen storage diseases and glycogenic hepatopathy.[5] This abnormal glycogen accumulation visible on routine H&E, termed glycogenosis, is characterized by faint grey to light pink cytoplasmic pallor and rarefication.

The observation by our group that some hepatocytes in NAFLD contained glycogenosis, led us to a systematic investigation. Our aim was to describe the prevalence of hepatic glycogenosis in adults and children enrolled in the National Institutes of Health-sponsored Nonalcoholic Steatohepatitis Clinical Research Network (NASH CRN) and to evaluate the associated clinical, laboratory and histopathological variables.

2 | MATERIALS AND METHODS

Haematoxylin & eosin, Masson trichrome and Prussian blue stained slides from the NASH CRN Database Study, a noninterventional registry, were prospectively reviewed and scored by the NASH CRN Pathology Committee in a consensus manner. The NASH CRN Pathology Committee is comprised of nine expert liver pathologists who practice at US-based academic medical centres. All cases in this study were scored during group review, with a minimal number of three pathologists in attendance at any given scoring session. Final scores were determined by discussion and a majority opinion. Approval from the institutional review boards at each participating center and the data coordinating center had been obtained. Each case was evaluated according to the published and validated NASH CRN scoring system[6] In addition, glycogenosis was defined as a faint grey to light pink cytoplasmic pallor seen on routine H&E using light microscopy (Figure 1A,B). The degree of glycogenosis was scored as "none", "focal" or "diffuse". "None" was defined as complete absence of glycogenosis. "Focal" was defined as glycogenosis involving less than 50% of hepatocytes. "Diffuse" was defined as glycogenosis involving equal to or more than 50% of hepatocytes. Because Periodic acid-Schiff (PAS) and Periodic acid-Schiff with diastase (PASD) histochemistry is not included among the standard NASH CRN stains, the presence of glycogen was confirmed with PAS and PASD in a subset of cases (n = 4). Similarly, electron microscopy was used on 1 case to confirm the presence of glycogen.

Demographics and clinical data were collected at the time of patient entry into the NASH CRN. Laboratory values were collected within 6 months prior to or up to one month after the date of the biopsy.

The two primary comparisons were the presence of any glycogenosis vs none, and focal vs diffuse glycogenosis. Unadjusted comparisons were stratified by adults and children (age < 18 years

of age). Distributions were summarized using proportions for categorical variables and means and standard deviations for normally distributed variables, or medians and inter-quartile ranges [IQR] for non-normal data. *P*-values for the two primary comparisons were derived using the chi-square test for categorical variables and t-tests for normally distributed variables or Wilcoxon rank sum test for non-normally distributed variables. Multiple logistic regression was used to test for independent effects on any vs no glycogenosis and diffuse vs focal glycogenosis. Adults and children were combined for the latter two analyses. Results from stratified analyses were similar to the combined results (data not shown). Akaike's information criteria (AIC) were used to select the set of variables that maximized the amount of information from a candidate list of all variables in Table 1. P-values were two-sided, nominal and not adjusted for multiple comparisons. Analyses were performed using SAS version 9.3 (SAS Institute) and Stata version 14 (StataCorp).

3 | RESULTS

A total of 2047 liver biopsies were analysed, including 1348 from adults and 699 from children. Glycogenosis, focal or diffuse, was observed in 54% of adult cases and 53.5% of paediatric cases. Diffuse glycogenosis was identified in 28% of adults and 34% of children.

Glycogen was confirmed by PAS and PASD histochemistry in a subset of cases (n = 4, Figure 1C,D). Electron microscopy from a formalin fixed paraffin embedded liver biopsy was performed on one representative case (Figure 2).

3.1 | Clinical and laboratory features associated with glycogenosis in adults

Compared to those without glycogenosis, adults with any glycogenosis were older (mean age in years was 51.6 ± 11.7 vs 48.9 ± 12.3, $P < .0001$), predominantly female (68% vs 59%, $P = .0008$) and more frequently had diabetes mellitus (45% vs 39%, $P = .05$). There were only four adult subjects with type 1 diabetes mellitus, and all were in the glycogenosis group. BMI was not different between the two groups ($P = .51$). Patients in this cohort (both those with and without glycogenosis) were mostly White, and Hispanic ethnicity was reported in a small subset of cases in both groups (14% in those with glycogenosis vs 11% in those without glycogenosis, $P = .09$) (Table 1).

Patients with any glycogenosis demonstrated a higher serum glucose (115 mg/dL ± 42 vs 108 ± 39, $P = .002$) and HbA1c (6.4% ± 1.3% vs 6.2% ± 1.1, $P = .008$) in comparison to patients without glycogenosis. There were no significant differences in ALT, AST, alkaline phosphatase, insulin, HOMA-IR or lipoprotein levels between those with or without glycogenosis.

Patients with diffuse glycogenosis as opposed to those with focal glycogenosis were more frequently women (75% vs 60%, $P < .0001$), of Hispanic ethnicity (17% vs 11%, $P = .03$), and had lower ALT levels (64 U/L ± 47 vs 74 ± 49, $P = .009$), and higher HDL levels (46 mg/dL ± 13 vs 43 ± 12, $P = .02$), but there were no differences in glucose, insulin, HOMA-IR or other lipoprotein levels.

Layman Summary

- The global "epidemic" of obesity continues to be an important and growing health concern. Obesity and related conditions including hypertension, dyslipidaemia and diabetes mellitus work synergistically to damage the liver. This is called nonalcoholic fatty liver disease (NAFLD). NAFLD has a mild subtype (NAFL) and a serious subtype called nonalcoholic steatohepatitis (NASH) that can lead to liver scarring (cirrhosis), liver cancer and liver failure. Distinguishing NAFL from NASH currently requires a liver biopsy. Pathologists review the liver tissue under a microscope to look for fat, inflammation, liver cell death, and fibrosis, and use what they see to make a diagnosis.
- A group of NIH-sponsored expert liver pathologists reviewed a large number of NAFLD liver biopsies from adults and children, and for the first time described that not only were the liver cells stuffed with lipid, but many cases (more than half) also showed liver cells stuffed with glycogen ("glycogenosis"). Statistical analysis showed that adult NAFLD cases with glycogen were associated with older age, women, high blood levels of glucose and insulin. Furthermore, although cases with glycogenosis were associated with more cell injury and cell death, there was relatively less fat in the cells and importantly less scarring (fibrosis). This is a new insight; in NAFLD not only do the liver cells have abnormal lipid metabolism and storage, but they also have abnormal glycogen metabolism and storage. Our findings suggest the possibility that shunting of substrates away from lipid droplets towards glycogen deposition may protect the liver from scaring, and thus in the long term may protect from liver failure. More studies are needed so that we can better understand the interplay between lipid and glycogen metabolism inside the NAFLD liver cells, this may broaden our attempts to find good therapies for NAFLD.

