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Original Article

Sex hormone pathway gene polymorphisms are associated with risk of advanced hepatitis C-related liver disease in males

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Abstract: Background: Males have excess advanced liver disease and cirrhosis risk including from chronic hepatitis C virus (HCV) infection though the reasons are unclear. Goal: To examine the role variants in genes involved in androgen and estrogen biosynthesis and metabolism play in HCV-related liver disease risk in males. Methods: We performed a cross-sectional study evaluating single nucleotide polymorphisms (SNPs) in 16 candidate genes involved in androgen and estrogen ligand and receptor synthesis and risk of advanced hepatic fibrosis (F3/F4-F4) and inflammation (A2/A3-A3). We calculated adjusted odds ratios (ORs) using logistic regression and used multifactor dimensionality reduction (MDR) analysis to assess for gene-environment interaction. Results: Among 466 chronically HCV-infected males, 59% (n = 274) had advanced fibrosis and 54% (n = 252) had advanced inflammation. Nine of 472 SNPs were significantly associated with fibrosis risk; 4 in *AKR1C3* (e.g., *AKR1C3* rs2186174: OR_{adj} = 2.04, 95% CI 1.38-3.02), 1 each in *AKR1C2* and *ESR1*, and 1 in *HSD17B6*. Four SNPs were associated with inflammation risk, 2 in *SRD5A1* (e.g., *SRD5A1* rs248800: OR_{adj} = 1.86, 95% CI 1.20-2.88) and 1 each in *AKR1C2* and *AKR1C3*. MDR analysis identified a single *AKR1C3* locus (rs2186174) as the best model for advanced fibrosis; while a 4-locus model with diabetes, *AKR1C2* rs12414884, *SRD5A1* rs6555406, and *SRD5A1* rs248800 was best for inflammation. Conclusions: The consistency of our findings suggests *AKR1C* isoenzymes 2 and 3, and potentially *SRD5A1*, may play a role in progression of HCV-related liver disease in males. Future studies are needed to validate these findings and to assess if similar associations exist in females.

Keywords: Epidemiology, hepatology, endocrinology, infectious diseases, digestive system, carcinogenesis, genetics

Introduction

HCV infection is a major public health problem globally. In the U.S, approximately 1.4% of the population is estimated to have been infected [1], most (75-85%) with chronic infection [2]. HCV is now the leading cause of cirrhosis and end-stage liver disease [3], the 12th leading cause of death in the U.S, liver transplant [4], and of hepatocellular carcinoma (HCC) [5], a lethal and increasingly prevalent malignancy.

Males have excess risk of progression to advanced liver disease or cirrhosis and HCC

across diverse populations as well as etiologies for liver disease, including HCV. In addition to gender-based differences in prevalence of other established risk factors for liver disease progression like alcohol abuse and greater visceral adiposity, differences in male (androgen) and female (estrogen) sex hormone signaling has also been suggested as a potential contributory factor. Some experimental cell-line research is supportive of a role of both androgens and estrogens or their receptors in HCV-related disease progression. For example, estradiol or E2 (a known powerful antioxidant and the most ubiquitous estrogen prior to menopause) has been shown to inhibit HCV virion production in an estrogen receptor dependent manner [6].

Only a few epidemiological studies have evaluated the association between serum levels of testosterone and risk of advanced HCV-related liver disease [7, 8], with findings of both significant association and no association reported. The association with E2 levels has also been evaluated in a few studies, with decreased risk of HCV-related cirrhosis reported in an Italian study with 355 HCV+ women [9], and lack of association with advanced fibrosis reported in a study in 35 Australian HCV+ males [7]. However, a major limitation of these studies is the potential for reverse causation bias to confound results given the presence of highly advanced liver disease may itself substantially alter serum sex hormone levels. Increased androgen synthesis due to gain-of-function mutations of steroidogenic enzymes has been described in other organs such as the prostate, where a point mutation of 3-hydroxysteroid 3-beta dehydrogenase 1 (HSD3B1) was responsible for recurrence of castration resistant prostate cancer [10]. Less is known about the role of genetic variants in sex hormone pathway synthesis and receptor signaling and risk of HCV-related cirrhosis and necroinflammatory activity. A single Italian case-control study with N = 387 HCV cases (22% female, 24% with concomitant HCC) evaluated a functional SNP in each of 3 genes involved in sex steroid hormone synthesis (CYP17A1, 5SRDA2, and COMT) and found significant association only for CYP17A1 (-34) T to C SNP, with effects predominantly limited to women, particularly postmenopausal women [11]. Evaluating published germline variants offers an alternative method of examining the role of sex hormones in advanced liver disease. Furthermore, given that cirrhosis persists in previously chronically infected HCV cases, and the risk of HCC remains elevated even 10 years after achieving a treatment-associated sustained virological response [12], identification of inherited germline genetic variants that are associated with HCV-related liver disease progression are likely to continue to play an important role for clinical risk stratification and targeted prevention and treatment.

In this study, we performed a novel genetic association study in a large and well-characterized cohort of U.S. male veterans with chronic HCV to test the hypothesis that host germline variation in 16 *a priori* selected genes in androgen and estrogen steroid sex hormone synthesis and signaling (androgen receptor [*AR*], aldo-keto reductase family 1 (AKR1) member C2 [*AKR1C2*], AKR1C3, cytochrome P450 (CYP) family 17, subfamily A, polypeptide 1 [*CYP17A1*] and *CPY19A1*, estrogen receptor (ESR) alpha [*ESR1*] and beta [*ESR2*], G protein-coupled estrogen receptor 1 [*GPER*], hydroxysteroid (HSD) 17-beta dehydrogenase 6 [*HSD17B6*], *HSD3B1,* and *HSD3B2,* sex hormone binding globulin [*SHBG*], steroid-5-alpha-reductase, alpha (SRD5A) polypeptide 1 [*SRD5A1*] *SRD5A2,* and UDP glucuronosyltransferase 2 family, polypeptide B17 [*UGT2B17*]) are associated with risk of advanced hepatic fibrosis and inflammation.

