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HSD17B13 is a Hepatic Retinol Dehydrogenase Associated with Histological Features of Non-Alcoholic Fatty Liver Disease

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YM, YR, and TJL designed the research studies; YM, OVB, KF, PMB, KV, SK, CK, CN, ET, DEK, JMZ, and AJS conducted experiments and acquired data; YM, OVB, PMB, YSdB, EKS, YC, VC, SKH, XD, NYK, and YR analyzed data; YM and YR wrote the manuscript; AJS and TJL critically reviewed the data and manuscript; YR supervised the study.

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is a common cause of chronic liver disease. A single nucleotide polymorphism (SNPs), rs6834314, was associated with serum liver enzymes in the general population, presumably reflecting liver fat or injury. We studied rs6834314 and its nearest gene, *HSD17B13* (17-beta hydroxysteroid dehydrogenase 13) to identify associations with histological features of NAFLD, and to characterize the functional role of HSD17B13 in NAFLD pathogenesis. The minor allele of rs6834314 was significantly associated with increased steatosis, but decreased inflammation, ballooning, Mallory-Denk bodies, and liver enzyme levels in 768 adult Caucasians with biopsy-proven NAFLD, and with cirrhosis in the general population. We found two plausible causative variants in the *HSD17B13* gene. rs72613567, a splice-site SNP in high linkage with rs6834314 ($r^2=0.94$) generates novel splice variants and shows a similar pattern of association with NAFLD histology. Its minor allele generates simultaneous expression of exon 6-skipping and G-nucleotide insertion variants. Another SNP, rs62305723 (encoding a P260S mutation) is significantly associated with decreased ballooning and inflammation. Hepatic expression of *HSD17B13* is 5.9-fold higher ($p=0.003$) in patients with NAFLD. HSD17B13 is targeted to lipid droplets, requiring the conserved AA22-28 sequence and AA71-106 region. The protein has a retinol dehydrogenase (RDH) activity, with enzymatic activity dependent on lipid droplet targeting and co-factor binding site. The exon 6-deletion, G-insertion, and newly-described naturally-occurring P260S mutation, all confer loss of enzymatic activity. In conclusion, we demonstrate the association of variants in *HSD17B13* with specific features of NAFLD histology, and identify the enzyme as a lipid droplet-associated RDH. Our data suggest that HSD17B13 plays a role in NAFLD through its enzymatic activity.

INTRODUCTION

The rise of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) has sparked considerable interest in pathogenic mechanisms of the disease. Racial and ethnic

variability and a known heritable component (1,2) hinted at a genetic contribution. Genome-wide association studies (GWAS) identified loci associated with NAFLD, most predominantly rs738409, a single nucleotide polymorphism (SNP) in the *PNPLA3* gene (3-5). Subsequently, we and others (6-8) demonstrated that rs738409 is strongly associated with histological features and severity of NAFLD. Although the association is highly significant statistically, the effect size with regards to liver fat accumulation (9) is quite weak. Similarly, the major allele of the *TM6SF2* SNP rs58542926 is associated with decreased liver fat (10), but conversely, with higher serum lipid levels and increased risk of atherosclerosis and cardiovascular disease (11). *In vivo* and *in vitro* studies provided evidence that *TM6SF2* regulates VLDL secretion and acts as a determinant of liver damage or cardiovascular disease under metabolic stress (10-14). Of other genes associated with NAFLD by GWAS only a few (such as *MBOAT7* (15-17)) were studied in large histologically-characterized NAFLD cohorts, and in even fewer has their mechanism been elucidated.

A recent large-scale GWAS identified new loci associated with plasma liver enzyme levels (18). In unselected population surveys, elevated transaminases occur concomitantly with metabolic syndrome risk factors (19,20) and are presumed to reflect hepatic fat accumulation and related injury. However, a direct association between these SNPs and NAFLD has not yet been shown. We hypothesized that plasma transaminase activity in the GWAS reflected the presence of fatty liver and associated injury, and that genetic associations with transaminases could identify novel genes involved in NAFLD pathogenesis. Our goals were to establish which of these newly-described SNPs is associated with histological features of NAFLD, identify the genes associated with these SNPs, and elucidate the mechanism by which these genes affect NAFLD.

In this work we demonstrate that SNPs in or near *HSD17B13* are associated with histological features of NAFLD, identify *HSD17B13* as a novel retinol dehydrogenase, and characterize the impact of genetic variants on its enzymatic activity. Our study provides a link between genetic variants, retinoid metabolism, and NAFLD pathogenesis, and identifies *HSD17B13* as a potentially druggable target in NAFLD.

METHODS

STUDY DESIGN

We initially genotyped in a cohort of subjects with biopsy-proven NAFLD 38 SNPs described by Chambers (18), to identify loci associated with histological features. After identifying a locus of interest, we genotyped additional SNPs in the candidate gene locus in the same cohort and confirmed associations in additional cohorts. Finally, we performed functional analyses to characterize the gene product and identify the functional impact of gene variants (Supplementary Figure 1).

STUDY POPULATIONS

The NAFLD cohort was described in detail previously (6). Briefly, we included patients from the NASH Clinical Research Network (NASH-CRN) observational database (21) and PIVENS (22) studies, as well as patients with biopsy-proven NAFLD seen at the NIH Clinical Center. Analysis was limited to Caucasians aged ≥ 18 years at the time of liver biopsy. All subjects had histological evidence of NAFLD or NASH on liver biopsy. Liver histology was scored semi-quantitatively using the NASH-CRN scoring system (23).

Confirmatory analyses were performed in a cohort of patients with chronic hepatitis C and available liver histology, and in two population-based cohorts – the Michigan Genomics Initiative (MGI) and the UK Biobank.

HEPATIC GENE EXPRESSION

Deidentified normal and NASH human liver samples were obtained from the Liver Tissue Cell Distribution System and from clinical trials at the NIH Clinical Center. Gene expression was quantified by qPCR.

INTRA-CELLULAR LOCALIZATION

Cells were stained with primary antibodies against organelle markers, LipidTox Red (Life Technologies, Carlsbad, CA) for lipid droplets, and Hoechst 33342 for nuclei. Staining was visualized using confocal microscope.

RETINOL DEHYDROGENASE (RDH) ACTIVITY

RDH activity was tested in a cell-based assay as previously described (24). Briefly, HEK293 cells were transfected with protein expression vectors and treated with all-trans-retinol for 8 hours, followed by retinaldehyde and retinoic acid (RA) quantification by HPLC and protein quantification by western blot.

STATISTICAL ANALYSIS

Associations between genotypes and histology were determined by a two-step method. Univariate analysis was performed using the Jonkheere-Terpstra test for ordered differences. SNPs with univariate significance level <0.1 were selected for multivariate ordinal regression, with age, BMI, gender and type 2 diabetes as covariates. We utilized the Benjamini-Hochberg (25) false discovery rate (FDR) to correct for multiple comparisons, using the calculator by Pike (26), with $q < 0.05$ considered significant. P-values are reported from the multivariate ordinal regression. Odds ratios (OR) of associations with histology were calculated using binary logistic regression. An additive inheritance model was used. Student's *t*-test was used for 2-group differences and one-way ANOVA followed by Dunnett's multiple comparison test were used for >2 groups in the *in vitro* enzymatic assays. All significance testing was two-sided. Statistical analyses were performed using SPSS Statistics V.19 (IBM) and Prism V.7 (GraphPad).

ETHICS

Human subject data was obtained from studies conducted according to the Declaration of Helsinki principles that were reviewed by Institutional Review Boards. All subjects gave written informed consent prior to inclusion.

