THE *SOD2* C47T POLYMORPHISM INFLUENCES NAFLD FIBROSIS SEVERITY: EVIDENCE FROM CASE-CONTROL AND INTRA-FAMILIAL ALLELE ASSOCIATION STUDIES

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Conflict of Interest

The authors have no conflicts of interest to declare.

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ABSTRACT

Background & Aims: Non-alcoholic fatty liver disease (NAFLD) is a complex disease trait where genetic variations and environment interact to determine disease progression. The association of *PNPLA3* with advanced disease has been consistently demonstrated but many other modifier genes remain unidentified. In NAFLD, increased fatty acid oxidation produces high levels of reactive oxygen species. Manganese-dependent superoxide dismutase (MnSOD), encoded by the *SOD2* gene, plays an important role in protecting cells from oxidative stress. A common non-synonymous polymorphism in *SOD2* (C47T; rs4880) is associated with decreased MnSOD mitochondrial targeting and activity making it a good candidate modifier of NAFLD severity.

Methods: The relevance of the *SOD2* C47T polymorphism to fibrotic NAFLD was assessed by two complementary approaches: we sought preferential transmission of alleles from parents to affected children in 71 family trios and adopted a case-control approach to compare genotype frequencies in a cohort of 502 European NAFLD patients.

Results: In the family study, 55 families were informative. The T allele was transmitted on 47/76 (62%) possible occasions whereas the C allele was transmitted on only 29/76 (38%) occasions, p=0.038. In the case control study, the presence of advanced fibrosis (stage >1) increased with the number of T alleles, p=0.008 for trend. Multivariate analysis showed susceptibility to advanced fibrotic disease was determined by *SOD2* genotype (OR 1.56 (95%CI 1.09-2.25), p=0.014), *PNPLA3* genotype (p=0.041), type 2 diabetes mellitus (p=0.009) and histological severity of NASH ($p=2.0x10^{-16}$).

Conclusions: Carriage of the SOD2 C47T polymorphism is associated with more advanced fibrosis in NASH.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD), widely considered to be the hepatic manifestation of the metabolic syndrome, encompasses a spectrum of liver disease from simple steatosis (fatty liver) through non-alcoholic steatohepatitis (NASH) to fibrosis and ultimately cirrhosis in the absence of alcohol abuse[1]. The prevalence of NAFLD is rapidly increasing and it is now the most common cause of chronic liver disease in western countries[2]. Despite its high prevalence, only a minority of patients with steatosis progress to develop inflammation and less than a quarter of subjects with NAFLD ever progress beyond steatosis to significant fibrosis and cirrhosis[3, 4].

NAFLD is best considered a complex disease trait where subtle inter-patient variations including host genetic factors and environment interact to produce disease phenotype and determine disease progression[5-7]. Basic science is helping to elucidate the mechanisms perpetuating liver cell injury and fibrosis in NAFLD through in vitro and in vivo studies. Attention is currently focused on the role of increased free fatty acid (FFA) flux on a background of insulin resistance as key drivers of pathogenesis through hepatocellular oxidative stress secondary to reactive oxygen species (ROS) production during β- and ω-FFA oxidation, direct lipotoxicity, cytokine release and endoplasmic reticulum stress. Consequent cellular damage triggers a mixture of immune mediated hepatocellular injury and both necrotic and apoptotic cell death pathways[8-11]. Persistence of these processes culminates in hepatic fibrosis[12].

Whilst the reasons for the apparent variation in individual susceptibility to progressive disease are incompletely understood, family/ethnic studies suggest that genetic factors play a significant role[13, 14]. Accordingly, genes encoding proteins influencing the magnitude of these different forms of cellular stress are obvious candidates as genetic factors contributing to susceptibility to progressive NAFLD[5]. Single nucleotide polymorphisms (SNPs) in several candidate genes involved in inflammation, oxidative stress and fibrogenesis have been associated with the severity of liver damage in NAFLD (reviewed[5]). As yet, only the

association of *PNPLA3* with advanced NASH has been consistently replicated in large studies[5].