3.2 | Clinical and laboratory features associated with glycogenosis in children

Compared to those without glycogenosis, children with any glycogenosis were younger (12.2 years ± 2.8 vs 12.8 ± 2.8, $P = .008$) had a lower BMI (31.3 kg/m^2 ± 6.0 vs 32.6 ± 6.8, $P = .008$), were less frequently White (59% vs 74%, $P < .0001$) and mainly Hispanic

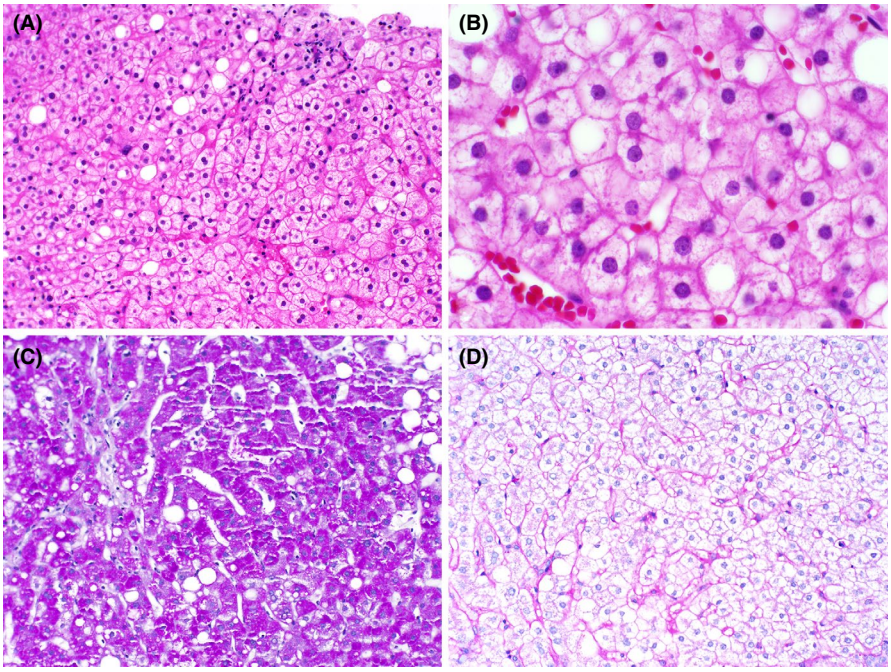

FIGURE 1 Glycogenosis as seen on routine light microscopy. (A) Medium to low power H&E showing diffuse cytoplasmic glycogen accumulation in hepatocytes (200x). (B) Higher power magnification shows cytoplasmic pale grey to faint pink glycogen collections (600x). (C) PAS histochemistry highlights the presence of diffuse cytoplasmic glycogen (200x). (D) PAS with diastase shows complete glycogen removal

(77% vs 66%, $P = .001$). A similar and small subset of patients in both groups carried a diagnosis of type 2 diabetes mellitus (6% vs 6%, $P = .75$). There were three children with type 1 diabetes mellitus, all of whom had diffuse glycogenosis (Table 2).

Paediatric subjects with any glycogenosis had higher levels of alkaline phosphatase (232 U/L ± 101 vs 216 ± 110, $P = .04$), glucose (92 mg/dl ± 30 vs 87 ± 11), HbA1c (5.6% ± 1.1 vs 5.4 ± 0.4, $P = .002$) and triglycerides (159 mg/dL ± 88 vs 143 ± 73, $P = .009$). ALT, AST, insulin, HOMA-IR and other lipoprotein levels were not statistically different between the two groups.

After stratifying patients with glycogenosis into "focal" and "diffuse" groups, most of the clinical and laboratory variables were not significantly different except for age and alkaline phosphatase. Children with diffuse glycogenosis were slightly younger (12.0 years ± 2.7 vs 12.7 ± 2.8, $P = .02$) and had higher levels of alkaline phosphatase (241 U/L ± 102 vs 217 ± 98, $P = .02$).

3.3 | Histological associations of glycogenosis in adults

Compared to those without glycogenosis, patients with any glycogenosis had lower steatosis grades (1.69 ± 0.89 vs 1.88 ± 0.90, $P = .0001$), higher ballooning score (1.07 ± 0.84 vs 0.90 ± 0.85, p 0.0002), and a higher proportion of acidophil bodies (49% vs 43%, $P = .03$), megamitochondria (33% vs 23%, $P < .0001$) and Mallory-Denk bodies (36% vs 29%, $P = .009$).Those with any glycogenosis were more frequently diagnosed with definite steatohepatitis (61% vs 51%, $P = .0007$) compared to borderline NASH or nonalcoholic fatty liver (NAFL, isolated steatosis). Even though many histologic features of severe hepatocyte injury were more frequent in those with any glycogenosis vs those with none, the NAFLD Activity Score (NAS) and fibrosis stage were not significantly different between the two groups. Furthermore, no significant differences were noted between those with any glycogenosis and those with no glycogenosis in lobular inflammation, portal inflammation, microvesicular steatosis, glycogenic nuclei and iron deposition (Table 1).

When compared to focal glycogenosis, cases with diffuse glycogenosis were characterized by a milder steatosis grade (1.44 ± 0.85 vs 1.96 ± 0.85, $P < .0001$), less frequent microvesicular steatosis (9% vs 15%, $P = .01$) and lower lobular inflammation score (1.49 ± 0.70 vs 1.62 ± 0.72, $P = .02$). As a consequence, those with diffuse glycogenosis had lower NAS (4.0 ± 1.7 vs 4.7 ± 1.7, $P < .0001$) and lower frequency of definite steatohepatitis (58% vs 63%, $P = .0003$) in comparison to focal glycogenosis cases.