Additional details are found in the Supplementary Methods.

RESULTS

We obtained DNA from 768 adult Caucasians with biopsy-proven NAFLD (mean age 49, mean BMI 34.6 kg/m^2 , 28% diabetic). Histologically, 59.5% had NASH and 12.1% were cirrhotic (Supplementary Table 1). We genotyped in these subjects 38 SNPs described by Chambers (18) (Supplementary Table 2). The associations between SNPs and histological features of NAFLD are detailed in Supplementary Table 3. Multivariate ordinal logistic regression adjusting for age,

gender, diabetes and BMI found 9 SNPs to be significantly associated with steatosis grade, 4 SNPs associated with degree of ballooning degeneration, and 3 SNPs associated with Mallory-Denk bodies. No SNP was associated with severity of inflammation or fibrosis. Associations were independent of the *PNPLA3* rs738409 genotype (Supplementary Table 3). Of the SNPs associated with NAFLD histology, only rs6834314 was also associated with serum transaminases in patients with NAFLD (Supplementary Table 4) and thus was the focus of subsequent study.

rs6834314 IS ASSOCIATED WITH NAFLD HISTOLOGY

rs6834314 is located on chromosome 4, 11kb downstream of the 17-beta hydroxysteroid dehydrogenase 13 (*HSD17B13*) gene. Each copy of the minor G-allele increased the risk of having liver fat content $\geq 33\%$ (OR=1.32, CI 1.02-1.70, Figure 1A, Supplementary Figure 2A). The mean steatosis grade in subjects with rs6834314G/G genotype was 0.2 units higher than in those with A/A (1.88 \pm 0.87 vs. 1.68 \pm 0.89), an effect similar to the impact of the *PNPLA3* rs738409 in the same subjects (0.21 units; 1.59 \pm 0.84 for rs738409C/C vs. 1.80 \pm 0.89 for G/G) (6). In contrast to its association with increased liver fat, the minor G-allele was significantly associated with lower risks for inflammation (OR=0.77 for total inflammatory score ≥ 3 , CI 0.60-0.99), significant ballooning (OR=0.67 for ballooning score >1 , CI 0.51-0.87), and the presence of Mallory-Denk bodies (OR=0.68, CI 0.51-0.91) and showed a non-significant trend for reduced fibrosis (OR=0.79, CI 0.60-1.05) on binary logistic regression. The associations with inflammation, ballooning, and Mallory-Denk bodies were independent of the association with steatosis and in fact, were stronger when steatosis grade was included in the regression model (Supplementary Table 5). The minor allele of rs6834314 was also associated with lower serum transaminases and GGT (Figure 1B). NAFLD has been previously associated with genetic variants in *PNPLA3* (rs738409), *TM6SF2* (rs58542926), *MBOAT7* (rs626283), and *GCKR* (rs1260326). After adjusting for these variants, rs6834314 remained significantly associated with steatosis (p=0.013) ballooning (p=0.031) and Mallory-Denk bodies (p=0.030), and trended for association with inflammation (p=0.067, Supplementary Table 6). rs6834314 genotype did not differ between patients with NASH and those with steatosis only.

To validate the association of rs6834314 with steatosis, we genotyped it in an independent sample of 317 Caucasian patients with chronic hepatitis C and available liver histology

(Supplementary Table 7). Liver fat was present in 179 (57.2%) subjects. As with the NAFLD cohort, rs6834314 genotype was significantly associated with the degree of steatosis in univariate ($p=0.032$, Jonkheere-Terpstra test) and multivariate ($p=0.028$, ordinal regression adjusted for BMI, age, and gender) analyses. Each copy of the minor G-allele was significantly associated with the presence of liver fat (OR=1.67, CI 1.11-2.51, $p=0.015$; Figure 1C, Supplementary Figure 2B). As expected, rs6834314 genotype was not associated with HCV-driven inflammation or fibrosis.

As further confirmation, we determined the association of rs6834314 with liver enzymes and diagnoses in the MGI cohort (Supplementary Table 8). rs6834314-G was associated with lower ALT ($p=3.3 \times 10^{-4}$) and AST ($p=5.1 \times 10^{-5}$) and with a lower risk of cirrhosis diagnosis (OR=0.79, $p=7.5 \times 10^{-4}$, Supplementary Figure 2C). The MGI cohort is population-based and histological data is not available for detailed analyses.

OTHER VARIANTS IN *HSD17B13* ARE ASSOCIATED WITH NAFLD

rs6834314 is downstream of *HSD17B13* and in high LD with multiple adjacent SNPs, overlapping parts of the *HSD17B13* gene. To determine whether the associations of rs6834314 reflect a biological effect of *HSD17B13*, we selectively genotyped additional variants in the gene region, including 2 non-synonymous coding SNPs, an insertion/deletion (indel) polymorphism in a splice site, and a frame-shift coding SNP, as well as 7 SNPs that capture most of the common variants in the gene region (Table 1). The tag SNP, rs6834314, is in strong LD ($D'=0.995$, $r^2=0.93$) with the indel rs72613567, located immediately adjacent to the 3'-end of exon 6 (Supplementary Figure 3). As expected, this indel had a similar pattern of association with NAFLD histology and liver enzymes as rs6834314 (Figure 1D). The minor allele, rs72613567-A was associated with increased steatosis (OR=1.36, CI 1.04-1.77) but with decreased inflammation (OR=0.74, CI 0.57-0.96), and trended towards decreased ballooning (OR=0.78, CI 0.60-1.03), Mallory-Denk bodies (OR=0.77, CI 0.57-1.03), and fibrosis (OR=0.77, CI 0.58-1.03, Supplementary Figure 4A).

rs72613567 was not genotyped in the MGI cohort but was genotyped in the UK Biobank cohort (Supplementary Table 9). The minor allele A-insertion was significantly less common in the NAFLD cohort (19.1%) compared to the population-based UK Biobank (27.9%, $p<0.001$) and was associated with a reduction in the risk of fibrosis and cirrhosis (OR=0.83, $p=9 \times 10^{-4}$,

Supplementary Figure 4B). rs72613567-A was also associated with lower risk of diagnoses of liver disease (OR=0.93, $p=8 \times 10^{-4}$), other inflammatory liver diseases (a diagnostic code that includes NASH, OR=0.82, $p=0.0012$) and alcoholic liver disease (OR=0.87, $p=0.015$). Histological data or liver enzymes are not available for this cohort.

Another SNP, the non-synonymous coding rs62305723G>A in exon 6, encodes a P260S substitution and is not in LD with rs6834314 ($r^2=0.02$). The rs62305723 minor A-allele is significantly associated with decreased inflammation (OR=0.46, CI 0.28-0.74), ballooning (OR=0.48, CI 0.30-0.76), and Mallory-Denk bodies (OR=0.51, CI 0.28-0.91), but, in contrast to rs72613567/rs6834314, is not associated with steatosis (Figure 1E, Supplementary Figure 5A). The minor A-allele was associated with a lower risk of NASH compared to steatosis (OR=0.39, CI=0.23-0.68, $p=0.001$). In the UK Biobank cohort, rs62305723 was not associated with liver-related diagnoses. Interestingly, the minor A-allele was associated with a diagnosis of blindness or low vision (OR=1.3, $p=0.007$, Supplementary Figure 5B).