The mitochondrial enzyme manganese-dependent superoxide dismutase (MnSOD), encoded by the nuclear *SOD2* gene, plays an important role in protecting cells from superoxide radicals[15]. *SOD2* is subject to a common polymorphism (C47T, rs4880) which results in an amino acid substitution in the signal sequence targeting the enzyme to the mitochondrion (Ala16Val) and may induce a conformational change in the protein tertiary structure [16]. The presence of alanine at position -9 in this sequence (C47 allele) has been demonstrated to be associated with more efficient protein import than valine (T47 allele) by *in vitro* expression studies and is predicted to result in higher enzyme activity[17, 18]. This *SOD2* polymorphism (rs4880) has been investigated as a possible susceptibility factor in NASH and several other diseases where oxidative stress is considered to play a role in pathogenesis including hereditary hemochromatosis[19] and drug induced liver injury[20]. A small study in 63 Japanese patients found a significantly increased prevalence of the lower activity homozygous T genotype among cases[21]. More recently, the homozygous T genotype was reported to be associated with an increased incidence of diabetic nephropathy in large studies of type 1 diabetics from Sweden[22] and Denmark[23] and with susceptibility to pancreatic cancer in a US-based study[24]. *SOD2* has also been investigated in relation to susceptibility to alcoholic liver disease but the results have been inconsistent and inconclusive[25, 26].

To further examine this biologically plausible association between *SOD2* genotype and susceptibility to fibrosing steatohepatitis, we have used two complementary approaches. Firstly, we have carried out a family study studying trios consisting of children with fibrotic NAFLD and their two parents in which we have performed transmission disequilibrium test (TDT) analysis to determine whether there is preferential transmission of a particular parental allele to the affected children[27]. Secondly, we performed a classical case-control allelic association study in unrelated patients with NAFLD of varying severity including 5

times more patients than that reported previously[21].

MATERIALS AND METHODS

Patients

A large patient cohort was recruited across centres in UK and Italy. The study had all the necessary ethical approvals in both the countries and all participants (or their parents) gave informed consent.

The Italian Family Study, collected DNA from 71 Italian family 'trios' in Rome (210 individuals) each comprising two living parents and an index child with biopsy-proven fibrotic NAFLD. Other causes of liver disease were excluded, including increased alcohol intake, chronic viral hepatitis, autoimmune hepatitis, hereditary hemochromatosis, α1-antitrypsin deficiency, Wilson's disease and drug induced liver disease. Detailed clinical data concerning these children has been presented previously[28].

For the case-control study, 502 European Caucasian patients with biopsy-proven NAFLD of different stages of disease were enrolled (338 patients from Newcastle upon Tyne, UK and a further 164 patients from Milan, Italy). Baseline characteristics of the cohort are shown in Table 1. These were unrelated patients with NAFLD, derived from a patient population originally identified as having ultrasographically detected bright liver and abnormal biochemical tests (ALT and GGT) between January 1999 and January 2007[29]. Alternate diagnoses were excluded, including increased alcohol intake (males and females consuming greater than 21/14 units of alcohol per week [>30/20g/day ethanol] respectively were excluded), as were any individuals with chronic viral hepatitis (hepatitis B and hepatitis C), autoimmune liver diseases, hereditary hemochromatosis, α1-antitrypsin deficiency, Wilson's disease and drug induced liver disease.

Clinical and laboratory data were collected on the date a diagnostic liver biopsy was performed. Body mass index (BMI) was calculated using the formula: weight (kilograms)/height (m^2). The presence of diabetes mellitus (fasting glucose ≥7.1 mmol/L mg/dl or treatment with anti-diabetic drugs) and hypertension (blood pressure ≥130/85 or on

treatment for previously diagnosed hypertension) was recorded. Laboratory evaluation included routine liver biochemistry (alanine and aspartate aminotransferase, total bilirubin, albumin, alkaline phosphatase and gamma glutamyl transpeptidase); complete blood count; total- and HDL-cholesterol and total triglycerides; fasting glucose; fasting insulin; viral serology for hepatitis B and C infection and autoantibodies.