3.4 | Histological associations of glycogenosis in children

Similar to adults, paediatric patients with any glycogenosis also had lower steatosis grades (1.89 ± 0.95 vs 2.23 ± 0.87, $P < .0001$). Unlike adults, there was no difference in the diagnostic classification between glycogenosis cases and those without ($P = .07$). The NAS was slightly lower in children with glycogenosis vs those with none (3.8 ± 1.6 vs 4.2 ± 1.5, $P = .0001$). No significant differences were observed between children with and without glycogenosis in lobular inflammation, portal inflammation, microvesicular steatosis, megamitochondria, ballooning grade, the presence of Mallory-Denk bodies, acidophil bodies or iron deposition. The fibrosis stage was not different in those children with glycogenosis compared to those without glycogenosis (Table 2).

TABLE 1 Demographics, laboratory data and histologic features by glycogenosis in 1348 adults

	None (n=620)	Any (n=728)	None vs Any P^a	Focal (n=348)	Diffuse (n=380)	Focal vs Diffuse P^a	None vs Diffuse P^a
	N (%) or Mean±SD	N (%) or Mean±SD		N (%) or Mean±SD	N (%) or Mean±SD		
Demographics							
Age at biopsy –yrs	48.9±12.3	51.6±11.7	<.0001	51.1±11.9	52.1±11.6	.28	.0001
Body mass index–kg/m^2	34.1±6.4	34.3±6.6	.51	34.3±6.4	34.4±6.9	.84	.51
Male Sex	254 (41)	233 (32)	.0008	139 (40)	94 (25)	<.0001	<.0001
Race			.52			.54	.32
White	497 (80)	579 (80)		281 (81)	298 (78)		
Black	29 (5)	27 (4)		14 (4)	13 (3)		
Other	94 (15)	122 (17)		53 (15)	69 (18)		
Ethnicity							
Hispanic	69 (11)	104 (14)	.09	39 (11)	65 (17)	.03	.007
Diabetes Mellitus (any)	244 (39)	325 (45)	.05	152 (44)	173 (46)	.65	.05
Type 2	243 (39)	321 (44)	.08	151 (43)	170 (45)	.77	.08
Laboratory measures							
AST, U/L	50±35	50±32	.86	53±33	48±32	.07	.42
ALT, U/L	68±54	69±48	.90	74±49	64±47	.009	.22
Alkaline phosphatase, U/L	83±52	83±33	.84	81±33	85±32	.06	.38
Glucose, mg/dL	108±39	115±42	.002	116±42	114±42	.66	.02
Insulin, μU/mL	24±30	27±29	.06	26±23	29±33	.13	.03
HOMA-IR, μU/mL^{b}mg/dL/405	7.1±12.2	8.2±10.1	.07	7.8±8.4	8.7±11.5	.22	.05
HbA1c, %	6.2±1.1	6.4±1.3	.008	6.5±1.3	6.4±1.2	.41	.08
Total cholesterol, mg/dL	191±48	189±42	.57	191±44	188±41	.29	.31
Triglycerides, mg/dL	178±154	180±158	.81	187±193	172±117	.21	.58
HDL, mg/dL	43±12	45±13	.13	43±12	46±13	.02	.01
LDL, mg/dL	115±40	112±37	.25	114±37	110±36	.12	.07
Histological features							
Biopsy Length, mm (range)	20.4±10.0 (1-70)	20.2±9.3 (3-64)	.70	20.3±9.7 (4-64)	20.1±8.8 (3-58)	.81	.65
Steatosis Grade	1.88±0.90	1.69±0.89	.0001	1.96±0.85	1.44±0.85	<.0001	<.0001
0 (<5%)	32 (5)	56 (8)	.001	10 (3)	46 (12)	<.0001	<.0001
1 (5-33%)	198 (32)	268 (37)		103 (30)	165 (43)		
2 (33-67%)	205 (33)	251 (34)		126 (36)	125 (33)		
3 (>67%)	185 (30)	153 (21)		109 (31)	44 (12)		
Steatosis location^b							
Zone 3 Predominant	316 (51)	334 (46)	.005	155 (45)	179 (48)	.04	.0004
Zone 1 Predominant	4 (1)	8 (1)		3 (1)	5 (1)		

(Continues)

TABLE 1 (Continued)

	None (n=620)	Any (n=728)	None vs Any P^a	Focal (n=348)	Diffuse (n=380)	Focal vs Diffuse P^a	None vs Diffuse P^a
	N (%) or Mean±SD	N (%) or Mean±SD		N (%) or Mean±SD	N (%) or Mean±SD		
Azonal	160 (26)	248 (34)		112 (32)	136 (36)		
Panacinar	137 (22)	131 (18)		78 (22)	53 (14)		
Steatosis macrovesicular type			.002			.05	.0002
Large droplet	444 (72)	576 (80)		265 (76)	311 (83)		
Mixed large and small droplet	157 (25)	126 (17)		71 (20)	55 (15)		
Small droplet	16 (3)	19 (3)		12 (3)	7 (2)		
Microvesicular Steatosis	69 (11)	84 (12)	.86	51 (15)	33 (9)	.01	.22
Lobular inflammation score	1.53±0.70	1.55±0.71	.53	1.62±0.72	1.49±0.70	.02	.45
0 (no foci)	11 (2)	13 (2)	.93	3 (1)	10 (3)	.08	.78
1 (<2 foci/20x hpf)	334 (54)	380 (52)		172 (49)	208 (55)		
2 (2-4 foci/20x hpf)	211 (34)	254 (35)		128 (37)	126 (33)		
3 (>4 foci/20x hpf)	66 (10)	81 (11)		45 (13)	36 (9)		
Portal inflammation score	1.12±0.58	1.18±0.61	.07	1.16±0.61	1.19±0.61	.45	.06
0-None	72 (12)	83 (11)	.07	41 (12)	42 (11)	.72	.06
1-Mild	402 (65)	434 (60)		211 (61)	223 (59)		
2- More than mild	145 (23)	211 (29)		96 (28)	115 (30)		
Ballooning injury score	0.90±0.85	1.07±0.84	.0002	1.11±0.84	1.03±0.84	.19	.02
0-None	259 (42)	236 (32)	.0009	106 (30)	130 (34)	.41	.05
Few	167 (27)	208 (29)		98 (28)	110 (29)		
2-Many	194 (31)	284 (39)		144 (41)	140 (37)		
Classic ballooning[c]	161 (26)	230 (32)	.02	114 (33)	116 (31)	.52	.12
Acidophil Bodies	268 (43)	358 (49)	.03	183 (53)	175 (46)	.09	.38
Megamitochondria	143 (23)	239 (33)	<.0001	104 (30)	135 (36)	.11	<.0001
Mallory Bodies	182 (29)	263 (36)	.009	125 (36)	138 (36)	.94	.02
Fibrosis stage	1.55±1.25	1.59±1.30	.57	1.62±1.28	1.57±1.32	.58	.86
0: None	152 (25)	192 (26)	.54	88 (25)	104 (27)	.14	.19
1A: Mild perisinusoidal only	71 (11)	80 (11)		33 (10)	47 (12)		
1B: Moderate perisinuosoidal	82 (13)	85 (12)		42 (12)	43 (11)		
1C: Periportal only	26 (4)	23 (3)		8 (2)	15 (4)		
2: Periportal and perisinusoidal	125 (20)	128 (18)		75 (22)	53 (14)		