Conditional analysis found rs62305723 and rs72613567 to associate with NAFLD histology independently of each other (Supplementary Table 10), suggesting both are causal variants. Including the *PNPLA3* rs738409 in the model did not impact significance (Supplementary Table 10) and the association of *HSD17B13* SNPs with histology and ALT was seen for all rs738409 genotypes (Supplementary Figure 6). We found no significant interaction between rs738409 and *HSD17B13* SNPs, although our study was not powered to detect such an effect. Two additional non-coding SNPs (rs6531975, rs10022237), neither in LD with rs6834314, were associated with histological features and rs10022237 was also associated with ALT. The detailed mapping supports a functional role for *HSD17B13* in the pathogenesis of NAFLD.

HEPATIC *HSD17B13* EXPRESSION IS ELEVATED IN NASH PATIENTS

To determine whether *HSD17B13* is expressed differentially in NAFLD, we quantified its expression in human NASH (n=43) or normal (n=14) liver samples. Hepatic expression of *HSD17B13* is 5.9-fold higher in NASH patients compared with controls ($p=0.003$, Figure 2A).

In the GTEx database, rs6834314 genotype did not affect hepatic *HSD17B13* expression (Supplementary Figure 7A, $p=0.6$) and neither did rs72613567 nor rs62305723 (data not shown).

We confirmed this in a pooled analysis of our liver tissue samples (Figure 2B). Since *HSD17B13* expression is upregulated in NAFLD, we examined whether rs6834314 modulates gene expression only in these subjects but found no significant impact of genotype on hepatic *HSD17B13* expression in either NASH (Supplementary Figure 7B) or normal liver (Supplementary Figure 7C), although nearly all high-expression samples had A/A genotype. To confirm that rs6834314 does not affect *HSD17B13* expression in hepatocytes, as liver tissue samples contain other cell types, we quantified *HSD17B13* mRNA in primary human hepatocytes from 22 donors and genotyped them for rs6834314. No donor was homozygous for the minor G-allele and there was no difference in *HSD17B13* expression between A/A and A/G genotypes (Supplementary Figure 7D). Thus, hepatic *HSD17B13* expression is upregulated in NAFLD but the association between rs6834314 genotype and NAFLD phenotype is not due to changes in expression levels and is more likely reflecting a change in protein function.

HSD17B13 LOCALIZES TO LIPID DROPLETS

To better understand the function of HSD17B13, we determined its subcellular localization by transfecting *HSD17B13*-GFP into HepG2 cells and staining for cellular organelle markers. HSD17B13-GFP co-localizes with the lipid droplets (Figure 3A) and is detected on the surface of purified droplets from transfected cells (Figure 3B) but does not co-localize with other organelles (Supplementary Figure 8A).

There are two described transcript variants for the human *HSD17B13* gene, formed by alternative splicing: variant A contains all 7 exons while variant B is missing exon 2 (Supplementary Figure 3) resulting in deletion of amino acids 71-106 (Figure 3C). Transfected HSD17B13(B)-GFP localized to the cytoplasm but not to lipid droplets (Figure 3C-D). By site-directed mutagenesis, we identified the N-terminal amino acids 22-28 of HSD17B13 as essential for lipid droplet targeting (Figure 3C). This 7-amino acid sequence is conserved within ADRP and TIP47 which are also lipid droplet-associated proteins (27) (Supplementary Figure 8B) and appears to be crucial for protein stability, as its deletion results in low protein expression.

HSD17B13 DOES NOT AFFECT HEPATOCYTE LIPID CONTENT

To assess the functional role of HSD17B13 in the liver, we investigated whether its expression level affects fat content of HepG2 cells incubated with a high concentration of fatty acids. The

expression of HSD17B13 protein was dose-dependently induced by doxycycline using Tet-on inducible expression system (Supplementary Figure 9A) with no effect on lipid content (Supplementary Figure 9B-C). Similarly, stable HSD17B13 overexpression or knockout in HepG2 cells did not affect their lipid content (Supplementary Figure 9D-E), suggesting HSD17B13 does not regulate hepatocyte lipid content in a direct manner.

HSD17B13 HAS RETINOL DEHYDROGENASE ACTIVITY *IN VITRO*

HSD17B13 is a member of the 17 β hydroxysteroid dehydrogenase (HSD) family, 14 structurally-related enzymes implicated in steroid and fatty acid metabolism (28); the substrate of HSD17B13 is currently unknown. Given the association of *HSD17B13* with NAFLD, we hypothesized that its functional role is due to its enzymatic activity and that SNPs modulate that activity. Beyond its similarity to the 17 β -HSD family, HSD17B13 is structurally similar to other members of the short-chain dehydrogenase/reductases (SDR16C) family (29), known to play a critical role in retinoid metabolism through their retinol dehydrogenase enzymatic activity, catalyzing the oxidation of retinol to retinaldehyde, the rate-limiting step in all-trans-RA biosynthesis (Figure 4A). Cells transfected with HSD17B13(A) (variant A) demonstrated RDH activity comparable to that of the positive control RDH10 (Figure 4B-D). In contrast, HSD17B13(B) had no RDH activity (Figure 4E-G). HSD17B13- Δ 22-28, which does not target lipid droplets, also lacked RDH activity although its expression was markedly lower than variants A and B, possibly because of low protein stability (Supplementary Figure 10). Thus, HSD17B13 demonstrates *in vitro* RDH activity that requires lipid droplet targeting for its stability and enzymatic function.

HSD17B13 contains the conserved TGXXXGXXG NAD(P)(H) co-factor binding site (31) common to SDRs (32). We generated a mutation within this motif (HSD17B13-G47/G49A) which resulted in loss of RDH activity (Figure 4H-J), confirming the need for cofactor binding for enzymatic activity.

THE IMPACT OF GENETIC VARIANTS IN *HSD17B13* ON ENZYMATIC ACTIVITY

The non-synonymous coding SNP rs62305723G>A encodes a P260S substitution. P260 is highly conserved in vertebrate HSD17B13 orthologs and in other SDR16C family members (Figure 5A), even though it is outside the predicted catalytic or cofactor binding sites, suggesting its

importance for protein function. We generated and tested this mutant form for enzymatic activity. Although the mutant protein HSD17B13(A)-P260S retains lipid droplet localization (Figure 5B) similarly to wild-type protein, it lacks RDH activity (Figure 5C-E). Thus, the minor allele of rs62305723 is generating a loss-of-function mutation that is associated with decreased hepatic inflammation and injury in patients with NAFLD.

The indel rs72613567 was associated with histological and serological features of hepatic fat and injury (Table 1). The indel is located in the 5' end of the intron between exon 6-7, with the minor allele (A-insertion) modifying a conserved splice donor sequence (GTAAGT to GTAAAGT). To determine whether this SNP affects splicing, we amplified and sequenced the surrounding regions from DNA and RNA of 36 human liver samples. We identified two novel *HSD17B13* splice variants – one with a single G-nucleotide insertion (G-insertion) between exons 6-7 causing a frameshift and premature stop-codon, and another with exon 6 skipping (delta-6) (Figure 6A). Both the delta-6 and the G-insertion variants retain localization to lipid droplets (Figure 6B) but have no RDH activity (Figure 6C) and were only found in subjects with the minor rs72613567A allele (Figure 6D-E), who, as expected, also carried the rs6834314 minor G-allele (Supplementary Figure 11A-B). Delta-6 was expressed simultaneously with exon 6-containing variants at a relatively fixed ratio (Supplementary Figure 11C-D).