Methods

Study population and design

Research assistants (RAs) obtained participant's witnessed written informed research consent prior to study participation. This research including written informed consent form was jointly approved by Institutional Review Boards for the Baylor College of Medicine and the Michael E. DeBakey VA Medical Center in Houston, Texas. Study details have been previously published [13].

Briefly, we prospectively recruited consecutive HCV-infected veterans prior to their previously scheduled HCV clinic visit at a large tertiary care VA medical center between May 1, 2009 and December 31, 2012. Patients completed a research assistant (RA) administered survey

interrogating medical and risk factor history including lifetime alcohol use, had anthropometric measurements taken, and completed a fasting venipuncture for performance of the FibroSure-ActiTest as a measure of hepatic pathology. We restricted our current analysis to individuals who were: (1) African American and White male veterans between 18 and 70 years as they collectively compose ~ 90% of the underlying target population; (2) had no history of HCC, liver transplant, decompensated liver disease including ascites, dementia, or psychosis; (3) were serologically-confirmed to have chronic HCV and to be negative for both HIV and active HBV infection; (4) were not currently receiving anti-HCV pharmacotherapy; and (5) had both FibroSURE testing and germline DNA genotyping completed by February 1, 2013.

Outcome and confounder variables

Our primary outcomes were FibroSURE-ActiTest (hereafter referred to as FibroSURE) determined hepatic fibrosis and inflammation. The FibroSURE test (also known as FibroTest, BioPredictive, France) uses a proprietary algorithm incorporating serum levels of α2-macroglobulin, apolipoprotein A1, haptoglobin, total bilirubin, and γ-glutamyl-transpeptidase to determine fibrosis level, and adds ALT to determine necroinflammatory activity level. Test scores are categorized into METAVIR biopsy-based equivalent degrees of hepatic pathology. FibroSURE has demonstrated good concordance with biopsy-assessed level of METAVIR pathology in diverse HCV-infected populations [14-17]. We defined advanced fibrosis cases as individuals with FibroSURE-determined fibrosis stage F3/F4-F4, cirrhosis, and mild fibrosis controls as individuals with stages F0-F3; and advanced inflammatory activity cases as individuals with inflammatory grades A2/A3-A3, severe, and mild activity controls as A0-A2. For purposes of sensitivity analyses limited to individuals with extreme phenotypes, we defined extreme phenotypes for fibrosis as F4, cirrhosis as advanced cases vs. F0, no fibrosis-F0/F1 as mild controls, and A3, severe activity as advanced vs. A0, no activity as mild. We also measured several potential confounders including overweight/obesity, diabetes, chronic alcohol abuse, HCV genotype and viral load. Patient's weight and height were converted into their body mass index (BMI) using the Quetelet index formula, with overweight/obesity defined as a BMI of 25 or higher. Diabetes was defined as present based on a positive history from either EMR review or patient self-report of prior physician diagnosis, or if fasting glucose was > 120 in an individual with no history of diabetes. Chronic alcohol abuse was defined as self-reported history of consuming 3 or more drinks a day for at least 10 consecutive years. Viral load was classified into tertiles and HCV viral genotypes were categorized into categories (genotypes 1, 2 or 3, and 4).

Selection of genes, SNPs and genotyping assays

Sample preparation and array staining were performed in a blinded manner in batches with randomly selected cases and controls and in accordance with Illumina protocols at the Baylor College of Medicine Laboratory for Translational Genomics. Arrays were scanned on an Illumina iScan system with SNP clustering and initial basic quality control performed using GenomeStudio software, version 2010 (Illumina, Inc, San Diego, CA, U.S.A.). To evaluate the possibility of undisclosed familial relationships or duplicated samples, we also performed identity-by-state clustering on the basis of autosomal genotypes, followed by multidimensional scaling analysis of the resulting matrix of identity-by-state pairwise distances using PLINK.

A total of 610 SNPs were identified for our 16 candidate genes on the Illumina HumanOmni 2.5-8 microarray (Illumina, San Diego, CA). After excluding monomorphic SNPs and those with minor allele frequency (MAF) < 0.05, the vast majority of our final 472 tagging SNPs were located in flanking and intronic regions.

Statistical analysis

We assessed for Hardy-Weinberg equilibrium (HWE) in the controls and compared allelic frequency between advanced cases vs. mild controls with a X^2 test with *p*-values calculated based on 10,000 permutations. For the primary analysis, we employed logistic regression analysis to evaluate the association between individual SNPs and advanced hepatic fibrosis (advanced = F3/F4-F4, cirrhosis vs. mild = F0-F3) and advanced hepatic inflammation

Table 1. Sociodemographic and clinical characteristics of male veterans with chronic hepatic C virus (HCV) infection based on FibroSURE-determined hepatic fibrosis and inflammation

