HEPATITIS

Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1

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Background: Interferon α (IFN-α) activated cellular signalling is negatively regulated by inhibitory factors, including the suppressor of cytokine signalling (SOCS) family. The effects of host factors such as obesity on hepatic expression of these inhibitory factors in subjects with chronic hepatitis C virus (HCV) are unknown.
Objectives: To assess the independent effects of obesity, insulin resistance, and steatosis on response to IFN-α therapy and to determine hepatic expression of factors inhibiting IFN-α signalling in obese and non-obese subjects with chronic HCV.
Methods: A total of 145 subjects were analysed to determine host factors associated with non-response to antiviral therapy. Treatment comprised IFN-α or peginterferon alpha, either alone or in combination with ribavirin. In a separate cohort of 73 patients, real time-polymerase chain reaction was performed to analyse hepatic mRNA expression. Immunohistochemistry for SOCS-3 was performed on liver biopsy samples from 38 patients with viral genotype 1 who had received antiviral treatment.
Results: Non-response (NR) to treatment occurred in 55% of patients with HCV genotypes 1 or 4 and 22% with genotypes 2 or 3. Factors independently associated with NR were viral genotype 1/4 ($p<0.001$), cirrhosis on pretreatment biopsy ($p=0.025$), and body mass index $\geq$ 30 kg/m^2 ($p=0.010$). Obese subjects with viral genotype 1 had increased hepatic mRNA expression of phosphoenolpyruvate carboxy kinase ($p=0.01$) and SOCS-3 ($p=0.047$), in comparison with lean subjects. Following multivariate analysis, SOCS-3 mRNA expression remained independently associated with obesity ($p=0.023$). SOCS-3 immunoreactivity was significantly increased in obesity ($p=0.013$) and in non-responders compared with responders ($p=0.014$).
Conclusions: In patients with chronic HCV viral genotype 1, increased expression of factors that inhibit interferon signalling may be one mechanism by which obesity reduces the biological response to IFN-α.

Despite an improvement in the efficacy of antiviral treatment in recent years, approximately 50% of patients infected with hepatitis C virus (HCV) genotype 1 and 20% of those with HCV genotype 3 fail to achieve sustained viral clearance.[1] Along with viral genotype and load,[2] various host genetic and biological factors have a role in the resistance to interferon alpha (IFN-α) therapy. These host factors include sex, age, ethnicity, and genetic variation in human leucocyte antigens and cytokine production (reviewed by Gao and colleagues[3]). In addition, patients with advanced fibrosis have a decreased response to antiviral treatment.[4]

In order to increase the number of patients achieving a sustained virological response (SVR), there is a need to identify modifiable risk factors that impact on treatment efficacy. As a result, there is increasing interest in the role of obesity and hepatic steatosis in this setting. Steatosis may adversely affect the response to antiviral therapy.[5 6] In patients with non-genotype 3 infection, the presence of steatosis was a predictor of failed treatment[7 8] and those with less steatosis were more likely to achieve SVR.[6] However, it is unlikely that steatosis intrinsically impairs antiviral efficacy as steatosis associated with viral genotype 3 does not appear to adversely affect the response to treatment.[6] Other investigators found that an elevated body mass index (BMI) in the obese range of >30 kg/m^2, rather than steatosis per se, was associated with therapeutic non-response.[9]

Obesity and steatosis are clearly interrelated and it remains unclear whether their reported effects are truly independent.[10] A number of studies have found that patients with a higher body weight have reduced response rates following antiviral therapy.[11–14] In the absence of weight based dosing, treatment failure in obese patients may be due to inadequate drug doses leading to lower serum levels of IFN-α.[15] Other mechanisms by which obesity may impair antiviral response such as by reducing the biological response to IFN-α are not well understood. Interaction of IFN-α with a cell surface receptor leads to a series of intracellular reactions that result in transcriptional induction of several antiviral and immunoregulatory genes.[3] This IFN-α-activated signalling is negatively regulated by a number of inhibitory factors, including the suppressor of cytokine signalling (SOCS)