DISCUSSION

In otherwise-normal subjects, elevated plasma liver enzymes are often considered a surrogate for the presence of liver fat but can also reflect other liver processes, derive from non-hepatic sources, or reflect variability in the genes for the enzymes themselves. Chambers et al (18) found at the population level several genetic variants that associate with liver enzyme levels. We aimed to identify which of these associations actually reflects fatty liver or injury by assessing the association of the variants with histological features of NAFLD. We identified several SNPs that associated with NAFLD features and by focusing on one of them, rs6834314, identified HSD17B13 as a lipid-droplet enzyme associated with the disease.

Other 17 β -HSDs act as molecular switches, modulating the activation of steroid hormone receptors in target tissues (28). *In vivo*, some 17 β -HSDs may have targets other than steroid hormones, such as prostaglandins, bile acids, and cholesterol and some have been implicated in

fatty acid metabolism (34). Compared to other family members that are restricted to gonads or ubiquitously expressed, HSD17B13 is predominantly expressed in the liver, and the protein is targeted from the ER to lipid droplets by its N-terminal region (27,31,35). We confirmed HSD17B13's lipid-droplet localization and identified a conserved 7 amino acids sequence (AA22-28) that is crucial for lipid droplet targeting. Interestingly, a naturally occurring splice variant, *HSD17B13(B)* that lacks exon 2 is not targeted to lipid droplets, despite the presence of AA22-28. In overexpression experiments using equal amounts of plasmid, the Δ 22-28 variant and *HSD17B13(B)* demonstrate lower protein levels than wild-type, suggesting that proper targeting is crucial for protein stability and protection from degradation; a similar phenotype was described for TM6SF2 variants (10).

We found *HSD17B13* to be highly expressed in NAFLD patients, consistent with a recent report (35). However, its specific target and physiological function have not been defined so far and neither was its role in NAFLD pathogenesis. We establish that HSD17B13 has RDH activity, and that its enzymatic activity depends on lipid-droplet targeting and cofactor binding. HSD17B13 clearly has RDH activity *in vitro* and this assay can be used to assess the impact of genetic variations on enzymatic function. Although *in vivo* the enzyme may have other substrates in addition to retinol, retinol is still a plausible *in vivo* substrate. Retinoids are stored as retinyl esters in the lipid droplets of hepatic stellate cells that, when activated, play critical roles in hepatic fibrogenesis. There is significant evidence that vitamin A metabolites (retinaldehyde and RA) and retinol binding protein (RBP4) are associated with pathogenesis of hepatic steatosis, fibrosis, adipogenesis, and insulin resistance (36-41). Enzymes involved in retinol metabolism are upregulated in hepatic lipid droplets in NAFLD (35). Furthermore, PNPLA3, strongly associated with NAFLD (3), acts as a retinyl-palmitate lipase to control serum retinol and RBP4 level in NAFLD patients and to modulate stellate cell fibrogenesis (42-45). This supports the critical role retinoid metabolism plays in NAFLD progression. The association of the P260S substitution with a diagnosis of blindness or low vision further supports a role for HSD17B13 in retinoid metabolism. In addition, the putative substrate binding sites (L153/L156, K208, L199/E202), homodimer interaction sites, and the P260 residue found in this study are all crucial for enzymatic activity and are conserved within RDH10, DHRS3, and HSD17B11, but not with other 17 β -HSDs (Supplementary Figure 12). In addition, a recent study in mice heterozygous for *Rdh10* knockout found increased steatosis on high fat diet despite only a modest decrease in

hepatic RA (46). This suggests unique substrate specificity and is consistent with retinoids being the substrate for HSD17B13. Ongoing *in vivo* experiments are aimed at confirming these findings. Hepatic HSD17B13 expression appears limited to hepatocytes, with no detectable expression in quiescent or activated stellate cells (data not shown). Whether HSD17B13 acts directly on the hepatocyte via modulation of hepatocyte RA synthesis, or indirectly through a paracrine or autocrine effect, remains to be shown. Our data suggest that HSD17B13 is a hitherto undescribed hepatic RDH and that its effect on NAFLD pathogenesis relates to its enzymatic activity and possibly to its impact on retinoid homeostasis. Further characterization of the enzymatic activity of HSD17B13 *in vitro* and *in vivo* can help clarify its role. The increased levels of HSD17B13 in NAFLD patients may reflect a separate pathological process in the multifactorial pathogenesis of NAFLD.

The genetic association of variants in *HSD17B13* with features of NAFLD is complex, with different SNPs associated with different phenotypic patterns. We identified two different and independent SNPs that result in loss-of-function variants and reduction in NAFLD-associated injury and inflammation and are likely causal variants. The rs62305723 SNP encodes a P260S mutation that abolishes the RDH activity *in vitro*. Conservation of P260 among vertebrates in the *HSD17B13* gene and in the SDR16C family indicates this site is essential for protein function. A second SNP, rs72613567, generates two simultaneously-expressed novel splice variants that are also devoid of enzymatic function, and are also associated with decreased injury. This SNP is in high LD with the tag SNP rs6834314, and likely explains the associations found with it, as well as the association with ALT reported by Chambers (18) and Xu (33), especially since we demonstrate that rs6834314 is not an expression quantitative trait locus (eQTL). Association with fibrosis stage, the major determinant of prognosis in NASH, was weaker, but rs6834314 and rs72613567 are associated with cirrhosis in the MGI and UK Biobank cohorts, respectively, consistent with their impact on liver injury. Interestingly, both rs62305723 and rs72613567 are located in or near exon 6 of the gene, away from the conserved catalytic and co-factor binding motifs of the 17 β -HSD family (28). Overall, our data supports a role for HSD17B13 in mediating NAFLD-associated injury through its enzymatic activity.

Some variants in *HSD17B13* were associated with histological degree of steatosis, including rs6834314, its associated splice SNP rs72613567, and other independent SNPs. The association of rs6834314 with steatosis was confirmed in an independent cohort of patients with chronic

hepatitis C. The alleles that associate with increased hepatic fat are those that associate with decreased injury, and the association with injury was independent of the association with steatosis. However, the loss-of-function P260S variant is not associated with steatosis. This suggests that the association with hepatic fat may be unrelated to the enzymatic activity and could be driven by another mechanism. This is also supported by our *in vitro* data, where knocking-out or overexpressing *HSD17B13* in hepatoma cells does not affect their fat-storage capacity. Consistent with our data, Abul-Husn (47) reported that *HSD17B13* overexpression does not change cellular lipid content. In contrast, Adam (48) recently found that *Hsd17b13* knock-out mice showed hepatic steatosis only at an older age, indicating *HSD17B13* might affect hepatic fat metabolism *in vivo*; the underlying mechanism is yet to be explored.

In a proteomic analysis of hepatic lipid droplets, Su (35) recently identified upregulation of *HSD17B13* in subjects with NAFLD, consistent with our results. They demonstrated an increase in lipid droplet size in cells over-expressing *HSD17B13* tagged with GFP; we obtained similar results but were not able to replicate them when transfecting with *HSD17B13* with a smaller tag (Flag epitope), suggesting the large GFP-tag on lipid-droplet associated *HSD17B13* causes non-specific lipid aggregation (data not shown). *HSD17B13* expression appears to be regulated by LXR and SREBP-1c (49), which can explain our finding of its upregulation in patients with NASH and is consistent with a functional role in NAFLD pathogenesis. Additional evidence supporting a role for *HSD17B13* in lipid metabolism comes from a recent GWAS (50), in which rare variants in *HSD17B13* were found to impact the effect of fenofibrate on plasma triglycerides and high-density lipoprotein.