		Inflammation				Fibrosis				Inflammation-Extreme Phenotypes only				Fibrosis-Extreme Phenotypes Only			
	All	Mild (A0-A2)	Advanced (A2/A3-A3)			Mild (F0-F3)	Advanced (F3/F4-F4)			Low (A0)	Advanced inflammation (A3)			Low (F0)	Advanced (F4)		
Risk Factor	N = 466	n = 252	n = 214	OR (95% CI)	*P*-value	n = 274	n = 192	OR (95% CI)	*P*-value	n = 133	n = 141	OR (95% CI)	*P*-value	n = 130	n = 177	OR (95% CI)	*P*-value
Race/Ethnicity					0.620				0.228				0.346				0.019
White	314 (67)	167 (66)	147 (69)	1		191 (70)	123 (64)	1		93 (70)	106 (75)	1		98 (75)	111 (63)	1	
Black	152 (33)	85 (34)	67 (31)	0.9 (0.59-1.35)		83 (30)	69 (36)	1.3 (0.86-1.94)		40 (30)	35 (25)	0.77 (0.43-1.35)		32 (25)	66 (37)	1.8 (1.1-3.1)	
Age-group					0.258				0.029				0.225				0.002
≤ 55	193 (41)	98 (39)	95 (44)	1		125 (46)	68 (35)	1		54 (41)	68 (48)	1		72 (55)	65 (37)	1	
> 55	273 (59)	154 (61)	119 (56)	0.8 (0.54-1.17)		149 (54)	124 (65)	1.5 (1.0-2.3)		79 (59)	73 (52)	0.73 (0.44-1.22)		58 (45)	112 (63)	2.1 (1.3-3.5)	
Chronic alcohol abuse^,&					0.702				0.499				0.534				0.906
Negative	179 (38)	94 (37)	85 (40)	1		101 (37)	78 (41)	1		48 (36)	57 (40)	1		53 (41)	71 (40)	1	
Positive	283 (61)	155 (62)	128 (60)	0.91 (0.62-1.35)		169 (62)	114 (59)	0.87 (0.59-1.30)		84 (63)	84 (60)	0.84 (0.5-1.4)		76 (58)	106 (60)	1 (0.64-1.70)	
Diabetes					0.381				0.098				0.199				0.056
Negative	355 (76)	196 (78)	159 (74)	1		216 (79)	139 (72)	1		107 (80)	104 (74)	1		106 (82)	127 (72)	1	
Positive	110 (24)	55 (22)	55 (26)	1.2 (0.78-1.94)		57 (21)	53 (28)	1.4 (0.92-2.27)		26 (20)	37 (26)	1.5 (0.8-2.7)		24 (18)	50 (28)	1.7 (0.97-3.16)	
Overweight/obese#					0.007				0.0111				0.004				0.001
Negative	126 (27)	81 (32)	45 (21)	1		86 (31)	40 (21)	1		52 (39)	32 (23)	1		49 (38)	36 (20)	1	
Positive	339 (73)	170 (67)	169 (79)	1.8 (1.2-2.8)		187 (68)	152 (79)	1.7 (1.1-2.8)		81 (61)	109 (77)	2.2 (1.3-3.8)		81 (62)	141 (80)	2.4 (1.4-4.1)	
Viral Load^					0.371				0.067				0.382				0.042
Low	153 (33)	81 (32)	72 (34)	1		97 (35)	56 (29)	1		51 (38)	46 (33)	1		51 (39)	53 (30)	1	
Medium	154 (33)	78 (31)	76 (36)	1.1 (0.68-1.76)		79 (29)	75 (39)	1.6 (1.0-2.7)		38 (29)	51 (36)	1.5 (0.8-2.8)		34 (26)	70 (40)	2 (1.1-3.6)	
High	152 (33)	89 (35)	63 (29)	0.8 (0.49-1.28)		94 (34)	58 (30)	1.1 (0.65-1.75)		44 (33)	44 (31)	1.1 (0.6-2.1)		45 (35)	54 (31)	1.2 (0.64-2.08)	
HCV genotype^					0.270				0.320				0.882				0.214
1	378 (81)	209 (83)	169 (79)	1		218 (80)	160 (83)	1		106 (80)	111 (79)	1		104 (80)	152 (86)	1	
2 and 3	81 (17)	39 (15)	42 (20)	1.3 (0.8-2.2)		52 (19)	29 (15)	0.76 (0.44-1.28)		27 (20)	30 (21)	1.1 (0.57-1.99)		26 (20)	25 (14)	0.66 (0.34-1.26)	

^Numbers do not add up to the column totals due to missing values. &Defined as 3 + drinks/day for at least 10 consecutive years. #Overweight/obesity (BMI ≥ 25).

(advanced = A2/A3-A3 vs. mild = A0-A2). We also performed a sensitivity analysis where we restricted the analysis to individuals with extreme phenotypes (F4, cirrhosis, as advanced cases vs. F0, no fibrosis as mild controls for fibrosis; A3, severe activity as advanced cases vs. A0, no activity as mild controls for hepatic activity, respectively). The Akaike's Information Criterion was used to determine the genetic model for each SNP. [18] Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated in multivariate analysis adjusting for age, race/ethnicity, overweight/obesity (BMI ≥ 25), diabetes, chronic alcohol abuse, viral load tertile, and HCV genotypes. We also compared our race-adjusted models to race-stratified models to assess adequacy of race-adjustment. To reduce the potential impact of spurious findings associated with multiple comparisons, we used significance threshold of $p \leq 0.005$ in the main effect analysis. To further evaluate the chance of obtaining a false-positive association, we used the false-positive report probability (FPRP) test. We used the moderate range of prior probabilities 0.01-0.05 and FPRP cutoff value 0.2, as previously suggested [19].

Next, to evaluate for potential dose-response effects, we performed a joint effect analysis for the significant SNPs identified from the main effects analysis ($P \leq 0.005$) by adding up the number of adverse alleles. Adverse alleles were defined as the minor allele of the at-risk SNPs (OR > 1) and the common allele of the protective-effect SNPs (OR < 1). To assess for and characterize potential epistasis including potential nonlinear interactions among individual SNPs or between SNPs and other risk factors (e.g., race and diabetes), we employed the non-parametric multifactor dimensionality reduction (MDR) method [20, 21]. It combines attribute selection and classification with cross-validation and permutation testing to identify the single best combined risk factor model for predicting disease risk. We selected the model with highest test accuracy and cross-validation consistency (CVC) and further evaluated it using permutation testing that shuffles mild and advanced disease cases 10,000 times and repeats the MDR analysis on each randomized dataset. This analysis was performed using MDR version 2.0 beta 8.4 that is freely available online (http://www.epistasis.org/software.html).

We similarly assessed the association between haplotypes created from SNPs for all genes with more than one SNP in strong linkage disequilibrium (LD) that had been significantly associated with increased risk of advanced fibrosis or advanced inflammation in single locus multivariate analysis. Pairwise LD among SNPs was examined using Lewontin's standardized coefficient *D'* and LD coefficient r^2, [22] with "strong LD" defined as the one-sided upper 95% CI boundary on *D'* is > 0.98 and the lower boundary is > 0.7. We performed haplotype analysis using HAPLO.STATS as developed by Schaid *et al*. (http://www.mayo.edu/hsr/Sfunc.html) and implemented in R statistical software [23]. Haplotypes with a frequency of less than 0.03 were pooled into a combined group and empirical *P* values based on 10,000 simulations were computed for all tests.