Abbreviations: SOCS, suppressor of cytokine signalling; HCV, hepatitis C virus; IFN-α, interferon α; IFN-R1, interferon receptor 1; PEPCK, phosphoenolpyruvate carboxy kinase; NR, non-response to antiviral treatment; RES, response to antiviral treatment; SVR, sustained virological response; BMI, body mass index; RT-PCR, real time-polymerase chain reaction; TNF-α, tumour necrosis factor α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HOMA, homeostasis model of assessment; STAT, signal transducer and activator of transcription

family.[3 16–18] Several studies have found that patients with high tumour necrosis factor α (TNF-α) levels have a poor response to IFN-α therapy[19–21] and this may occur via induction of SOCS proteins[22] that interfere with the interaction between the IFN-α receptor and signalling proteins.[16] Obesity and steatosis are known to be associated with elevated levels of TNF-α[23 24] and more recent studies have shown increased hepatic expression of SOCS proteins in insulin resistant states.[25 26]

The aims of this study were to evaluate the independent effects of obesity, hepatic steatosis, and obesity related metabolic factors on the response to antiviral therapy in patients with chronic HCV infection. In addition, we examined hepatic expression of the IFN-α receptor 1 (IFN-R1) and factors that may inhibit IFN-α signalling (TNF-α, SOCS-3), and phosphoenolpyruvate carboxy kinase (PEPCK) as a marker of hepatic insulin sensitivity in obese and non-obese subjects with chronic HCV.

MATERIALS AND METHODS

Study populations

This study was a retrospective review of patients with chronic HCV seen at a single centre between 1995 and 2004. Subjects were included in the current study if they fulfilled the following criteria: (a) chronic HCV with circulating HCV RNA (detected by Amplicor HCV Monitor assay; Roche, New Jersey, USA) and abnormal serum aminotransferase levels for at least six months; (b) liver biopsy consistent with chronic hepatitis; (c) compensated liver disease; and (d) written informed consent for inclusion in the study. Patients with other forms of chronic liver disease or antibodies to human immunodeficiency virus were not considered for the analysis. The study protocol was approved by both the Princess Alexandra Hospital Research Ethics Committee and the University of Queensland Research Ethics Committee.

A total of 145 subjects received antiviral therapy. Treatment comprised IFN-α or peginterferon alpha (PEG-IFN-α) either alone or in combination with ribavirin. As the purpose of the study was to determine the effect of host factors on treatment outcome, only subjects who completed 80% of the intended doses of antiviral therapy were evaluated. In an additional group of 73 untreated patients with chronic HCV, liver tissue (2–3 mm) was immediately frozen in liquid nitrogen at the time of biopsy and stored at −80℃ until extraction of RNA was performed.

Details on weight, height, and average alcohol intake (g/day) were obtained from all patients at the time of treatment and/or liver biopsy. Information regarding average alcohol intake (g/day) prior to the last six months was also obtained. Subjects receiving antiviral therapy were required to consume <70 g ethanol per week for ⩾ 6 months prior to treatment and were abstinent during treatment.

Sex, ethnicity, and age at treatment were also recorded. On the basis of BMI and ethnicity, subjects were classified as lean (Caucasian <25 kg/m^2, Asian <22.5 kg/m^2), overweight (Caucasian 25–29.9 kg/m^2, Asian 23–24.9 kg/m^2), or obese (Caucasian ⩾30 kg/m^2, Asian ⩾25 kg/m^2).

Histopathological examination

Liver biopsy sections were analysed by an experienced hepatopathologist (AC) who was blinded to the laboratory parameters and clinical data. The degree of inflammation was graded according to the method of Ishak[27] and fibrosis was staged according to the method of Scheuer.[28] Steatosis was graded as follows: 0 (<5% hepatocytes affected); 1 (mild, 5–29% of hepatocytes affected); 2 (moderate, 30–70% of hepatocytes affected); or 3 (severe, >70% of hepatocytes affected).