Recently, Abul-Husn (47) also reported that the splice-site SNP rs72613567 is associated with levels of ALT and AST and with the risk of chronic liver disease. Their findings, including the presence of loss-of-function splice variants associated with rs72613567, are consistent with ours and together highlight the role of *HSD17B13* in liver injury. Whether the association with injury is specific to fatty liver disease is unclear. A major strength of our study compared to (47) is our focus on genetic associations with the separate specific histological components of NAFLD, allowing us to highlight the different impact on hepatic fat and injury. Our findings suggest that *HSD17B13* affects injury and inflammation in NAFLD differently than in viral hepatitis, and that the association with steatosis, not previously reported, may be due to a different mechanism. Furthermore, beyond the data on the impact of truncated or unstable forms generated by the

splicing SNP, also demonstrated by Abul-Husn (47), we describe for the first time a novel single amino acid mutation of HSD17B13 that confers loss of enzymatic activity despite stable protein expression and proper cellular localization, and associate this novel mutation with histological severity of NAFLD. This confirms the link between enzymatic function and phenotype.

In conclusion, we confirmed that the association of rs6834314 with ALT reflects its association with NAFLD and that *HSD17B13* represents the affected gene of interest. Our data establish HSD17B13 as a lipid-droplet associated protein and point to its RDH activity as a key aspect of function, with a potential pathogenic link to NAFLD. Genetic variations in HSD17B13 have complex consequences that vary by type and locus, and further investigation into the genotype-phenotype association is warranted.

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FIGURE LEGENDS

Figure 1. *HSD17B13*-related SNPs are associated with liver disease. Association of rs6834314 genotype with (A) liver histology scores and (B) blood levels of liver enzymes in subjects with NAFLD. (AA, n=485; GA, n=226; GG, n=41). (C) Association of rs6834314 genotype with liver histology scores in subjects with chronic hepatitis C. (AA, n=165; GA, n=125; GG, n=23). (D) Association of rs72613567 genotype with liver histology scores in subjects with NAFLD. (--, n=503; -A, n=220; AA, n=35). (E) Association of rs62305723 genotype with liver histology scores in subjects with NAFLD. (GG, n=615; GA/AA, n=76). Mean±SEM. P-values for histology from multivariate ordinal regression adjusted for age, gender, and BMI. P-values for enzymes from linear regression of log-transformed enzyme activity, adjusted for age, gender, and BMI.

Figure 2. Hepatic expression of *HSD17B13*. (A) Expression levels of *HSD17B13* by qPCR in liver samples from healthy controls (n=14) and NASH patients (n=43). (B) Expression levels of *HSD17B13* by qPCR according to rs6834314 genotype in liver samples from control and NASH patients (A/A genotype: n=45; and A/G or G/G genotype: n=12). rs6834314A/G and G/G were pooled together due to lower frequency. Lines denote median and interquartile range and are normalized to controls. Mann-Whitney test was used for significance testing.

Figure 3. *HSD17B13* localizes with lipid droplets. (A) HepG2 cells were transfected with *HSD17B13*(A)-GFP and stained for the lipid droplet marker protein ADRP (Perilipin-2). (B)

Lipid droplets were isolated from HSD17B13(A)-GFP transfected cells and stained with the lipid droplet specific dye LipidTox. (C) Mutations were generated and tested for cellular localization with LipidTox used to stain lipid droplets. (D) HSD17B13(A)-GFP and HSD17B13(B)-Flag were co-transfected into HEK293 cells. Immunofluorescence staining was used to visualize HSD17B13(B)-Flag. Nuclei were counterstained with Hoechst 33342. One representative image from repeated experiments is shown. Bars indicate 10 μ m.

Figure 4. HSD17B13 is a retinol dehydrogenase. (A) Oxidation of retinol to retinaldehyde by retinol dehydrogenases (asterisk) is the rate limiting step of retinoic acid synthesis. HEK293 cells transfected with HSD17B13(A), empty vector, or RDH10 as positive control were treated with retinol (5 μ M) and levels of synthesized retinal (B) and retinoic acid (C) were measured by HPLC. Transfection of HSD17B13(B) variant does not lead to increased retinaldehyde (E) nor retinoic acid (F) synthesis. Co-factor binding site mutation protein HSD17B13(A)-G47/49A has no RDH activity (H and I) in HEK293 cells treated with 2 μ M retinol. Anti-Flag Western blot used to determine protein transfection efficiency in each experiment (D, G, and J). Data are presented as Mean \pm SEM.

Figure 5. The P260S mutation in HSD17B13 (rs62305723G>A) is associated with loss of RDH activity despite lipid droplet targeting. (A) The P260S substitution is located within a highly conserved region of HSD17B13 orthologues and human homologues. (B) In HepG2 cells transfected with HSD17B13-P260S-GFP, the mutant protein, similar to the wild type protein, can be detected in lipid droplets. Transfection of HEK293 cells with HSD17B13-P260S-Flag does not lead to increased retinaldehyde (C) or retinoic acid (D) synthesis in the presence of 2 μ M retinol despite adequate transfection efficiency (E). Data are presented as Mean \pm SEM.

Figure 6. HSD17B13 delta 6 and G-insertion variants localize with lipid droplets, lose RDH activity, and are associated with rs72613567. (A) Schema of HSD17B13 variants A, delta 6, and G-insertion. (B) In HepG2 cells transfected with HSD17B13-delta 6-Flag or HSD17B13-G insertion-Flag the mutant proteins can be detected in lipid droplets stained by LipidTox. (C) Transfection of HEK293 cells with HSD17B13-delta 6 or HSD17B13-G insertion does not lead to increased retinoic acid levels. The delta 6 (D) and G-insertion (E) variants are only detected in subjects who carry the rs72613567 minor A allele.

Table 1 – P-Values for Association of SNPs in the *HSD17B13* Gene with Histological Features and ALT in the NAFLD Cohort. Arrows Denote Direction of Effect for the Minor Allele

SNP	SNP Type	Alleles	Minor Allele Frequency ^A	Genotype Frequency ^A	R ² vs. rs6834314 genotype ^B	Fat ^C	Inflammation ^C	Ballooning ^C	Mallory-Denk Bodies ^C	Fibrosis ^C	ALT ^D
rs72613567	Splice site	-/A	0.194	0.66/0.29/0.05	0.938	0.008* ↑	0.063 ↓	0.202	0.062 ↓	0.373	4.2X10 ⁻⁴ * ↓
rs62305723	Non-synonymous coding	G/A	0.056	0.89/0.11/0.003	0.022	0.326	5X10 ⁻⁵ * ↓	0.002* ↓	0.025 ↓	0.065 ↓	0.217
rs10433937	Non coding	T/G	0.199	0.65/0.29/0.05	0.979	0.019* ↑	0.062 ↓	0.042 ↓	0.013 ↓	0.323	2.3X10 ⁻⁴ * ↓
rs6531975	Non coding	G/A	0.202	0.64/0.32/0.05	0.019	0.016* ↓	0.098	0.728	0.641	0.392	0.744
rs10022237	Non coding	C/T	0.428	0.33/0.49/0.18	0.403	0.004* ↑	0.021 ↓	0.05 ↓	0.100	0.090	0.033 ↓
rs6531973	Non coding	C/A	0.238	0.57/0.39/0.05	0.091	0.535	0.621	0.853	0.321	0.402	0.207
rs6531972	Non coding	A/C	0.404	0.35/0.49/0.16	0.171	0.067	0.648	0.532	0.993	0.603	0.333
rs7692397	Non coding	C/A	0.241	0.58/0.36/0.06	0.104	0.074	0.068	0.257	0.231	0.227	0.215
rs9647458 ^E	Non coding	A/G	0.096	0.82/0.18/0.008	0.035	0.646	0.696	0.137	0.472	0.661	0.579