Results

Characteristics of the study population

Sample characteristics for the 466 HCV+ male veterans included in our analysis are reported in **Table 1**. Mean age was 59 years, with 67% White and 33% African American. Prevalence of FibroSURE-determined advanced fibrosis (F3/F4-F4, cirrhosis) was 59% (n = 274) and prevalence of advanced inflammatory activity (A2/A3-A3, severe) was 54% (n = 252). Presence of overweight/obesity (BMI ≥ 25) was the single measured potential confounder consistently associated with significant excess risk of advanced hepatic fibrosis and inflammation risk.

Association between individual SNP and advanced hepatic fibrosis and inflammatory activity risk

All genotype call rates were > 0.99. Of the 472 SNPs evaluated in the analysis, there were nine SNPs in four genes (*AKR1C3*, *AKR1C2*, *HSD17B6*, *ESR1*) that were significantly associated with advanced fibrosis risk (all permuted allelic *p*-values < 0.02) (**Table 2**). *AKR1C3* had four SNPs that were significantly associated with advanced fibrosis (F3/F4-F4) risk, with three of four associated with approximately two-fold excess advanced fibrosis risk in primary race-adjusted multivariate analyses (e.g., OR_{adj} for rs2154306 = 1.95, 95% CI 1.32-2.88, $p = 0.0007$). In contrast, both SNPs in *AKR1C2* and *ESR1* were associated with significantly decreased risk of advanced fibrosis (e.g., OR_{adj} for rs6909023 for *ESR1* = 0.39, 0.22-0.70, *p* =

Table 2. Genotype frequencies of SNPs and the associations with advanced hepatic fibrosis or inflammation risk in patients with active HCV infection ($P \leq 0.005$)

SNP and model[ǂ]	Gene	Location	Coordinate	Minor Allele	MAF	Permutation	Logistic regression*	*P* value	False Positive Report Probability Prior Significance Level[$]	
					Advanced/Mild	*P*[&]	OR (95% CI)		*0.05*	*0.01*
Advanced Inflammation	*Risk (A2/A3-A3 vs. A0-A2)*									
rs2211623[d]	*AKR1C3*	Intergenic	5105141	C	0.06/0.03	0.0029	3.04 (1.47-6.31)	0.0028	**0.15**	0.48
rs12414884[r]	*AKR1C2*	Intergenic	5063398	C	0.38/0.47	0.0015	0.48 (0.29-0.79)	0.0039	**0.08**	0.34
rs6555406[r]	*SRD5A1*	Intergenic	6694692	G	0.41/0.34	0.0105	2.15 (1.27-3.63)	0.0043	**0.10**	0.36
rs248800[d]	*SRD5A1*	Intron	6660400	A	0.16/0.10	0.002	1.86 (1.20-2.88)	0.0051	**0.10**	0.37
Extreme Inflammation	*Phenotype Risk (A3 vs. A0 only)*									
rs10163138[d]	*CYP19A1*	Intergenic	51614124	A	0.03/0.10	0.02	0.23 (0.08-0.61)	0.0032	0.31	0.7
rs879332[r]	*HSD3B2*	Intergenic	119940918	G	0.39/0.42	0.006	0.38 (0.19-0.73)	0.004	**0.13**	0.45
rs248800[d]	*SRD5A1*	Intron	6660400	A	0.17/0.09	0.027	2.34 (1.30-4.23)	0.0048	**0.14**	0.45
rs6555406[r]	*SRD5A1*	Intergenic	6694692	G	0.44/0.35	0.0018	2.88 (1.43-5.80)	0.003	**0.14**	0.46
Advanced Fibrosis	*Risk (F3/F4-F4 vs. F0-F3)*									
rs2186174[d]	*AKR1C3*	Intergenic	5176727	C	0.29/0.21	0.0022	2.04 (1.38-3.02)	0.0003	**0.008**	**0.04**
rs2398203[d]	*AKR1C3*	Intergenic	5176783	A	0.25/0.18	0.0043	2.00 (1.35-2.97)	0.0005	**0.013**	**0.06**
rs2154306[d]	*AKR1C3*	Intron	5143902	G	0.32/0.22	0.0007	1.95 (1.32-2.88)	0.0007	**0.016**	**0.08**
rs4559587[d]	*AKR1C3*	Intron	5142855	C	0.02/0.07	0.0008	0.30 (0.13-0.67)	0.0035	**0.20**	0.57
rs2801904[r]	*AKR1C2*	Intergenic	5083884	C	0.42/0.49	0.017	0.45 (0.28-0.74)	0.0018	**0.04**	**0.19**
rs2096421[r]	*AKR1C2*	Intergenic	5097405	C	0.37/0.45	0.0114	0.43 (0.25-0.74)	0.0021	**0.06**	0.27
rs4237805[r]	*HSD17B6*	Intergenic	57249600	A	0.35/0.32	0.0102	2.43 (1.36-4.35)	0.0028	**0.09**	0.34
rs6909023[r]	*ESR1*	Intron	152153697	A	0.06/0.12	0.0046	0.39 (0.22-0.70)	0.0015	**0.06**	0.25
rs6920483[d]	*ESR1*	Intron	152131321	A	0.04/0.08	0.0169	0.36 (0.18-0.71)	0.0032	**0.14**	0.45
Extreme Fibrosis Phenotype	*Risk (F4 vs. F0 only)*									
rs4237805[r]	*HSD17B6*	Intergenic	57249600	A	0.35/0.31	0.0006	4.12 (1.82-9.37)	0.0007	**0.10**	0.38

Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval; FPRP, False positive report probability. [ǂ]The Akaike's information criterion was used to determine the genetic model for each SNP. [d], dominant; [r], recessive. [&]*P* value for difference in allele distributions between patients with mild (≤ F3, ≤ A2) vs. advanced (> F3, > A2) hepatic fibrosis or inflammation. Generated by permutation test with 10,000 runs. *Adjusted for age in years, race/ethnicity, presence of overweight/obesity (BMI ≥ 25), chronic alcohol abuse, diabetes, viral load, and HCV genotypes. [$]Bold have a FPRP < 0.2 values at OR 1.5 level suggesting a true association.