(Continues)

TABLE 1 (Continued)

	None (n=620)	Any (n=728)	None vs Any P^a	Focal (n=348)	Diffuse (n=380)	Focal vs Diffuse P^a	None vs Diffuse P^a
	N (%) or Mean±SD	N (%) or Mean±SD		N (%) or Mean±SD	N (%) or Mean±SD		
3: Bridging fibrosis	119 (19)	161 (22)		74 (21)	87 (23)		
4: Cirrhosis	44 (7)	58 (8)		27 (8)	31 (8)		
Diagnostic classification							
Not NAFLD	28 (5)	34 (5)	.0007	4 (1)	30 (8)	.0003	.004
NAFLD, Not steatohepatitis	146 (24)	144 (20)		76 (22)	68 (18)		
Borderline, Zone 3 pattern	123 (20)	95 (13)		41 (12)	54 (14)		
Borderline, Zone 1 pattern	6 (1)	14 (2)		8 (2)	6 (2)		
Definite steatohepatitis	317 (51)	441 (61)		219 (63)	222 (58)		
NAFLD activity score							
0	5 (1)	9 (1)	.33	1 (0)	8 (2)	<.0001	.02
1	26 (4)	25 (3)		4 (1)	21 (6)		
2	79 (13)	93 (13)		33 (9)	60 (16)		
3	93 (15)	119 (16)		63 (18)	56 (15)		
4	147 (24)	141 (19)		57 (16)	84 (22)		
5	107 (17)	143 (20)		69 (20)	74 (19)		
6	83 (13)	112 (15)		61 (18)	51 (13)		
7	58 (9)	71 (10)		47 (14)	24 (6)		
8	22 (4)	15 (2)		13 (4)	2 (1)		
Mean±SD	4.3±1.8	4.3±1.8	.94	4.7±1.7	4.0±1.7	<.0001	.003

[a]Derived from chi-squared test for nominal variables and t-test with unequal variance for continuous variables

[b]10 cases had no steatosis; 166 cases are missing microgranulomas, large lipogranulomas, pigmented macrophages and glycogen nuclei–no longer scored

[c]Severe ballooning and presence of Mallory bodies

Compared to children with focal glycogenosis, those with diffuse glycogenosis demonstrated a lower steatosis grade (1.80 ± 0.93 vs 2.04 ± 0.96, $P = .02$), a higher frequency of megamitochondria (14% vs 7%, $P = .03$) and a slightly lower NAS (3.6 ± 1.6 vs 4.0 ± 1.6, $P = .04$).

3.5 | Covariates of glycogenosis (none, any, focal, diffuse) on multiple logistic regression analyses in adults

Older age at biopsy (OR 1.012, 95% CI 1.006, 1.027, $P = .003$), female sex (OR 1.31 95% CI 1.1, 1.71, $P = .04$), high glucose (OR 1.005, 95% CI 1.001, 1.009, $P = .01$), higher insulin (OR 1.013, 95% CI 1.002, 1.025, $P = .02$), and lower HOMA-IR (OR 0.97, 95% CI 0.94, 1.02, $P = .06$) significantly increased the risk for any glycogenosis. Female sex (OR 2.04, 95% CI 1.41, 2.95, $P < .001$) and use of insulin (OR 1.009, 95% CI 1.001, 1.017, $P = .02$) were associated with diffuse glycogenosis (Table 3).

Of the histological features, lower steatosis grade (OR 0.82, 95% CI 0.71, 0.94, $P = .006$), lower fibrosis stage (OR 0.84, CI 95% 0.75, 0.95, $P = .005$) were associated with higher risk for glycogenosis. The presences of megamitochondria (OR 1.48. 95% CI 1.13, 1.95, $P = .004$), acidophil bodies (OR 1.32, 95% CI 1.03, 1.69, $P = .03$), and higher ballooning grade (OR 1.30, 95% CI 1.09, 1.55, $P = .003$) were associated with higher odds of finding glycogenosis on histological exam. Diffuse glycogenosis compared to focal glycogenosis was associated with lower steatosis (OR 0.53, 95% CI 0.43, 0.64, $P < .001$) and less microvesicular steatosis (OR 0.56, 95% CI 0.33, 0.97, $P = .04$).

3.6 | Covariates of glycogenosis (none, any, focal, diffuse) on multiple logistic regression analyses in children

Hispanic ethnicity (OR 1.53, 95% CI 1.03, 2.25, $P = .03$), elevated triglycerides (OR 1.004, 95% CI 1.002, 1.007, $P = .001$) and high