^A Allele and genotype frequencies from the NAFLD cohort

^B R^2 for linkage between SNPs and rs6834314 from LDLink, limited to populations of European ancestry

^C Multivariate ordinal logistic regression adjusted for age, BMI, gender and diabetes

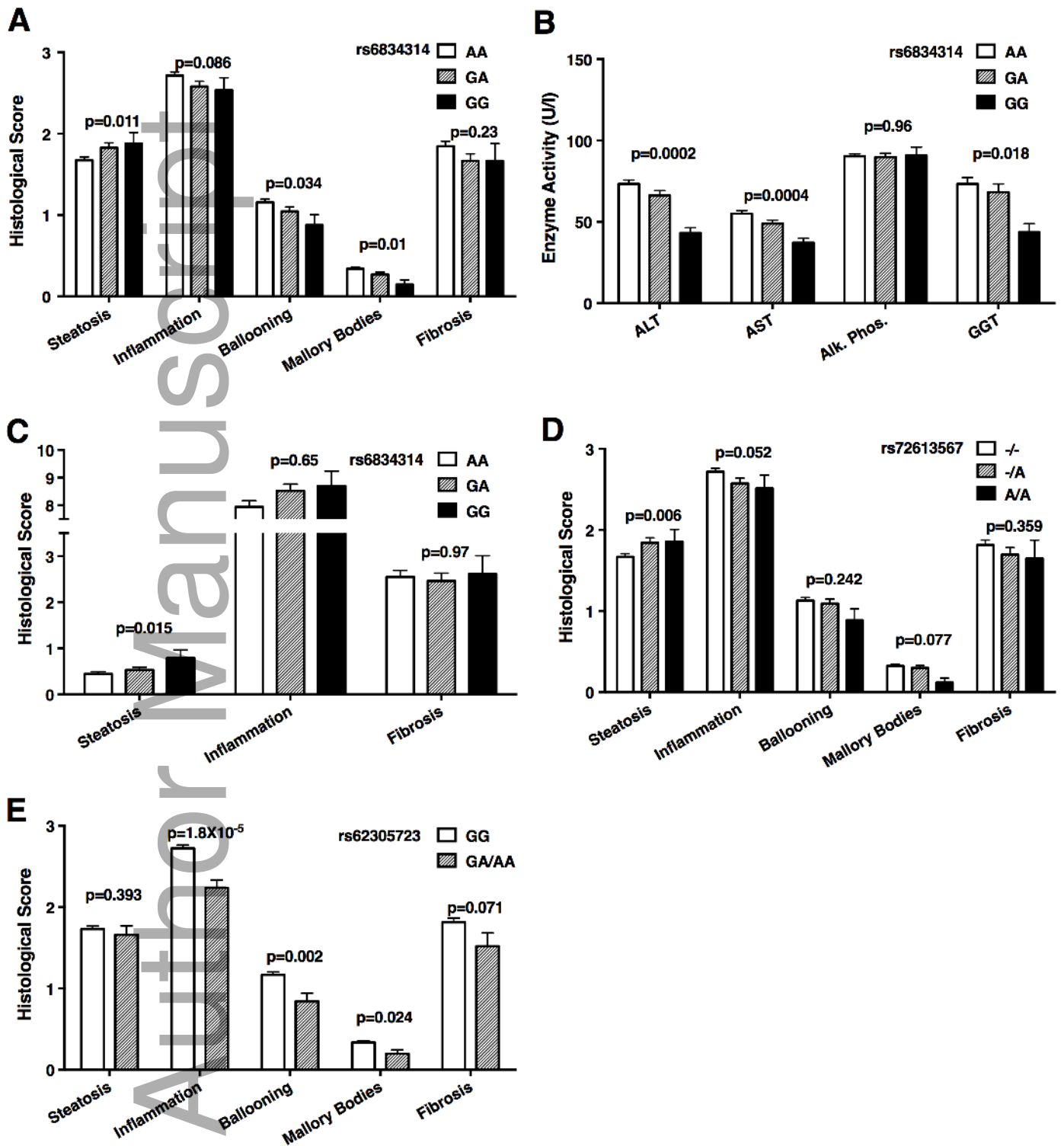
^D Linear regression of log-transformed enzyme activity adjusted for age, BMI, gender and diabetes

^E rs76926692 (non-synonymous coding) and rs75049937 (frameshift) were genotyped but omitted for minor allele frequency $\leq 0.3\%$