Liver biopsy

Ultrasound guided liver biopsy was performed in all patients. Specimens (at least 1.6cm length and 5μm thick) were fixed in formalin for evaluation. Tissue sections were stained with hematoxylin and eosin, impregnated with silver for visualizing reticulin framework and stained with trichrome for visualizing collagen. Liver biopsies were reviewed by a single expert liver pathologist at each participating centre, unaware of clinical or genetic data. The severity of steatosis, necroinflammatory grade and stage of fibrosis were scored according to modified Brunt criteria[30]. For fibrosis, stage 0 = no fibrosis; stage 1 = isolated perisinusoidal or portal fibrosis; stage 2 = perisinusoidal and portal/periportal fibrosis; stage 3 = septal or bridging fibrosis; and stage 4 = cirrhosis. As the main aim of this study was to evaluate the possible role of the polymorphism of *SOD2* in predicting patients with progressive NAFLD, *a priori* we considered the presence of stage 2 fibrosis in adults to be indicative of a more progressive disease phenotype. Given the younger age and therefore limited duration of disease exposure of paediatric patients, the presence of fibrosis stage 1 or greater was considered indicative of a progressive disease phenotype in the family study.

DNA Preparation

Venous blood was collected at the time of liver biopsy and DNA was prepared from peripheral blood lymphocytes as described previously[31]. Genotyping was performed by personnel unaware of clinical status or histology of patients.

SOD2 rs4880 Genotyping

SOD2 genotype was determined by PCR-RFLP analysis using a minor modification of our previously described method.[26] The final volume (20µl) of the PCR reaction contained 0.5-1 µg genomic DNA, 0.625 units Taq DNA polymerase, 1x Taq DNA polymerase reaction buffer (50mM potassium chloride, 10mM Tris-HCL pH 9.0, 0.1% (v/v) Triton X-100, 1.5mM $MgCl_2$), 0.1mM dNTPs and 0.25µM both forward and reverse primers (5'-CAGCCCAGCCTGCGTAGACGG-3' and 5'-GCGCGTTGATGTGAGGTTCCAG-3'). Amplification (35 cycles of denaturation at 95°C for 1 minute, annealing at 63°C for 1 minute and extension at 72°C for 1 minute) was then performed in a GeneAmp PCR system 9700 thermal cycler. After successful amplification, PCR products (6µl) were diluted with the appropriate restriction enzyme buffer to a final volume of 20µl and 5 units *Bsa*WI added. The digest was then incubated at 60°C for 3 hours. The presence of T at the polymorphic site results in creation of a restriction site for *Bsa*WI. Digests were analyzed by polyacrylamide gel electrophoresis on 10% polyacrylamide gels in 1xTBE buffer and visualised by staining with ethidium bromide.

Statistical Analysis

Statistical analyses were performed using various packages including 'genetics', 'combinat' and 'dgc.genetics' running in the R software environment (R version 2.7.1)[32]. Transmission Disequilibrium Tests (TDT) were performed to determine preferential transmission of alleles and statistical significance in the family study. This approach is not subject to many of the potential confounding effects inherent in case control studies and is significantly more powerful at detecting true associations[27]. In the cohort study, we evaluated by univariate and multivariate analysis the capability of *SOD2* to predict progressive NAFLD (fibrosis 2-4). Parametric and non-parametric data were presented as means ± standard deviation and percentage when appropriate. The statistical analysis was performed using ANOVA, student t test, Pearson chi-square test and chi-square test for

trend when appropriate. Significance was taken as $p<0.05$. Hardy-Weinberg equilibrium was determined for each study population using the web-based calculator available at www.tufts.edu/ which confirmed that UK and Italian study populations were in equilibrium. The multivariate analysis was performed by logistic regression analysis to evaluate the factors associated with progressive NAFLD (fibrosis stage ≥2). The results of the multivariate analysis are expressed as odds ratio (OR) with 95% confidence intervals (CI).

RESULTS

The SOD2 C47T Allele is Preferentially Transmitted to Children with Fibrosing Steatohepatitis

In the Italian family study, 61 of the 71 children had fibrosing steatohepatitis (59 patients exhibited stage 1 fibrosis and 2 patients had stage 2 disease). Transmission disequilibrium testing (TDT)[27] was used to seek preferential transmission of either SOD2 allele to affected children in the family study. For *SOD2* rs4880, 55 of the 71 families were informative in that one or both parents were heterozygous for this SNP. In these families, the T allele was transmitted on 47/76 (62%) possible occasions whereas the C allele was transmitted on only 29/76 (38%) occasions, p=0.038.