Laboratory data

Viral genotyping was performed using the Inno-Lipa HCV II assay (Innogenetics, Zwijnaarde, Belgium). Circulating HCV RNA was detected by polymerase chain reaction (PCR) using the Amplicor HCV assay (Roche, New Jersey, USA). SVR was defined as undetectable HCV RNA at the end of 24 weeks of follow up after completion of treatment. Patients were responders (RES) if they had undetectable HCV RNA on completion of antiviral therapy. Patients who had detectable HCV RNA on treatment completion were defined as non-responders (NR). Serum was collected at the time of liver biopsy following an overnight fast for eight hours. Standard biochemical tests were performed using a Hitachi 747-100 Analyser (Roche, Australia). Circulating insulin and C peptide levels were determined using the Tosoh AIA600 analyser, two site immunoenzymometric assays (Tosoh Medics, San Francisco, California, USA). Insulin resistance was determined using the homeostasis model of assessment (HOMA).[29]

Real time-polymerase chain reaction (RT-PCR) methods

Steady state mRNA levels of PEPCK, TNF-α, SOCS-3, and IFN-R1 were assessed by semi quantitative RT-PCR assays, using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene. Total RNA was extracted from liver biopsy tissue (n = 73) according to the Trizol method (Invitrogen, Melbourne, Victoria, Australia). cDNA was prepared by reverse transcription, as previously described.[30]

The probe and primer sequences for GAPDH and TNF-α were as described previously.[30] Primer sequences for IFN-R1, SOCS-3, and PEPCK were designed using online software Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3www.cgi) (table 1) and were purchased from Proligo Australia Pty Ltd (Lismore, New South Wales, Australia). PEPCK and TNF-α mRNA levels were determined by RT-PCR using a TAMRA-FAM probe, as previously described.[30] mRNA expression of SOCS-3 and IFN-R1 was determined using SYBR green chemistry. Diluted (1/20) cDNA (5 μl) was added to a PCR mix containing 6.70 μl sterile water, 12.5 μl 2× SYBR mix (Qiagen, Clifton Hill, Victoria, Australia), and 0.4 μl each of forward and reverse primers to make up a final volume of 25 μl. Cycling conditions for amplification were 95℃ for 15 minutes, followed by 40 cycles of 94℃ for 15 seconds, 60℃ for 30 seconds, and 72° for 30 seconds in a Rotorgene 3000 (Corbett Robotics, Brisbane, Australia). Each assay was performed in duplicate and analysis was performed using Rotorgene Analysis Software (Corbett Robotics).

Table 1 Primer and probe sequences for real time-polymerase chain reaction

Gene	Forward	Reverse	Probe
SOCS-3	5′-CCCTCGCCACCTACTGAA-3′	5′-TCCGACAGAGATGCTGAAGA-3′	NA
IFN-R1	5′-GTGGAACAGGAGCGATGAGT-3′	5′-CAACCTCATACCATGAAGAAGTG-3′	NA
TNF-α	5′-CCCCAGGGACCTCTCTCTAA-3′	5′-CAGCTTGAGGGTTTGCTACA-3′	5′-AGCCCTCTGGCCCAGGCAGT-3′
PEPCK	5′-AGCTGGCAACATGGAGTCTT-3′	5′-CTTCCGGAACCAGTTGACAT-3′	5′-CCCTTCTTTGGCTACAACTTCGGCA-3′
GAPDH	5′-TGCACCACCAACTGCTTAGC-3	5′-GGCATGGACTGTGGTCATGAG-3′	5′-CCTGGCCAAGGTCATCCATGACAACTT-3′

SOCS, suppressor of cytokine signalling; IFN-R1, interferon receptor 1; TNF-α, tumour necrosis factor α; PEPCK, phosphoenolpyruvate carboxy kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NA, not applicable.

Relative concentrations of mRNAs present were determined as previously described.[30] For each gene, the average of the duplicate assays was obtained and normalised to the average amount of GAPDH for each sample to determine relative changes in mRNA expression.

Immunohistochemistry for SOCS-3

Formalin fixed paraffin embedded liver biopsy samples from 38 of the subjects infected with HCV genotype 1 or 4 who subsequently received antiviral therapy were used for immunohistochemical analysis of SOCS-3 expression, as previously described.[30] The primary antibody used in the study was anti-SOCS-3 (Fusion Antibodies, Belfast, Northern Ireland; dilution 1/100). Tissue sections were photographed using a PixeLink Colour Digital Camera (Total Turnkey Solutions, Mona Vale, New South Wales, Australia) mounted on an Olympus BX-40 microscope (Olympus Australia Pty Ltd, Mt Waverley, Victoria, Australia).