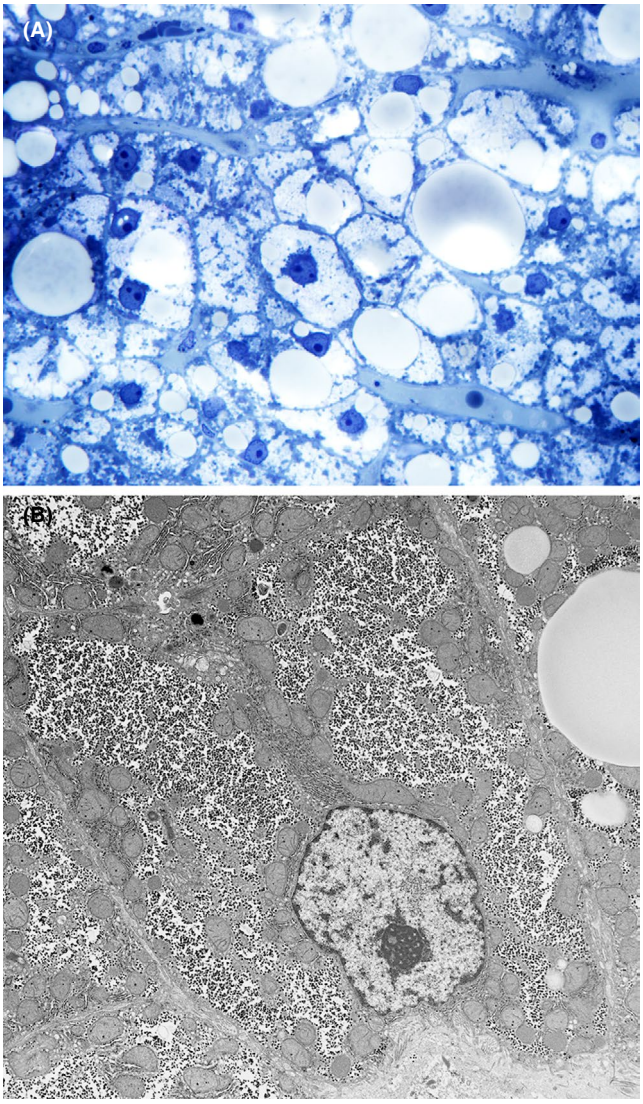

FIGURE 2 Glycogenosis as seen on electron microscopy. (A) Toluidine stained thick section reveals cytoplasmic glycogen (400x). (B) Ultrastructure of the same hepatocyte confirms cytoplasmic glycogen aggregates (1000x)

glucose (OR 1.017, 95% CI 1.005, 1.030, $P = .007$) increased the odds of finding any glycogenosis. In contrast, White race (OR 0.46, 95% CI 0.32, 0.67, $P < .001$), higher weight (OR 0.989, 95% CI 0.982, 0.996, $P = .002$), higher AST (OR 0.997, 95% CI 0.994, 1.000, $P = .04$), and higher steatosis grade (OR 0.65, 95% CI 0.54, 0.79, $P < .001$) reduced the odds of finding any glycogenosis. The diagnosis of diabetes (OR 0.2, 95%CI 0.05, 0.72, $P = .01$), and more acidophil bodies (OR 0.63, 95% CI 0.39, 1.00, $P = .05$) reduced the odds of having diffuse glycogenosis in children (Table 4).

4 | DISCUSSION

Glycogen is a branched polymer of glucose stored primarily in the liver and to a lesser extent in skeletal muscle.[7] It provides a readily available and easy to mobilize source of glucose. Hepatocellular glycogen can accumulate when hepatic carbohydrate metabolism becomes dysregulated in NAFLD.[1] As glycogen accumulates, the hepatocyte cytoplasm can take on a glassy, pale grey to light pink appearance, termed glycogenosis. To the best of our knowledge, this is the first study to systematically and prospectively characterize glycogenosis in the setting of NAFLD on a large scale in adults and children. We demonstrate that glycogenosis is common, occurring in more than half of both adults and children with NAFLD. Furthermore, diffuse glycogenosis is also common and was identified in approximately one third of both adults and children (28% and 34% respectively).

In adults, the presence of glycogenosis was associated with older age, female sex, higher serum glucose level, and higher insulin level. The histological variables associated with the presence of glycogenosis were lower steatosis grade, but greater hepatocellular injury (higher ballooning grade, the presence of acidophil bodies, Mallory-Denk bodies and megamitochondria).

Based on multiple logistic regression analysis in adults, a lower steatosis score and histologic features indicative of hepatocellular injury such as ballooning, megamitochondria and acidophil bodies were positively associated with glycogenosis in adults. Interestingly, however, despite increased hepatocyte injury, a lower fibrosis stage was associated with the presence of any glycogenosis. This suggests that glycogenosis may have a protective effect on disease progression.

Our study illuminated some curious observations. In some instances of glycogenosis, individual cells may become swollen and take on cytoplasmic pallor, and in this context the enlarged, pale, glycogen-filled hepatocytes may be confused with ballooned hepatocytes (Figure 1A,B). Accurate identification of hepatocyte ballooning is critical as ballooned hepatocytes are a hallmark histopathologic finding for the diagnosis of steatohepatitis and help distinguish the severe form of NAFLD, steatohepatitis, from nonalcoholic fatty liver.[6,8,9] Cytoskeletal alterations[9] and fat droplet accumulation in ballooned hepatocytes have been documented,[10] but to our knowledge abundant glycogen accumulation in ballooned hepatocytes has not previously been described. We have observed that ballooned hepatocytes may rarely contain abundant visible glycogen (Figure 3); the large majority of ballooned hepatocytes in our experience, however, do not contain visible glycogen. Glycogenosis in NAFLD can be diffuse, involving essentially every hepatocyte, or it can be focal, occurring in small groups of or individual hepatocytes. Accurate determination of the NAFLD hepatocellular ballooning grade can be challenging[9,11,12] and the presence of glycogenosis may further add to the difficulty. A raised awareness that glycogenosis is common in NAFLD may be advantageous for correct histopathological classification.

Glycogenosis in NAFLD is a distinct entity and should not be confused with glycogen storage diseases and glycogenic hepatopathy. Glycogenic hepatopathy has been best characterized in patients with type 1 diabetes mellitus and poor glycaemic control, and in patients following high dose corticosteroid therapy.[5,13,14] Glycogenic hepatopathy has rarely been reported in patients with type 2 diabetes

TABLE 2 Demographics, laboratory data and histologic features by glycogenosis in 699 children