Carriage of the C47T SOD2 Genotype is Associated with Advanced Fibrosis

To determine whether carriage of the *SOD2* rs4880 SNP influenced susceptibility to fibrosing steatohepatitis in an adult population, we examined whether it was associated with histological disease progression in a large cohort of NAFLD patients. The total study population of 502 patients with biopsy-proven NAFLD was genotyped for *SOD2* rs4880. Clinical details are reported in Table 1. Broadly, the UK study population exhibited a more severe metabolic syndrome and NASH phenotype than the Italian population. *SOD2* genotypes were in Hardy-Weinberg equilibrium. Similar SOD2 allele frequencies were observed at both the UK (C47: 49.4%) and Italian (C47: 48.4%) centres which were consistent with those observed in a cohort of North-Western European descent by the International HapMap project (www.ncbi.nlm.nih.gov/projects/SNP; rs4880).

Table 2 summarizes the relationship between *SOD2* genotype and a number of patient-specific and clinical parameters relevant to the disease. Both age and a fibrosis score >1 were significantly associated with *SOD2* genotype. In particular, a gene-dosage effect was observed with the incidence of advanced fibrosis (stage >1) increasing with the number of T (Val) alleles (p=0.008, X^2 for trend) (Figure 1). 72.2% of CC individuals had a fibrosis score

of 0 or 1 compared with 56.2% of homozygous T patients. Homozygosity for the T allele vs. C was associated with an odds ratio of 2.02 (95% CI 1.19-3.45; p=0.008) for the development of fibrosis of grade 2 or above. There was no significant difference for steatosis score or other clinical parameters, including the presence of diabetes, between *SOD2* genotype groups.

Multivariate analysis and relevance of PNPLA3 Genotype

Given the previous reports demonstrating a role for *PNPLA3* I148M [33, 34] as a modifier of disease progression in NAFLD, the cohort was also genotyped for this polymorphism. A multivariate logistic analysis was performed to control for the effect of *PNPLA3* together with other factors relevant to disease severity. In line with our previous findings (34), a dominant effect for the *PNPLA3* variant allele was assumed. Recruitment centre (UK vs. Italy) was included within the analysis to ensure that variations in environmental influences were controlled. In multivariate analysis, degree of steatosis was found to be significantly associated with BMI and *SOD2* genotype (odds ratio 1.35 (95% CI 1.01-1.80), p=0.039) as well as, in agreement with our previous findings, *PNPLA3* genotype (Table 3-A). Steatohepatitis was independently associated with a greater degree of steatosis, age, BMI and the presence of diabetes (Table 3-B). Consistent with its metabolic function and supporting the view that *SOD2* genotype exerts its modifier effect through direct modulation of fibrogenic response to oxidative stress rather than initiation of steatohepatitic injury, carriage of neither *SOD2* allele predicted severity of steatohepatitis (Table 3-B). The analysis confirmed that carriage of the *SOD2* rs4880 polymorphism was an independent risk factor for advanced fibrosis (OR 1.56 (95%CI 1.09-2.25), p=0.014) (Table 3-C). The effect of *SOD2* on fibrosis was comparable to that of *PNPLA3* (rs738409; I148M) (OR 1.69, p=0.041). Histological steatohepatitis grade also remained a strong predictor of fibrosis stage (OR 21.9 (95%CI 1.16-44.3), $p=2.0x10^{-16}$).