Image analysis was used to quantify the immunoreactivity of SOCS-3. A minimum of 30 non-overlapping fields at a magnification of ×400 were photographed. Image analysis software (Image Pro Plus 4.5; SciTech Pty Ltd, Preston, Victoria) was used to assess the mean immunoreactive area per biopsy. Per cent positive area was defined as the ratio of pixels set above the segmentation threshold to the total number of pixels within a defined area of interest. For each section, per cent positive area for replicate fields was averaged.

Statistical analysis

Continuous normally distributed variables were represented graphically as mean (SEM). Grade of steatosis, stage of fibrosis, and alcohol consumption were summarised using the median. The χ^2 or Fisher's exact tests were used to determine differences in patient distribution between RES and NR and between SVR and NR for variables such as sex, cirrhosis on pretreatment biopsy, presence of steatosis, and BMI categories. To compare the means of normally distributed variables, analysis of variance (ANOVA) or Student's *t* tests were performed. To determine differences between groups for non-normally distributed variables, medians were compared using the Mann-Whitney U test. Pearson's correlation coefficient was used to measure the degree of association between continuous normally distributed variables. The degree of association between non-normally distributed or ordinal variables was assessed using Spearman's non-parametric correlation. Binary logistic regression was used to determine discrete factors associated with NR, adjusting for age at treatment, sex, previous alcohol consumption, presence of steatosis, cirrhosis, type of treatment received, viral genotype, and BMI. ANCOVA was performed to identify predictors of normally distributed variables such as SOCS-3, adjusting for factors such as age, sex, BMI, HOMA, cirrhosis on pretreatment biopsy, previous alcohol consumption, and presence of steatosis. A backward elimination approach was used to remove non-significant variables and determine the most parsimonious model. All analysis was carried out using SPSS software version 11.0 (SPSS Inc., Chicago, Illinois, USA). Statistical significance was taken at the 95% confidence interval.

Table 3 Factors independently associated with non-response to treatment

Characteristic	p Value	OR (95% CI)
Viral genotype 1 or 4	<0.001	4.1 (1.9–8.9)
Cirrhosis on pretreatment biopsy	0.025	3.2 (1.2–9.0)
Obesity*	0.010	3.9 (1.4–11.2)

*Caucasian ⩾30 kg/m^2, Asian ⩾25 kg/m^2.
OR (95% CI), odds ratio (95% confidence interval).

RESULTS

Patient characteristics and antiviral regimes

Of a total of 218 patients, 145 (95% Caucasian, 5% Asian) received antiviral therapy and 73 (100% Caucasian) comprised the RT-PCR cohort. Overall, mean age of the patients was 41.2 (0.6) years and 156 (72%) were male. Viral genotype was 1 in 99 (45.4%), 2 in five (2.3%), 3 in 112 (51.3%), and 4 in two (1%). BMI was classified as lean in 101 patients (46.3%), overweight in 84 (38.5%), and obese in 33 (15.1%). Median prior alcohol intake was 20 (3–60) g/day. Thirty three patients (15.1%) had cirrhosis on liver biopsy. One hundred (45.9%) patients had no steatosis, 71 (32.6%) had mild steatosis, and 47 (21.5%) had moderate or severe steatosis. Mean fasting insulin level (available for 146 patients) was 9.1 (0.64) mU/l, mean HOMA score was 2.02 (0.19), and mean C peptide level was 0.73 (0.03) nmol/l. Within each viral genotype, there were no significant differences between patients who received antiviral therapy and those in the RT-PCR group, except that those who received therapy were slightly older (42.3 (0.7) *v* 39.0 (1.0) years, respectively; p = 0.018).

Table 2 Clinical and demographic characteristics of responders and non-responders to antiviral therapy