Table 3. Cumulative genetic risk analysis of adverse genotypes on advanced hepatic fibrosis or inflammation risk in patients with HCV

No. of adverse genotypes#	Advanced group (%)	Mild group (%)	Logistic regression*		*P* trend
			OR (95% CI)	*P*-value	
Advanced hepatic fibrosis risk (F3/F4-F4 vs. F0-F3)					< 0.0000001
1 ~4	21 (10.94)	92 (33.58)	1		
5	55 (28.65)	82 (29.93)	2.94 (1.64-5.27)	0.0002114	
6~7	51 (26.56)	52 (18.98)	4.30 (2.33-7.91)	0.0000015	
8~9	65 (33.85)	48 (17.51)	5.52 (3.02-10.08)	< 0.0000001	
Advanced hepatic inflammation risk (A2/A3-A3 vs. A0-A2)					< 0.0000001
0	21 (9.81)	43 (17.06)	1		
1	89 (41.59)	145 (57.53)	1.25 (0.7-2.25)	0.449	
2	80 (37.38)	59 (23.41)	2.77 (1.49-5.16)	0.0018	
3~4	24 (11.22)	5 (2.00)	9.83 (3.29-29.2)	0.000007549	

Abbreviations: OR, odds ratio; CI, confidence interval. #The cumulative genetic risk analysis was evaluated by adding up the number of adverse alleles of the significant SNPs identified from the main effects analysis. Adverse alleles were defined as the minor allele of the risk SNPs (fibrosis: rs2186174, rs2398203, rs2154306, rs4237805; inflammation: rs2211623, rs6555406, rs248800), and the common allele of the protective SNPs (fibrosis: rs4559587, rs2801904, rs2096421, rs6909023, rs6920483; inflammation: rs12414884, rs10163138, rs879332). *Adjusted for age in years, race/ethnicity, presence of overweight/obesity (BMI ≥ 25), chronic alcohol abuse, diabetes, viral load, and HCV genotypes.

0.002). Our parallel race-stratified analyses demonstrated that associations in White and African American HCV+ males were generally consistent in direction and magnitude with overall race-adjusted associations with the exception of single SNPs in *AKR1C3* (rs4559587) and *ESR1* (rs6909023) (Supplemental **Table 1**). However, formal statistical tests for interaction were not significant. In our sensitivity analysis restricted to individuals with extremes of fibrosis (F4 vs. F0, only), only a single SNP in *HSD17B6* (rs4237805) was significantly associated with advanced fibrosis risk (OR_{adj} = 4.12, 95% CI 1.82-9.37, p = 0.0007) (**Table 2**).

There were four SNPs in three genes (n = 1 *AKR1C2*, n = 1 *AKR1C3*, and n = 2 *SRD5A1*) that were significantly associated with advanced inflammatory risk (all permuted p-vales ≤ 0.01) (**Table 2**). The strongest signal in multivariate analysis was with *AKR1C3* rs2211623, which was associated with 3-fold excess risk of advanced inflammation (p = 0.0028). Similar to our findings for advanced fibrosis, the identified *AKR1C2* variant was associated with a decreased risk of advanced activity (OR_{adj} for rs123414884 = 0.48, 95% CI 0.29-0.79; p = 0.0039; OR_{adj} = 0.51, 0.30-0.89, 95% CI 0.018; and OR_{adj} = 0.45, 95% CI 0.23-0.87, p = 0.018 for the overall sample, for White HCV+ males only, and African-American HCV+ males, respectively) (Supplemental Table 1). In our sensitivity analyses restricted to individuals with extreme inflammatory activity phenotypes (A3 vs. A0 only), four SNPs in three genes (n = 1 each in *CYP19A1* and *HSD3B2* and n = 2 in *SRD5A1*) were significantly associated with advanced inflammatory activity in our overall multivariate analyses (e.g., OR_{adj} for *SRD5A1* rs248800 = 2.34, 95% CI 1.30-4.23, 95% CI 0.0048) (**Table 2**).

To further evaluate the robustness of these findings, we calculated FPRP value at two levels of prior probabilities (0.05 and 0.01) for the nine SNPs significantly associated with advanced fibrosis and the six SNPs significantly associated with advanced inflammatory activity (**Table 2**). At a prior probability level of 0.05, all but one remained noteworthy (FPRP ≤ 0.2, prior α = 0.05), while at a lower prior probability of 0.01, only 4 SNPs (AKR1C3 rs2186174, AKR1C3 rs2398203, AKR1C3 rs2154306, and AKR1C2 rs2801904) remained noteworthy associated with fibrosis with FPRP ≤ 0.2.

Association between the cumulative effects of the SNPs and advanced hepatic fibrosis or inflammation risk

We assessed for potential dose-response or cumulative effects of the significant main effect SNPs (nine for advanced fibrosis and four for

Table 4. Haplotype analysis for advanced fibrosis risk in males with chronic HCV (N = 466)

		Proportion		OR (95% CI)*	*P*-value	Global haplotype association *p*-value#
AKR1C2^	All	F0-F3	F3/F4-F4			
AA	0.5192	0.4858	0.5667	1	---	0.071
CC	0.4069	0.4397	0.3607	0.72 (0.55-0.94)	0.017	
CA	0.0546	0.0515	0.0586	0.95 (0.52-1.72)	0.86	
AC	0.0194	0.0231	0.014	0.53 (0.18-1.56)	0.25	
AKR1C3^^	All	F0-F3	F3/F4-F4			
AAAG	0.6898	0.7115	0.6602	1	---	0.0084
AGCA	0.2049	0.1733	0.25	1.65 (1.17-2.33)	0.0047	
rare&	0.1053	0.1152	0.0898	0.84 (0.54-1.32)	0.45	
ESR1^^^	All	F0-F3	F3/F4-F4			
GA	0.8946	0.8682	0.9322	1	---	0.0085
AA	0.0596	0.074	0.0389	0.53 (0.29-0.95)	0.034	
rare&	0.0459	0.0578	0.0289	0.47 (0.23-0.96)	0.038	

Note-The single gene (SRD5A1) with more than 1 SNP associated with advanced fibrosis had SNPs in weak LD, and thus can't perform haplotype analysis for inflammation risk. Abbreviations: OR, odds ratio; CI, confidence interval; N.A, not available. ^Loci chosen for fibrosis risk and *AKR1C2* haplotype analysis: rs2801904 rs2096421; ^^Loci chosen for fibrosis risk and AKR1C3: rs2154306 rs4559587 rs2186174 rs2398203; ^^^Loci chosen for fibrosis risk and ESR1: rs6909023 rs6920483. *Adjusted for age in years, race/ethnicity, presence of overweight/obesity (BMI ≥ 25), chronic alcohol abuse, diabetes, viral load, and HCV genotype. #Generated by permutation test with 10,000 times. &Haplotypes with a frequency less than 0.03 were pooled into one mixed group.

advanced inflammation risk) in a joint effects analysis. We treated the minor allele of the risk-effect SNPs and the common allele of the protective-effect SNPs as adverse alleles. As shown in **Table 3**, a strong gene-dosage effect on risk for advanced fibrosis and advanced inflammation was observed when the SNPs were analyzed in combination in multivariate analyses (*p*-value for trend < 0.0000001 for both advanced fibrosis and advanced inflammation risk).