DISCUSSION

Using both case-control and intra-familial association methodologies, we have shown a consistent association between a functional SNP in the mitochondrial targeting sequence of *SOD2* and fibrosis severity in NAFLD. These results provide persuasive genetic evidence that mitochondria-derived oxidative stress is important in the pathogenesis of advanced NAFLD. In particular, use of TDT analysis in a young NAFLD population is a novel feature. In addition, we have also demonstrated a strong association with *SOD2* genotype in a European case-control study population ($p=0.008$, X^2 for trend). The central role of MnSOD in cellular protection against oxidative stress has been well demonstrated by the finding that deficiency of this enzyme is lethal in mice[35]. Homozygous knockout mice typically die within the first 10 days post-natally, with impairment of function of several organs, particularly the heart[36]. Hepatic steatosis is also observed in this model, reflecting the interaction between ROS production and exacerbation of hepatic insulin resistance and lipid accumulation. An effect that was also observed in the current study where increased steatosis with *SOD2* genotype was found ($p=0.039$), possibly reflecting oxidative stress mediated Apolipoprotein B degradation and impaired VLDL excretion[37]. Heterozygous animals have a normal lifespan but evidence for increased hydroperoxide accumulation in hepatocyte mitochondria followed by apoptosis has been reported[36]. More recently, conditional knockouts with deficiency of MnSOD only in hepatocytes have been generated. These animals show a number of signs of liver failure as well as loss of metabolic zonation[38].

The presence of alanine at position -9 in the *SOD2* sequence (C47 allele) has been demonstrated to be associated with more efficient protein import than valine (T47 allele) by *in vitro* expression studies and is predicted to result in greater *SOD2* enzyme activity[17, 18]. Expression of the *SOD2* gene is inducible by a variety of modulators ranging from cytokines to UV irradiation[15] but it is likely that even when induced, the difference between the two allelic variants will be maintained. Our association of T allele carriage with greater NAFLD

severity is consistent with the previous small study in a Japanese population[21] and also with studies on other diseases where oxidative stress is likely to be relevant to disease pathogenesis including diabetic nephropathy[22], pancreatic adenocarcinoma[24], exocrine pancreatic insufficiency in chronic alcoholic pancreatitis[39] and diabetic retinopathy[40]. *SOD2* is a relatively small gene of 14 kb and consists of a single haplotype block. SNPs other than rs4880 have been described but most of those studied previously, for example rs2855116[41], are in strong linkage disequilibrium with the rs4880 SNP, which is the only common non-synonymous polymorphism described in *SOD2*.

In mammalian liver, fatty acid oxidation occurs in three organelles; β-oxidation takes place in the mitochondria and peroxisomes and cytochrome P4504A mediated ω-oxidation takes place in the microsomes[42, 43]. The synthesis of reactive oxygen species by fatty acid metabolism and the mitochondrial respiratory chain is increased in patients with NAFLD[44]. ROS are able to induce lipid peroxidation of the expanded lipid stores, compromise mitochondrial integrity and promote apoptotic cell death[44]. There is increasing evidence from studies in both humans and rodents that fibrotic NAFLD is associated with increased levels of ROS and mitochondrial abnormalities[45]. Superoxide-generated lipid hydroperoxides degrade to several hydroxy-alkenals including 4-hydroxynonenal. Studies in both humans and animals suggest that 4-HNE induces and/or activates uncoupling protein 2 (*UCP2*)[46]. Though this process results in uncoupling of substrate oxidation from ATP synthesis and should avoid a further increase in mitochondrial hydrogen peroxide production, ATP depletion makes cells more vulnerable to damage if exposed to further insults. The high activity (C47) form of MnSOD is likely to protect mitochondria better from superoxide exposure better than the T47 form, thus avoiding the need to uncouple the electron transfer process.

As with many other complex genetic diseases, susceptibility to fibrotic NAFLD is likely to determined by epistatic interaction of a number of different genes and environmental influences, indeed several genetic modifiers have been reported across a range of

progressive liver diseases[5, 34, 47-53]. In clinical studies it is difficult or impossible to control for all the subtle inter-patient variations, whether genetic or environmental, that interact to determine disease phenotype and progression[7] and so there remains a need for validation in independent patient cohorts, either through further candidate gene studies or large-scale genome-wide association studies. However, *SOD2* represents a good example of a biologically plausible candidate gene with a common, well-established, functionally significant polymorphism. This study clearly demonstrates the predicted association with disease susceptibility for fibrosing steatohepatitis and has several key methodological strengths: (i) it is the largest association study examining the influence of *SOD2* on NASH associated fibrosis; (ii) the cohort studied comprised patients with biopsy proven NASH; and (iii) the association was further demonstrated in a family study where preferential transmission of the low activity (T47) allele was seen in offspring with biopsy proven NASH. Much focus is given to changes in gene expression that increase ROS in NASH pathogenesis whilst the role of modifiers of host defense is too often ignored. The current study highlights the importance of this aspect of disease pathogenesis. Although the results of some therapeutic trials of anti-oxidants in NASH have been inconclusive[54-59], other large studies have been supportive[60]. Taken together with the data presented here, these provide a strong rationale for further investigation of the utility of anti-oxidants to ameliorate fibrosis progression in steatohepatitis.