	None (n=325)	Any (n=374)		Focal (n=138)	Diffuse (n=236)		None vs Diffuse
	N (%) or Mean±SD	N (%) or Mean±SD	None vs Any P^a	N (%) or Mean±SD	N (%) or Mean±SD	Focal vs Diffuse P^a	P^a
Demographics							
Age at biopsy–yr	12.8 ±2.8	12.2±2.8	.008	12.7±2.8	12.0±2.7	.02	.0005
Body mass index–kg/m^2	32.6±6.8	31.3±6.0	.008	31.6±6.4	31.1±5.8	.40	.006
Male Sex	233 (72)	257 (69)	.41	96 (70)	161 (68)	.82	.37
Race			<.0001			.89	.0006
White	241 (74)	219 (59)		80 (58)	139 (59)		
Black	9 (3)	14 (4)		6 (4)	8 (3)		
Other	75 (23)	141 (38)		52 (38)	89 (38)		
Ethnicity							
Hispanic	213 (66)	287 (77)	.001	105 (76)	182 (77)	.90	.004
Diabetes Mellitus, any	18 (6)	24 (6)	.75	5 (4)	19 (8)	.13	.24
Type 2	18 (6)	21 (6)	1.00	5 (4)	16 (7)	.25	.54
Laboratory measures							
AST, U/L	68±63	62±48	.16	60±45	63±50	.54	.33
ALT, U/L	117±110	107±94	.23	105±87	108±99	.77	.35
Alkaline phosphatase, U/L	216±110	232±101	.04	217±98	241±102	.02	.006
Glucose, mg/dL	87±11	92±30	.006	88±19	94±35	.06	.002
Insulin, μU/mL	33±29	35±46	.56	39±68	32±26	.29	.77
HOMA-IR, μU/mL[b]mg/dL/405	7.2±7.0	8.1±11.0	.20	8.7±14.9	7.8±7.9	.49	.41
HbA1c, %	5.4±0.4	5.6±1.1	.002	5.5±0.5	5.7±1.3	.08	.001
Total cholesterol, mg/dL	163±39	166±36	.26	163±39	168±35	.22	.11
Triglycerides, mg/dL	143±73	159±88	.009	159±93	159±84	.99	.02
HDL, mg/dL	40±10	40±9	.25	40±9	40±10	.75	.25
LDL, mg/dL	95±31	96±30	.71	93±29	97±30	.14	.33
Histological features							
Biopsy Length–mm, (range)	19.5±10.0 (4-60)	19.1±8.1 (3-49)	.58	19.1±8.3 (4-49)	19.0±8.1 (3-44)	.93	.60
Steatosis score	2.23±0.87	1.89±0.95	<.0001	2.04±0.96	1.80±0.93	.02	<.0001
0 (<5%)	12 (4)	24 (6)	<.0001	9 (7)	15 (6)	.04	<.0001
1 (5-33%)	57 (18)	118 (32)		33 (24)	85 (36)		

(Continues)

TABLE 2 (Continued)

	None (n=325)	Any (n=374)		Focal (n=138)	Diffuse (n=236)		None vs Diffuse
	N (%) or Mean±SD	N (%) or Mean±SD	None vs Any P[a]	N (%) or Mean±SD	N (%) or Mean±SD	Focal vs Diffuse P[a]	P[a]
2 (33-67%)	100 (31)	107 (29)		39 (28)	68 (29)		
3 (>67%)	156 (48)	125 (33)		57 (41)	68 (29)		
Steatosis location[b]							
Zone 3 Predominant	126 (39)	119 (32)	.02	58 (42)	61 (26)	.007	.0004
Zone 1 predominant	54 (17)	85 (23)		27 (20)	58 (25)		
Azonal	23 (7)	43 (12)		10 (7)	33 (14)		
Panacinar	122 (38)	124 (33)		42 (31)	82 (35)		
Steatosis macro-vesicular type			<.0001			.35	<.0001
Large droplet	216 (66)	308 (83)		110 (80)	198 (85)		
Mixed large and small droplet	101 (31)	59 (16)		25 (18)	34 (15)		
Small droplet	8 (3)	5 (1)		3 (2)	2 (1)		
Microvesicular Steatosis	6 (2)	5 (1)	.76	3 (2)	2 (1)	.36	.33
Lobular inflammation score							
0 (no foci)	2 (1)	2 (1)	.82	0 (0)	2 (1)	.21	.74
1 (<2 foci/20x hpf)	189 (58)	222 (59)		75 (54)	147 (62)		
2 (2-4 foci/20x hpf)	110 (34)	129 (34)		56 (41)	73 (31)		
3 (>4 foci/20x hpf)	24 (7)	21 (6)		7 (5)	14 (6)		
Mean (SD)	1.48±0.64	1.45±0.61	.55	1.51±0.59	1.42±0.62	.18	.26
Portal inflammation score	1.06±0.55	1.05±0.56	.76	0.99±0.59	1.08±0.54	.11	.64
0-None	40 (12)	50 (13)	.91	25 (18)	25 (11)	.11	.82
Mild	226 (70)	256 (69)		89 (65)	167 (71)		
2-More than mild	59 (18)	67 (18)		23 (23)	44 (19)		
Ballooning injury score							
0-None	201 (62)	245 (66)	.21	89 (64)	156 (66)	.95	.25
Few	84 (26)	98 (26)		37 (27)	61 (26)		
Many	40 (12)	31 (8)		(9)	19 (8)		
Mean (SD)	0.50±0.71	0.43±0.64	.13	0.44±0.65	0.42±0.64	.74	.14
Classic ballooning[c]	19 (6)	16 (4)	.34	7 (5)	9 (4)	.56	.28
Acidophil bodies	177 (54)	210 (56)	.70	84 (61)	126 (53)	.16	.80

(Continues)

TABLE 2 (Continued)