Association between haplotypes and risk of advanced hepatic fibrosis and advanced hepatic inflammation risk

Three genes (*AKR1C2*, *AKR1C3*, and *ESR1*) were significantly associated with advanced fibrosis risk in our main analyses, with each having more than 1 SNP in strong LD (D' > 0.7, see Supplemental Table 2). **Table 4** summarizes the haplotype frequencies and associations with mild and advanced fibrosis. Our haplotype evaluation identified associations between the respective individual haplotype profiles and advanced fibrosis risk for *ESR1* and *AKR1C3* that closely approached our significance criterion of $p < 0.005$ in multivariate analysis (global *p*-values = 0.0084 and 0.0085, respectively). Haplotype assessment was not possible for advanced inflammation risk because the only gene with more than 1 SNP significantly associated with advanced inflammation risk in our primary analysis (*SRD5A1*) had SNPs which were not in LD.

Gene-gene and gene-environment interactions

The clinical variables (race/ethnicity, age, chronic alcohol abuse, diabetes, overweight/obesity, viral load, and HCV genotype) and the noteworthy SNPs from our main effects analysis (nine for fibrosis and four for inflammation, respectively) were included in MDR analysis to assess for interaction. **Table 5** summarizes the best joint risk factor interaction models obtained for advanced fibrosis and for advanced inflammation risk, respectively. Firstly, in models interrogating advanced fibrosis risk, both the best one-locus model and the best overall model among the six best candidate joint effect or interaction models model for predicting advanced fibrosis risk was the model with *AKR1C3* rs2186174 (testing accuracy = 0.60, CVC = 100, $p = 0.0002$); while the next most predictive model, a two-locus model combining obesity and *AKR1C2* rs2096421, was nearly as predictive (testing accuracy = 0.58, CVC = 90, *p*

Table 5. Summary of Multifactor Dimensionality Reduction (MDR) results for interactions analysis on advanced hepatic fibrosis and inflammation risk

#Factors	MDR models	Testing Aucracy	CVC	*P*	OR	*footnote*
Advanced fibrosis risk (F3/F4-F4 vs. F0-F3)						
1	**rs2186174**	**0.60**	**100/100**	**0.0002**	**2.04 (1.40-2.97)**	***BEST***
2	obesity, rs2096421	0.58	90/100	< 0.0001	2.43 (1.64-3.59)	*BETTER*
3	diabetes, obesity, rs2096421	0.55	83/100	< 0.0001	2.91 (1.94-4.36)	*BETTER*
4	age, rs2096421, rs2186174, rs6909023	0.54	78/100	< 0.0001	3.36 (2.23-5.05)	*GOOD*
5	age, rs2801904, rs2096421, rs2186174, rs6909023	0.50	63/100	< 0.0001	4.13 (2.72-6.26)	*X*
6	diabetes, overweight/obesity, chronic alcohol abuse, rs2096421, rs4237805, rs2154306	0.56	65/100	< 0.0001	4.96 (3.31-7.44)	*GOOD*
Advanced inflammation risk (A2/A3-A3 vs. A0-A2)						
1	obesity	0.46	51/100	0.0054	1.81 (1.18-2.76)	*X*
2	obesity, rs12414884	0.60	100/100	0.0001	2.10 (1.43-3.08)	*BETTER*
3	diabetes, rs6555406, rs248800	0.50	65/100	< 0.0001	2.40 (1.64-3.50)	*X*
4	**diabetes, rs12414884, rs6555406, rs248800**	**0.63**	**100/100**	**< 0.0001**	**2.84 (1.94-4.16)**	***BEST***
5	diabetes, chronic alcohol abuse, rs12414884, rs6555406, rs248800	0.58	73/100	< 0.0001	3.58 (2.39-5.35)	*GOOD*
6	race, diabetes, overweight/obesity, chronic alcohol abuse, rs12414884, rs6555406	0.49	57/100	< 0.0001	3.77 (2.55-5.57)	*X*

#*P*-value based on 10,000 permutations. ^Cross-validation Consistency. Note-Best model with highest CVC and accuracy in bold.

< 0.0001) Secondly, in models for advanced inflammation risk, the best single locus model was with overweight/obesity (CVC = 51, testing accuracy = 0.46, p = 0.005); while the joint effect 4-locus model (i.e., diabetes, *AKR1C2* rs12414884, *SRD5A1* rs6555406, and *SRD5A1* rs248800) was the best overall with a perfect CVC and test accuracy of 0.63 (p < 0.0001).

Discussion

This is the first study to evaluate multiple genetic variants in androgen and estrogen synthesis and receptor pathways and risk of advanced hepatic fibrosis and inflammation in chronically HCV-infected males. We identified nine individual SNPs (rs2186174, rs2398203, 4s2154306, and rs4559587 in *AKR1C3*, rs2801904 and rs4s2096421 in *AKR1C2*, rs6909023 and rs6920483 in *ESR1*, and rs4237805 in *HSD17B6*) as significant predictors (p < 0.005) of advanced FibroSURE-determined fibrosis risk (F3/F4-F4 vs. F0-F3) in HCV+ males after accounting for multiple other factors that influence risk of advanced liver disease including age and obesity. *AKR1C3* had the most SNPs associated with advanced fibrosis risk; three of four SNPs were associated with significant 2-fold excess risk in race-adjusted analyses (all p-values < 0.005), with excess risk also observed in race-stratified analyses (all p-values ≤ 0.006 in African-American and ≤ 0.05 in White males, respectively). The consistency of allelic, genotypic, and haplotypic associations with *AKRC1C3* are supportive of a potential role for this gene in etiopathogenesis of advanced fibrosis in HCV+ males.