TABLE 1: COHORT CHARACTERISTICS

	All	UK	Italy	p-Value*
Number	502	338	164	-
Country/Ethnicity	Caucasian	UK Caucasian	Italy Caucasian	-
Sex (Male)	339 (67.5%)	210 (62.1%)	129 (78.6%)	
***SOD2* rs4880 C-Allele Frequency**	0.491	0.494	0.484	
Age, years	49.1 ± 12.3	49.7 ± 12.7	47.9 ± 11.4	0.1
BMI, kg/m^2	31.8 ± 5.7	34.05 ± 5.3	27.4 ± 3.8	$4.5x10^{-45}$
DM	166 (33.5%)	127 (38.3%)	39 (23.7%)	0.001
HOMA-IR	5.5 ± 5.3	6.3 ± 6.2	4.3 ± 3.5	0.0003
ALT, IU/L	75.1 ± 59.2	80.6 ± 64.05	63.7±46.1	0.001
Total Cholesterol	5.44 ± 1.5	5.6 ± 1.3	5.09 ± 1.7	0.0007
TG	2.42 ± 1.8	2.81 ± 2.0	1.64 ± 1.0	$2x10^{-16}$
Steatosis Score				
1	198 (40%)	96 (29%)	102 (62%)	<0.0001
2	178 (36%)	145 (44%)	33 (20%)	<0.0001
3	115 (24%)	86 (27%)	29 (18%)	0.03
NASH (yes)	263 (53%)	184 (55%)	79 (48%)	0.18
Fibrosis Score				
0	215 (43%)	129 (38%)	86 (52.4%)	0.002
1	108 (22%)	62 (18%)	46 (28.1%)	0.01
2	76 (15%)	56 (17%)	20 (12.1%)	0.23
3	62 (12%)	55 (16%)	7 (4.3%)	0.00008
4	41 (8%)	36 (11%)	5 (3.1%)	0.004

* Statistical analysis of UK vs. Italian recruitment centers.

TABLE 2: COMPARISON OF SELECTED PATIENT CHARACTERISTICS ACCORDING TO *SOD2* GENOTYPE

Phenotype		Combined Cohort			
		CC (n=119)	**CT (n=255)**	**TT (n=128)**	**p-Value**
Age	Yrs	49.6±12.6	47.9±12.1	51.3±12.1	0.036
BMI	kg/m^2	31.9±5.9	32.1±6.1	30.9±5.1	0.1
Gender					
Male		76 (0.64)	173 (0.68)	90 (0.70)	0.2
Female		43 (0.36)	82 (0.32)	38 (0.30)	
Diabetes					
Yes		32 (0.27)	87 (0.35)	47 (0.38)	0.07
No		87 (0.73)	164 (0.65)	78 (0.62)	
Fibrosis >1					
Yes		33 (0.19)	90 (0.50)	56 (0.31)	0.008
No		86 (0.27)	165 (0.51)	72 (0.22)	
NASH					
Yes		59 (0.22)	131 (0.5)	73 (0.27)	0.1
No		60 (0.26)	121 (0.52)	51 (0.22)	
Steatosis >1					
Yes		66 (0.23)	146 (0.5)	81 (0.27)	0.11
No		52 (0.26)	104 (0.53)	42 (0.21)	
Hypertension					
Yes		58 (0.23)	118 (0.47)	74 (0.3)	0.1
No		53 (0.24)	124 (0.56)	44 (0.2)	
ALT	IU/L	70.4±49.7	77.1±62.2	75.5±61.4	0.6
Glucose	mmol/L	35.4±41.7	37.8±49.2	40 ± 47	0.7
Insulin		21±18.8	20.3±17.8	19.2±14.6	0.8
HOMA-IR		5.6±6.2	5.6±5.2	5.1±4.5	0.8
Total Chol	mmol/L	5.3±1.4	5.5±1.4	5.2±1.6	0.08
TG	mmol/L	2.3±1.5	2.5±2.1	2.2±1.3	0.1

Population Hardy-Weinberg Calculation (UK: X^2 =0.58, p=0.44; Italy: X^2 =0.21, p=0.65; Combined: X^2 =0.13, p=0.72) i.e. in equilibrium.