	None (n=325)	Any (n=374)		Focal (n=138)	Diffuse (n=236)		None vs Diffuse
	N (%) or Mean±SD	N (%) or Mean±SD	None vs Any P^a	N (%) or Mean±SD	N (%) or Mean±SD	Focal vs Diffuse P^a	P^a
Megamitochondria	27 (8)	42 (11)	.21	9 (7)	33 (14)	.03	.03
Mallory bodies	22 (7)	20 (5)	.52	9 (7)	11 (5)	.48	.29
Fibrosis stage	1.07±1.05	0.98±0.99	.28	0.99±1.00	0.98±0.98	.90	.31
0: None	116 (36)	138 (37)	.54	50 (36)	88 (37)	.67	.51
1A: Mild perisinusoidal only	28 (9)	24 (6)		9 (7)	15 (6)		
1B: Moderate perisinusoidal	13 (4)	15 (4)		9 (7)	6 (3)		
1C: Periportal only	77 (24)	108 (29)		38 (28)	70 (30)		
2: Periportal and perisinusoidal	45 (14)	50 (13)		17 (12)	33 (14)		
3: Bridging fibrosis	42 (13)	35 (9)		13 (9)	22 (9)		
4: Cirrhosis	3 (1)	4 (1)		2 (1)	2 (1)		
Diagnostic classification							
Not NAFLD	10 (3)	23 (6)	.07	9 (7)	14 (6)	.31	.20
NAFLD, Not Steatohepatitis	94 (29)	124 (33)		49 (36)	75 (32)		
Borderline, Zone 3 pattern	59 (18)	46 (12)		16 (12)	30 (13)		
Borderline, Zone 1 pattern	97 (30)	106 (28)		31 (22)	75 (32)		
Definite steatohepatitis	65 (20)	75 (20)		33 (24)	42 (18)		
NAFLD activity score							
0	1 (0)	2 (1)	.003	0 (0)	2 (1)	.02	.0001
1	9 (3)	17 (5)		8 (6)	9 (4)		
2	30 (9)	72 (19)		23 (17)	49 (21)		
3	58 (18)	81 (22)		17 (12)	64 (27)		
4	90 (28)	80 (21)		35 (25)	45 (19)		
5	78 (24)	64 (17)		29 (21)	35 (15)		
6	42 (13)	45 (12)		22 (16)	23 (10)		
7	11 (3)	8 (2)		3 (2)	5 (2)		
8	6 (2)	5 (1)		1 (1)	4 (2)		
Mean±SD	4.2±1.5	3.8±1.6	0001	4.0±1.6	3.6±1.6	.04	<.0001

[a]Derived from chi-squared test for nominal variables and t-test with unequal variance for continuous variables

[b]3 cases had no steatosis; 136 cases are missing microgranulomas, large lipogranulomas, pigmented macrophages and glycogen nuclei–no longer scored

[c]Severe ballooning and presence of Mallory-Denk bodies

TABLE 3 Multiple logistic regression[a] of any vs. no glycogenosis and diffuse vs. focal glycogenosis on demographics, laboratory measures and histologic features in adults

	Any vs No glycogenosis			Diffuse vs Focal glycogenosis		
	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	*P*-value
Demographics						
Age at biopsy–yr	1.012	1.006, 1.027	.003			
Female sex vs male sex	1.31	1.01, 1.71	.04	2.04	1.41, 2.95	<.001
Hispanic (yes vs no)				1.58	0.97, 2.28	.07
Laboratory measures						
Glucose–mg/dL	1.005	1.001, 1.009	.01			
LDL–mg/dL				0.996	0.992, 1.001	.10
Insulin–μU/mL	1.013	1.002, 1.025	.02	1.009	1.001, 1.017	.02
HOMA-IR-μU/mL[b]mg/dL/405	0.97	0.94, 1.02	.06			
Histologic features						
Steatosis grade–score	0.82	0.71, 0.94	.006	0.53	0.43, 0.64	<.001
Steatosis type			.02			
Mixed vs Large droplet	0.67	0.50, 0.90				
Small vs Large droplet	1.09	0.53, 2.24				
Microvesicular Steatosis (present vs absent)				0.56	0.33, 0.97	.04
Ballooning grade–score	1.30	1.09, 1.55	.003			
Non-hepatocyte iron grade-score	1.34	1.10, 1.62	.003	1.26	0.97, 1.63	.08
Megamitochodria (present vs absent)	1.48	1.13, 1.95	.004	1.36	0.95, 1.94	.10
Acidophils (yes vs no)	1.32	1.03, 1.69	.03			
Fibrosis–stage	0.84	0.75, 0.95	.005			
Biopsy length–mm	0.99	0.98, 1.00	.14			

[a]Covariates were selected using AIC criteria from two multiple logistic models regressing any (n = 672) vs no (n = 547) glycogenosis and regressing diffuse (n = 346) vs focal (n = 326) glycogenosis in adults with non-missing data on candidate set of age, gender, ethnicity, white race, BMI, weight, ALT, AST, alkaline phosphatase, triglycerides, cholesterol , HDL, LDL, glucose, insulin, HOMA-IR, HbA1c, diabetes, ballooning, lobular inflammation, portal inflammation, iron grade, Mallory bodies, steatosis grade, location and type, fibrosis stage, microvascular steatosis, megamitochondria, acidophils, non-hepatocyte iron grade and biopsy length.

mellitus.[15] Glycogenic hepatopathy is associated with high transaminase elevations which may result in a liver biopsy. The diffuse hepatic glycogenosis seen in type 1 diabetes-associated glycogenic hepatopathy is rapidly reversible with good control of glucose levels. Adequate glucose control also results in reversal of hepatomegaly and return of transaminase levels to normal. Glycogenic hepatopathy typically shows diffusely pale hepatocytes and absence of histological evidence supporting of NAFLD. In this cohort, type 1 diabetes was rare (four adults and three children) and the overwhelming majority of subjects with diabetes had type 2. Even though in our adult cases variables of insulin resistance (serum glucose levels and insulin) were associated with glycogenosis, there was no elevation of transaminases vs the nonglycogenotic (control) population. None of the subjects had a diagnosis of glycogen storage disease which would be exclusionary for entry in this NASH CRN study.

Why is glycogenosis so common in NAFLD and associated with decreased steatosis? There is a complex interplay between hepatocellular carbohydrate and lipid metabolism. These pathways are

TABLE 4 Multiple logistic regression[a] of any vs no glycogenosis and diffuse vs focal glycogenosis on demographics, laboratory measures and histologic features in children

	Any vs No glycogenosis			Diffuse vs Focal glycogenosis		
	Odds ratio	95% CI	*P*-value	Odds ratio	95% CI	P-value
Demographics						
Hispanic vs non-Hispanic	1.53	1.03, 2.25	.03			
White vs non-white	0.46	0.32, 0.67	<.001			
Weight–kg	0.989	0.982, 0.996	.002			
Laboratory measures						
Triglycerides–mg/dL	1.004	1.002, 1.007	.001			
Total cholesterol–mg/dL				1.006	1.000, 1.023	.07
Alkaline phosphatase–U/L				1.002	1.000, 1.005	.10
AST–U/L	0.997	0.994, 1.000	.04			
Glucose–mg/dL	1.017	1.005, 1.030	.007			
Comorbidities						
Diabetes (yes vs no)				0.20	0.05, 0.72	.01
Histologic features						
Steatosis grade–score	0.65	0.54, 0.79	<.001	0.78	0.59, 1.03	.08
Steatosis type			<.001			
Mixed vs large droplet	0.40	0.27, 0.60				
Small vs large droplet	0.39	0.11, 1.32				
Steatosis location						.05
Zone 1 vs Zone 3				1.96	1.02, 3.76	
Azonal vs Zone 3				2.37	0.98, 5.74	
Panacinar vs Zone 3				2.06	1.13, 3.76	
Acidophils (yes vs no)				0.63	0.39, 1.00	.05