AKR1C3 (aldo-keto reductase family 1 member C3) is a member of the large aldo/keto reductase superfamily of enzymes acting upon diverse substrates including hormones, prostaglandins, and xenobiotics like polycyclic aromatic hydrocarbons. It is expressed in a broad range of tissue types including liver. AKR1C3 reciprocally catalyzes conversion of active androgens, estrogens and progestins to their respective non-active metabolites; and preferentially transforms the androgen precursor androstenedione to the most ubiquitous androgens, testosterone and dihydrotestosterone, respectively. AKR1C3 has also been recently shown to be a selective coactivator of the androgen receptor (AR) [24] and is among the most highly upregulated genes in castration-resistant prostate cancer, [25] and has been

identified as a therapeutic target to modulate androgen synthesis in prostate cancer [26]. Germline genetic polymorphisms (SNPs) in *AKR1C3* have been associated with cancer risk including in the prostate [27] and bladder [28].

We also found several SNPs in fellow aldo/keto reductase superfamily member, *AKR1C2*, which is most highly expressed in liver, were associated with decreased risk of advanced hepatic fibrosis (rs2801904 and rs2096421) and advanced hepatic inflammation (rs-12414884), respectively. AKR1C2 has high affinity binding for bile acids it also acts upon diverse substrates including prostaglandins and xenobiotics, and catalyzes inactivation of 5-alpha-dihydrotestosterone (DHT), the most potent androgen, to 5-alpha-androstane-3-alpha, 17-beta-diol. Expression levels of AKR1C2 and AKR1C3 in human adipose tissue have been shown to be significantly correlated with multiple measures of obesity level [29, 30] including visceral adiposity, with the best two locus model for HCV-related fibrosis risk with *AKR1C2* SNP rs2096421 and presence of increased adiposity (BMI $\geq$ 25). Although the potential functional relevance of the individual *AKR1C3* and *AKR1C2* SNPs that we identified as associated with risk of advanced HCV-related liver fibrosis and inflammation is currently unknown, one could hypothesize that their role in controlling androgen biosynthesis (AKR1C3) or catabolism (AKR1C2) may play a role in the strong observed gender differences in cirrhosis and HCC risk.

We also found evidence suggestive of a potential direct role of estrogen signaling in HCV-related fibrosis risk; 2 SNPs in *ESR1* were associated with greater than 2-fold decreased advanced fibrosis risk in males overall, with similar associations observed in race-stratified analyses (e.g., for rs6909023 OR_{adj} = 0.39 (p = 0.002), OR_{adj} = 0.41 (p = 0.012), OR_{adj} = 0.38 (p = 0.05) for males overall, for African American males, and for White males, respectively). *ESR1* (*ESR-alpha*) codes for the primary cognate estrogen receptor, a nuclear hormone receptor that when activated by its ligand 17B-estradiol or E2, co-regulates the expression of genes involved in cellular proliferation and differentiation in addition to its role in sexual dimorphism in multiple target tissues, including liver. A single large (n = 2,404) case-control study in China found that HBV+ individuals with cirrhosis were significantly more likely to carry two specific *ESR1* haplotypes compared to HBV+ individuals without cirrhosis, including one that was functionally associated with estrogen receptor regulatory variation ('C' allele transition in c. 453-397) [31]. However, the potential functional relevance of the *ESR1* SNPs identified as protective for HCV-related liver fibrosis in the current study is unknown.

We identified another four SNPs (*AKR1C3* rs221623, *AKR1C2* rs12414884, *SRD5A1* rs6555406, *SRD5A1* rs248880) as predictors of advanced inflammation risk (A2/A3-A3 vs. A0-A2). Interestingly, in single locus analysis for advanced inflammation risk, another *AKR1C3* SNP, rs2211623, was the single strongest signal conveying 3-fold excess relative risk (p = 0.003). Although the mechanisms by which SRD5A1 may influence risk of developing advanced HCV-related inflammatory activity are unknown, a recent study in male mice demonstrated that loss of SRD5A1 significantly decreased HCC risk even though accelerating hepatic steatosis [32]. The potential role *SRD5A1* variants may play in development of advanced HCV-related hepatic inflammatory activity is interesting in light of earlier cross-sectional research in HCV-infected male veterans which suggested that use of the 5-alpha-reductase inhibitor finasteride prescribed for treatment of benign prostatic hyperplasia was associated with a modest though non-significant decreased risk of both advanced hepatic inflammation and fibrosis [33]. However, as finasteride is a far more selective inhibitor of the SRD5A type 2 (SRD5A2) isoenzyme, such comparisons must be duly qualified.

Our study has several strengths. It was performed in a diverse and well-phenotyped cohort of HCV+ males with extensive data on multiple potential confounding factors for advanced liver disease including obesity, alcohol abuse, and diabetes. We had a relatively large sample size that provided good statistical power for several findings. We calculated the false positive report probability and also employed a more stringent criterion ($p \leq 0.005$) to assess statistical significance to help protect against Type I error inflation associated with multiple comparisons, and also used the MDR method, which collapses high-dimensional genotype

predictors (i.e., SNPs) into a single dimension, that provides greater power to assess for gene-gene and gene-environment interactions, including non-linear interactions [21]. An additional strength is our use of a comprehensive analytic approach that included single locus analysis, haplotype analysis, assessment for cumulative dosage effects, and MDR analysis. We also performed sensitivity analyses limited to individuals with the most extreme phenotypes (i.e., F0 vs. F4 for fibrosis risk and A0 vs. A3 for inflammation risk). As expected, SNPs in several genes that were associated with either advanced fibrosis or inflammatory activity risk in our overall analysis were even stronger risk factors in our extreme phenotype sensitivity analysis (e.g., rs4237805 in *HSD17B6* or hydroxysteroid (17-beta) dehydrogenase 6, which is most highly expressed in the liver and is important in the reverse conversion of 3 alpha-adiol to the potent androgen DHT, and excess risk advanced fibrosis; and rs248800 in *SRD5A1* or steroid 5-alpha-reductase 1, which codes for an enzyme that catalyzes the conversion of testosterone into DHT, and risk advanced inflammation).