* FDR q-value < 0.05

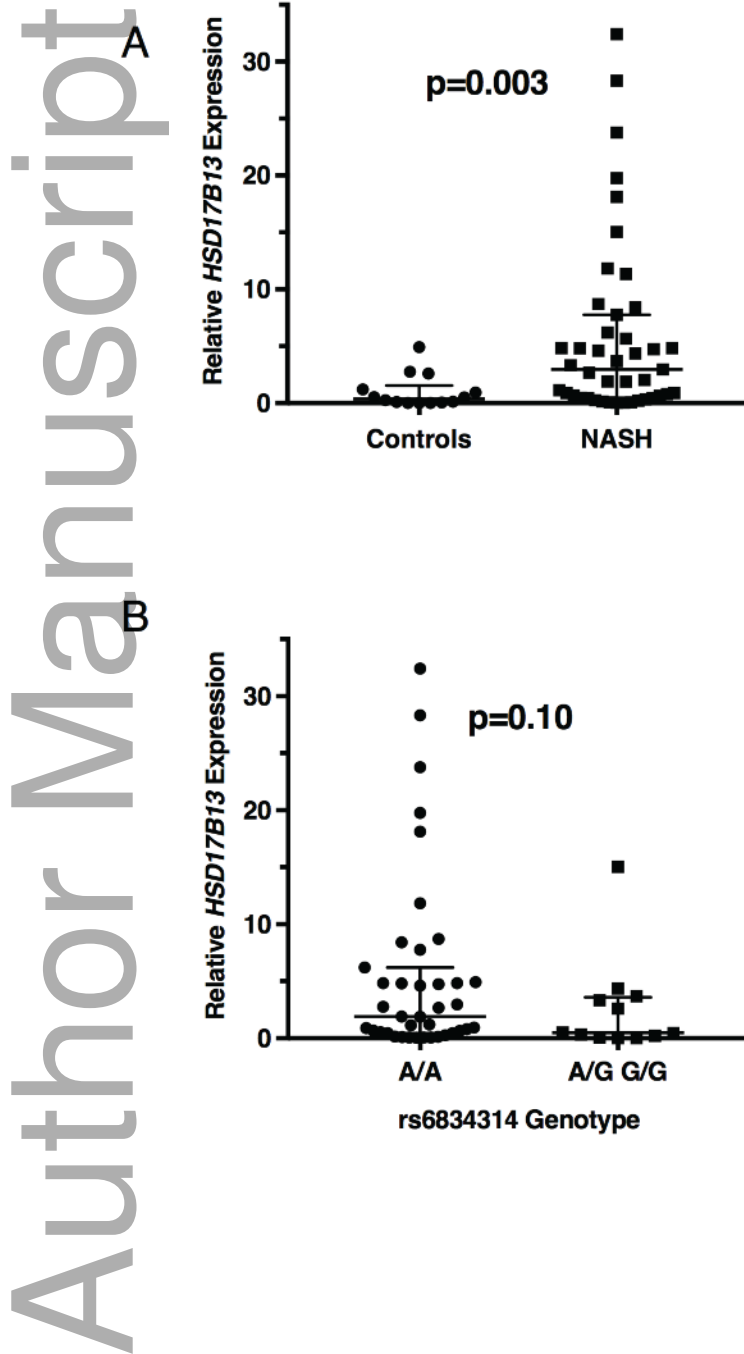
Author Manuscript

Figure 1



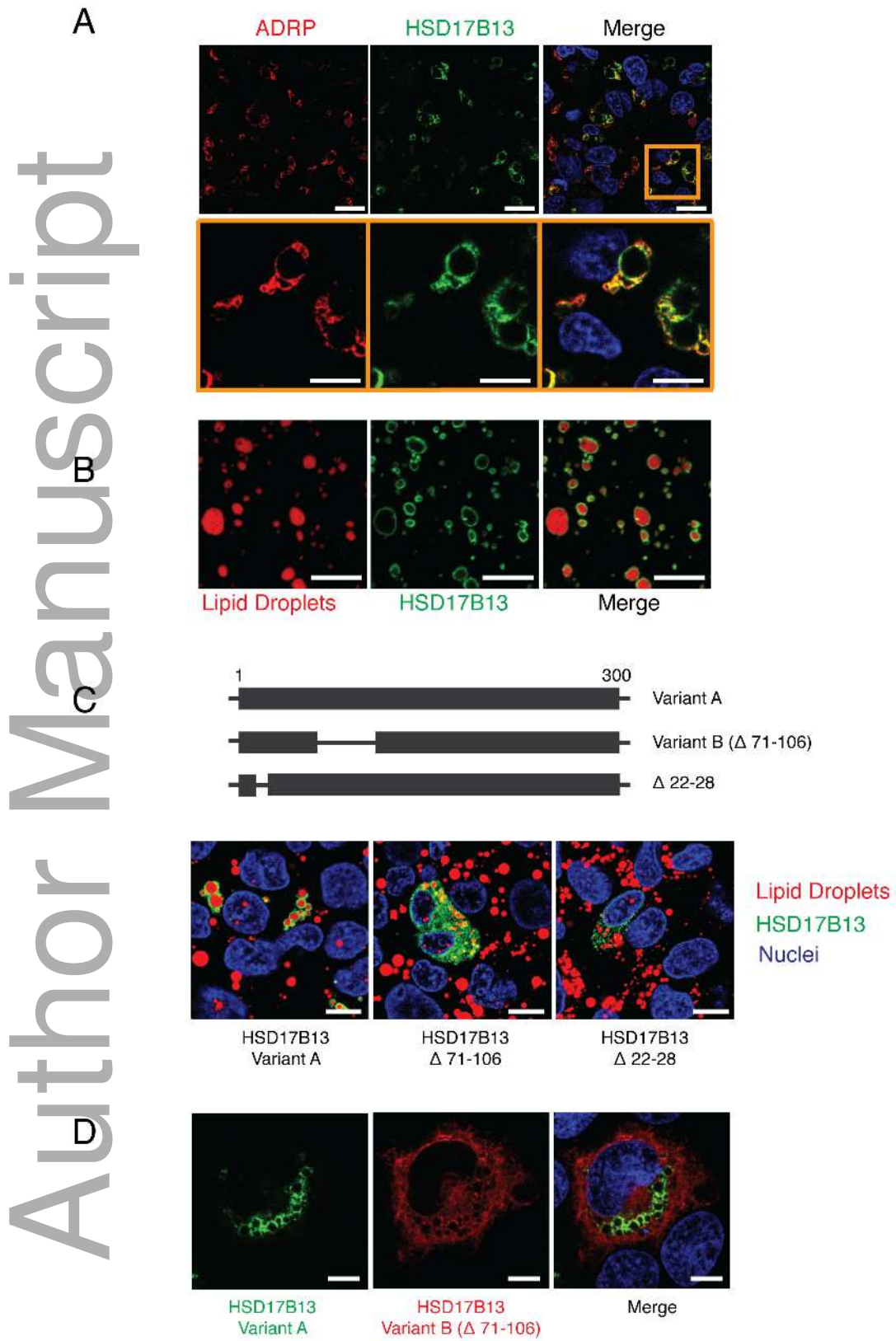
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Figure 2



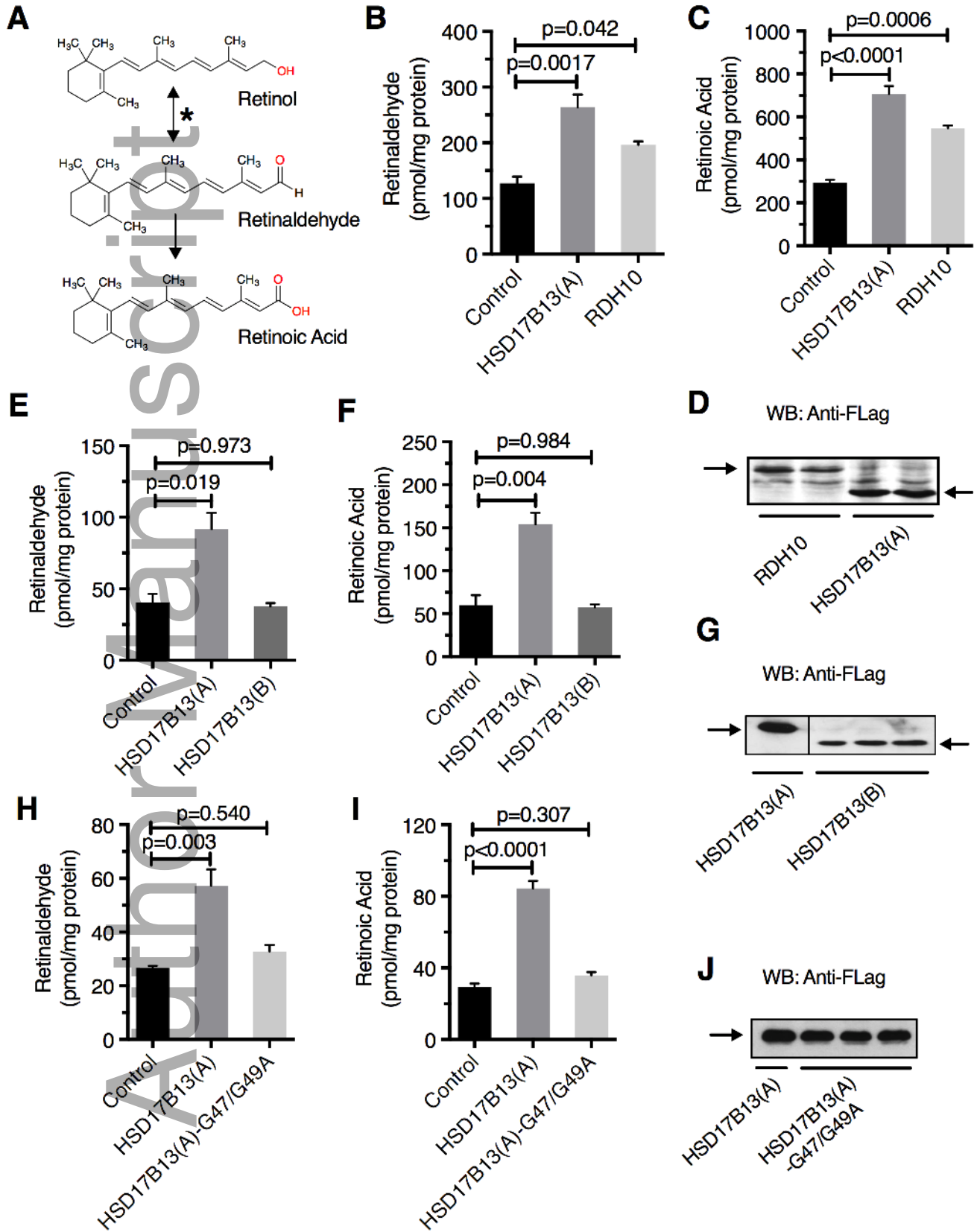
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Figure 3



hep_30350_f3.tif

Figure 4



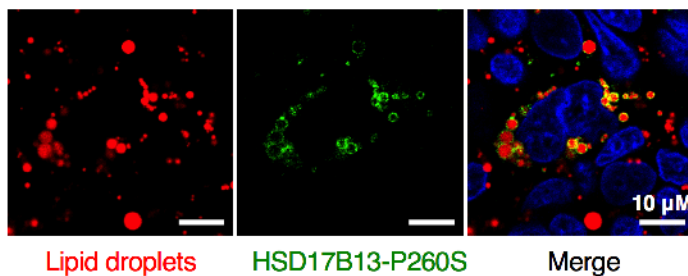
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Figure 5