[a]Covariates were selected using AIC criteria from two multiple logistic models regressing any (n = 355) vs no (n = 301) glycogenosis and regressing diffuse (n = 230) vs focal (n = 125) glycogenosis in children with non-missing data on candidate set of age, gender, ethnicity, white race, BMI, weight, ALT, AST, alkaline phosphatase, triglycerides, cholesterol , HDL, LDL, glucose, insulin, HOMA-IR, HbA1c, diabetes, ballooning, lobular inflammation, portal inflammation, iron grade, Mallory bodies, steatosis grade, location and type, fibrosis stage, microvascular steatosis, megamitochondria, acidophils, non-hepatocyte iron grade and biopsy length.

normally finely balanced, but they can become dysregulated and may shunt substrates from one pathway to another. For example, several studies have documented a link between high fructose diets and NAFLD.[16,17] In humans and rodents, fructose induces glycogen synthesis to a greater degree than glucose,[18-20] and a prior adult NASH CRN study showed an inverse relationship between fructose intake and the severity of steatosis.[21] Unfortunately no histological assessment of glycogenosis was available in that study, and data on fructose consumption was not available in the current study.

Furthermore, the inverse relationship between glycogenosis and steatosis is highlighted by a recent primate study. Baboons given a high-fat, high-simple-carbohydrate diet which induced maternal obesity during pregnancy resulted in offspring with more severe liver steatosis but a lesser degree of hepatic glycogen accumulation compared to offspring of controls.[22]

The interconnection of hepatic carbohydrate and lipid metabolism, and the downstream effects on NAFLD progression risk is further illustrated by a recent important finding. In individuals at high risk of NAFLD, genetic variation (rs4841132 G < A) in protein phosphatase 1 regulatory subunit 3B (PPP1R3B), a hepatic glycogen metabolism regulatory protein, is associated with reduced hepatic steatosis and protection against liver fibrosis, but results in increased liver glycogen.[23,24] The authors speculate that this variant upregulates PPP1R3B levels, favouring energy storage as glycogen by shunting glucose away from glycolysis while suppressing de novo lipogenesis. The authors suggest that this variant is protective against steatosis and fibrosis.

Some of the limitations of this study are the lack of dietary information, the lack of data on prior corticosteroid use, the lack of clinical data regarding the presence or absence of hepatomegaly, the lack of imaging studies specifically addressing the presence or absence of hepatic glycogen, the lack of quantitative analysis of hepatocellular glycogen and triglyceride levels in these samples, and the lack of PPP1R3B genetic data in this cohort.

In summary, this study demonstrates that glycogenosis is commonly seen in the context of adult and paediatric NAFLD. In adults, glycogenosis is associated with older age, female sex, and higher blood glucose and insulin levels. Furthermore, we show that

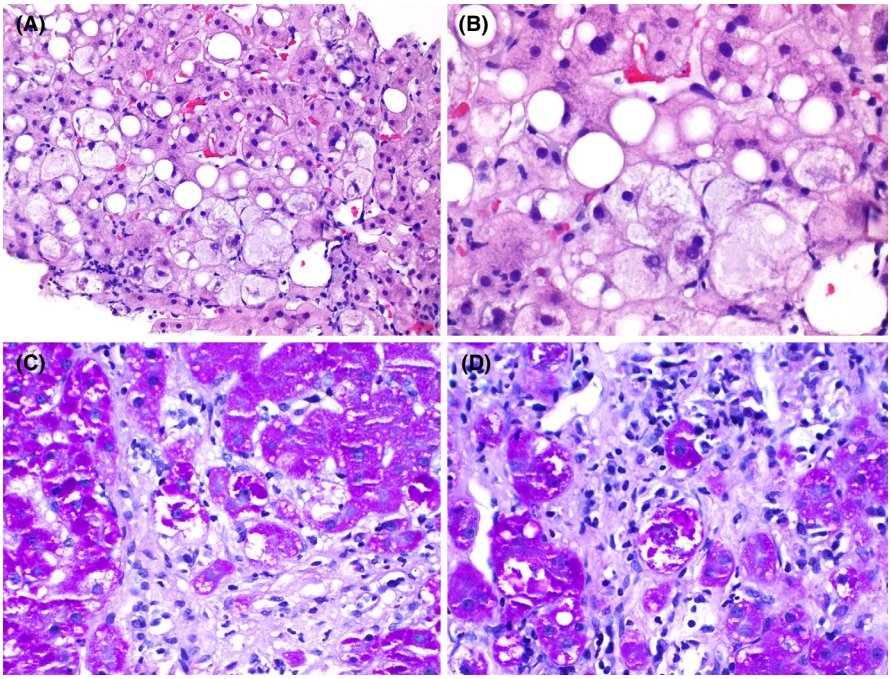

FIGURE 3 Ballooned hepatocytes with cytoplasmic glycogen. (A, B). H&E stained sections of ballooned hepatocytes with prominent glycogen aggregates (200x and 400x respectively). C and D. PAS stained section of ballooned hepatocytes with cytoplasmic glycogen (200x and 400x respectively)

glycogenosis is associated with a higher degree of ballooning, but a lower steatosis grade and lower fibrosis stage. NAFLD glycogenosis should not be confused with hepatocyte ballooning, glycogenic hepatopathy or glycogen storage disorders. Although dysregulated lipid metabolism has been well documented in the setting of fatty liver disease, further studies are warranted to better understand the causes and effects of altered carbohydrate metabolism in NAFLD.

CONFLICT OF INTEREST

There are no conflicts of interest.

ETHICS APPROVAL AND PATIENT CONSENT STATEMENT

Each participating institution obtained IRB approval to enrol patients into the parent NASH CRN studies. Written consents was signed by participating patients.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

No material is obtained/reproduced from other sources.

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APPENDIX

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