There are several limitations of our study that need to be considered. First, our study was cross-sectional. Although this prohibits drawing a causal inference, this is of less concern in our genetic association study as germline genotype by definition predates adult-acquired HCV-related liver disease phenotype. Second, our primary outcome variables were determined only by FibroSURE testing. Although liver biopsy may still be considered the 'gold standard', it is well-known to be subject to variability both in tissue sampling and in pathologist interpretation, and may be less useful in discriminating intermediate stages of disease [34]. Our use of the FibroSURE test which was uniformly ascertained for all study participants reduces the considerable selection bias that would have otherwise been introduced if we relied on liver biopsy, which is generally obtained only in a small minority of patients. Further, the FibroSURE test has been well-validated with good test performance characteristics in diverse HCV populations [14-17]. Also, given our study included only HCV+ men, it is unknown if these findings will generalize to HCV+ women. Additionally, we had reduced power for performance of race-stratified analyses, though associations were generally consistent in direction and magnitude with overall race-adjusted associations with the exception of 2 SNPs (*AKR1C3* rs4559587 and *ESR1* rs6909023), and our sample did not include HCV+ males of either Asian or Hispanic race/ethnicities. We also did not have a parallel measure of hepatic gene expression nor of circulating sex hormone levels; however, these measures (unlike our genetic variants) are subject to a strong potential for reverse causation bias or confounding (e.g., liver disease itself leads to changed hormonal levels as opposed to being caused by them, with circulating levels alterable by other host factors like obesity and alcohol use).

In summary, our findings suggest that inherited variants in several genes involved in sex hormone and receptor synthesis and metabolism may be associated with risk of advanced hepatic fibrosis and inflammation in males with chronic HCV. The potential functional mechanisms involved are not known at this time; however, as several of these genes are associated with androgen biosynthesis, if our results are replicated in other independent cohorts, it raises the intriguing possibility that these molecules may become targets for pharmacotherapies in addition to being potentially important markers for clinical risk stratification in HCV+ males.

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Abbreviations

HCV, hepatitis C virus; SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval; HCC, hepatocellular carcinoma; BMI, body mass index; MAF, minor allele frequency; HWE, Hardy-Weinberg equilibrium; FPRP, false-positive report probability; LD, linkage disequilibrium; MDR, multifactor dimensionality reduction; CVC, cross-validation consistency.

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Supplemental Table 1. Multivariate logistic regression analysis of the main effect SNPs genotypes on HCV-related liver disease risk in males according to race/ethnicity

Advanced Inflammation Risk (A2/A3-A3 vs. A0-A2)				
	White		African-American	
SNP	OR (95% CI)*	*p*-value	OR (95% CI)*	*p*-value
rs2211623	2.29 (0.76-6.96)	0.14	3.54 (1.35-9.30)	**0.01**
rs12414884	0.51 (0.30-0.89)	**0.02**	0.45 (0.23-0.87)	**0.02**
rs6555406	2.07 (0.97-4.42)	**0.0495**	0.87 (0.40-1.89)	0.73
rs248800	1.82 (1.08-3.08)	**0.02**	2.07 (0.92-4.67)	0.07
Extreme Inflammation Phenotype Risk (A3 vs. A0 only)				
	White		African-American	
rs6555406	2.71 (1.01-7.26)	**0.05**	0.76 (0.18-3.22)	0.71
rs10163138	0.67 (0.04-11.17)	0.78	0.19 (0.06-0.55)	**0.00**
rs879332	1.14 (0.58-2.23)	0.70	na	na
rs248800	2.04 (1.04-4.02)	**0.04**	4.04 (1.13-14.45)	**0.03**
Advanced Fibrosis Risk (F3/F4-F4 vs. F0-F3)				
	White		African-American	
rs2186174	1.73 (1.07-2.80)	**0.03**	2.90 (1.44-5.81)	**0.00**
rs2398203	1.61 (1.00-2.61)	**0.05**	3.15 (1.54-6.46)	**0.00**
rs2154306	1.71 (1.06-2.76)	**0.03**	2.64 (1.31-5.31)	**0.01**
rs4559587	0.23 (0.09-0.58)	**0.00**	1.19 (0.15-9.07)	0.87
rs2801904	0.47 (0.26-0.85)	**0.01**	0.41 (0.16-1.02)	**0.05**
rs2096421	0.47 (0.27-0.85)	**0.01**	0.24 (0.05-1.14)	**0.05**
rs4237805	2.57 (1.41-4.68)	**0.00**	0.87 (0.05-14.44)	0.92
rs6909023	0.38 (0.13-1.06)	**0.05**	0.41 (0.20-0.82)	**0.01**
rs6920483	0.48 (0.09-2.46)	0.38	0.34 (0.16-0.72)	**0.01**
Extreme Fibrosis Phenotype Risk (F4 vs. F0 only)				
	White		African-American	
rs4237805	4.17 (1.80-9.65)	**0.00**	na	na

Abbreviations: OR, odds ratio; CI, confidence interval; na, not applicable. Note: Bold associations *p*-value ≤ 0.05. *Adjusted for age, presence of overweight/obesity (BMI ≥ 25), chronic alcohol abuse, diabetes, viral load, and HCV genotype.

Supplemental Table 2. Linkage disequilibrium analysis and Fibrosis association*

D'/r statistic			
AKR1C2	rs2096421		
rs2801904	0.9216/0.8461		
AKR1C3	rs2154306	rs2186174	rs2398203
rs4559587	0.9959/-0.1334	0.9956/-0.127	0.9948/-0.1143
rs2154306	.	0.9934/0.9463	0.9926/0.8513
rs2186174	.	.	0.9997/0.9001
ESR1	rs6920483		
rs6909023	0.8629/0.7151		

*Note - The single gene (SRD5A1) with more than 1 SNP associated with advanced fibrosis had SNPs in weak LD, and thus can't perform haplotype analysis for inflammation risk.