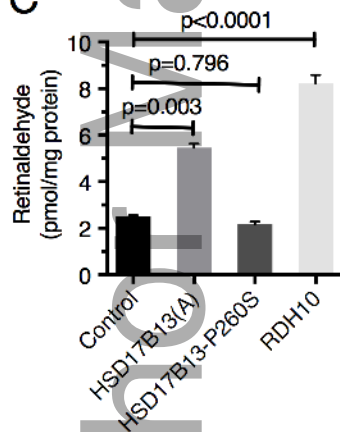
A

Human	240-	DEVVRS	LDGI	LTNKK	MIFV	PSYI	NI	FLRL
Chimpanzee	240-	DEVVRS	LDGI	LTNKK	MIFV	PSYT	NI	FLRL
Monkey	240-	DEVVRS	LDGI	LTNKK	MIFV	PSYI	NI	FLIL
Horse	240-	DEVARS	LDGI	LTNKK	MIFV	PSYLN	ISL	TTL
Pig	240-	DEVARS	LDGI	LTNKK	MIFV	PSYLN	ISL	TTL
Dog	240-	DTVARS	LDGI	LTNKK	MIFV	PSYNY	YLIL	
Mouse	240-	EEVARS	LNGI	LTNKK	MIFV	PSYI	NISL	LIL
Rat	240-	DEVARS	LDGI	LTNKK	MIFV	PSYI	NISL	IV
Frog	240-	EDVVK	CLME	GLT	NKKMI	IVP	SSV	KYSLIL
HSD17B13	240-	DEVVRS	LDGI	LTNKK	MIFV	PSYI	NI	FLRL
HSD17B11	240-	EEVVNR	LMHGI	LTEQ	KMIFI	PSSI	AFL	TTL
RDH10	270-	DYCVK	QAMK	AILTD	QPMI	CTP	RLMY	IVTFM
RDHE2	248-	KYAVE	KIVE	AI	LQEK	MYLY	MPK	LLYFMMFL
DHRS3	244-	ETVAR	RRTVE	AVQL	NQALL	LLLP	WTM	MHALVIL

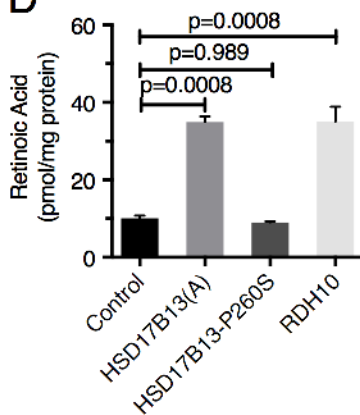
B



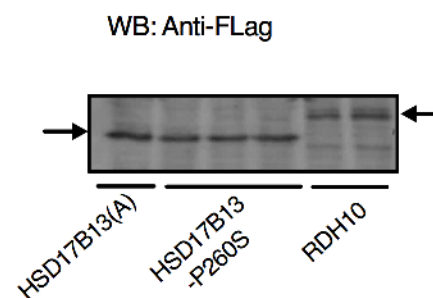
C



D

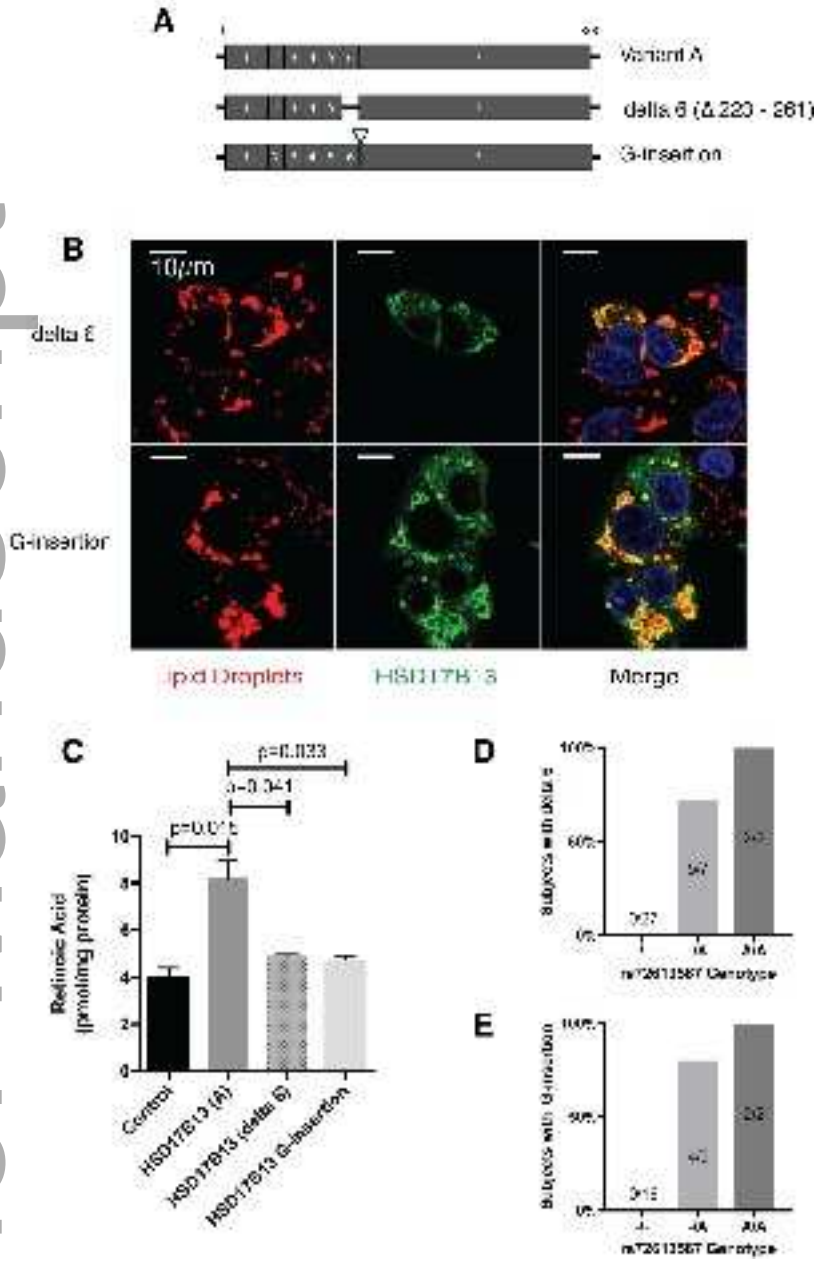


E



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Figure 6



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