Advanced Fibrosis with TT genotype vs. CC 2.02 (95% CI 1.19-3.45), p=0.008; CC vs. CT 1.4 (0.88-2.28), p=0.14.

TABLE 3: MULTIVARIATE ANALYSIS BY DISEASE ELEMENT

A. Steatosis 0+1 vs 2+3

Variables	**OR (95% CI)**	**p-value**
SOD2	**1.35(1.01-1.80)**	**0.039**
PNPLA3	**1.64(1.09-2.46)**	**0.016**
Age	**0.98(0.96-0.99)**	**0.040**
Sex	0.74(0.46-1.19)	0.222
BMI	**1.06(1.02-1.11)**	**0.003**
Diabetes	**1.46(0.94-2.27)**	**0.09**
Recruitment Centre	**0.33(0.20-0.54)**	**$1.2x10^{-5}$**

B. NASH 0 vs 1

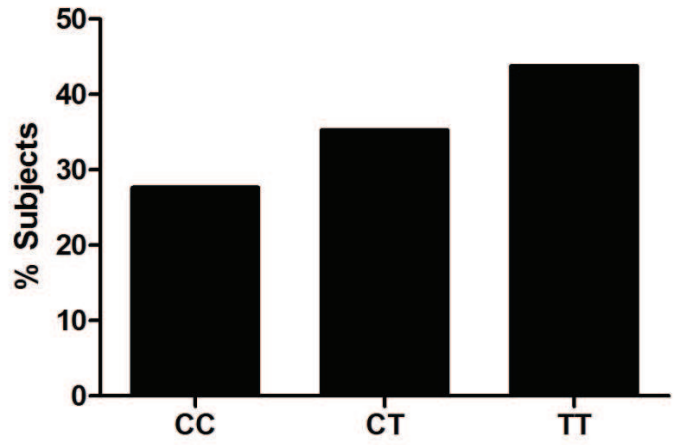
Variables	**OR (95% CI)**	**p-value**
SOD2	1.09(0.81-1.48)	0.538
PNPLA3	1.44(0.94-2.21)	0.091
Age	**1.02(1.00-1.04)**	**0.024**
Sex	0.74(0.44-1.22)	0.244
BMI	**1.06(1.01-1.11)**	**0.008**
Diabetes	**2.97(1.86-4.78)**	**$5.3\ x10^{-6}$**
Steatosis	**3.91(2.86-5.45)**	**$2x10^{-16}$**
Recruitment Centre	**2.21(1.27-3.91)**	**0.005**

C. Fibrosis 0+1 vs 2+3+4

Variables	**OR (95% CI)**	**p-value**
SOD2	**1.56(1.09-2.25)**	**0.014**
PNPLA3	**1.69(1.02-2.84)**	**0.041**
Age	1.02(0.99-1.04)	0.056
Sex	1.41(0.81-2.5)	0.231
BMI	1.03(0.97-1.08)	0.261
Diabetes	**1.94(1.17-3.21)**	**0.009**
Steatosis	0.81(0.56-1.16)	0.273
NASH	**21.9(1.16-44.3)**	**$2x10^{-16}$**
Recruitment Centre	**0.33(0.17-0.62)**	**0.0006**

Calculations assume a co-dominant model for *SOD2* and a dominant model for *PNPLA3* variants

FIGURE 1: GENE DOSAGE EFFFECT OF SOD2 C47T SNP ON NASH ASSOCIATED FIBROSIS



Prevalence of advanced fibrosis in 502 patients with biopsy proven NASH subdivided according to SOD2 C47T genotype. Chi-squared for trend p=0.008. CC vs CT+TT: OR 1.6 (1.02-2.5) p=0.038.

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