	Genotypes 1 and 4 (n = 66)			Genotypes 2 and 3 (n = 79)		
	RES	NR	p Value	RES	NR	p Value
No of patients	30 (45%)	36 (55%)		62 (78%)	17(22%)	
Sex (% male)	20 (67%)	31 (86%)	0.080*	43 (69%)	13 (76%)	0.40*
Age (y)	42.2 (1.3)	43.7 (1.0)	0.33†	40.8 (1.1)	45.5 (2.5)	0.062†
Pretreatment biopsy						
Cirrhosis	2 (7%)	14 (39%)	**0.003***	6 (10%)	2 (12%)	0.58*
Steatosis						
None	17 (57%)	15 (42%)		19 (31 %)	9 (53%)	
Mild	10 (33%)	15 (42%)		23 (37%)	4 (24%)	
Moderate/severe	3 (10%)	6 (17%)	0.45‡	20 (32%)	4 (24%)	0.23‡
BMI						
Non-obese	29 (97%)	25 (69%)		55 (89%)	13 (76%)	
Obese	1 (3%)	11 (31%)	**0.004***	7 (11%)	4 (24%)	0.24*
Fasting insulin (mU/l)	9.71 (2.47)	9.98 (1.50)	0.92†	8.45 (1.06)	7.73 (1.06)	0.75†
HOMA (mU/l)	1.95 (0.045)	2.31 (0.48)	0.62†	1.89 (0.26)	1.68 (0.45)	0.71†
C peptide (nmol/l)	0.77 (1.79)	0.86 (0.95)	0.60†	0.69 (0.56)	0.56 (0.75)	0.25†

Data are mean (SEM).
NR, non-response to antiviral treatment; RES, response to antiviral treatment; BMI, body mass index; HOMA, homeostasis model of assessment.
*Fisher's exact test; †Mann-Whitney U test; ‡χ^2 test.

Table 4 Treatment response, histological data, and metabolic risk factors in non-obese and obese patients with chronic hepatitis C virus

	Non-obese (n = 54)	Obese (n = 12)	p Value
Genotypes 1 and 4			
Response to treatment			
RES	29 (54%)	1 (8%)	
NR	25 (46%)	11 (92%)	**0.004***
Cirrhosis on pretreatment biopsy	10 (19%)	6 (50%)	**0.024***
Steatosis			
None	31 (57%)	1 (8%)	
Mild	20 (37%)	5 (42%)	
Moderate/severe	3 (6%)	6 (50%)	**<0.001†**
Glucose (mmol/l)	4.74 (0.09)	5.11 (0.46)	0.46‡
Insulin (mU/l)	8.41 (1.41)	14.63 (2.58)	**0.041‡**
C peptide (nmol/l)	0.73 (0.10)	1.14 (0.17)	**0.041‡**
HOMA	1.75 (0.29)	3.54 (1.05)	**0.026‡**
	Non-obese (n = 68)	**Obese (n = 11)**	**p Value**
Genotypes 2 and 3			
Response to treatment	55 (81%)	7 (64%)	
RES			
NR	13 (19%)	4 (36%)	0.24*
Cirrhosis on pretreatment biopsy	7 (13%)	1 (9%)	0.88*
Steatosis			
None	26 (38%)	2 (18%)	
Mild	25 (37%)	2 (18%)	
Moderate/severe	17 (25%)	7 (64%)	0.035†
Glucose (mmol/l)	4.78 (1.46)	4.81 (0.35)	0.95‡
Insulin (mU/l)	7.42 (0.85)	12.99 (3.23)	0.13‡
C peptide (nmol/l)	0.62 (0.41)	0.95 (0.17)	0.080‡
HOMA	1.60 (0.17)	3.06 (0.97)	0.18‡

Data are mean (SEM).
NR, non-response to antiviral treatment; RES, response to antiviral treatment; HOMA, homeostasis model of assessment.
*Fisher's exact test; †χ^2 test; ‡Mann-Whitney U test.

A total of 145 subjects fulfilled the criteria for the treatment arm of the study; 128 patients received combination therapy with ribavirin and either standard IFN-α (n = 108) or PEG-IFN-α (n = 20), and 17 patients received IFN-α (n = 14) or PEG-IFN-α (n = 3) monotherapy.

Effect of host factors on treatment outcome

NR was seen in 36 patients (55%) with HCV genotypes 1 or 4 and in 17 (22%) with viral genotypes 2 or 3 (table 2). Among patients with viral genotype 1 or 4, subjects with NR were more likely to have cirrhosis on pretreatment biopsy (p = 0.003) and to be obese (p = 0.004) but there was no difference in the prevalence or severity of steatosis or mean levels of circulating insulin, C peptide, or HOMA scores between patients with NR and RES. In patients with viral genotypes 2 or 3, there was no significant difference in host characteristics between subjects with NR or those with RES (table 2). Median alcohol intake did not differ between subjects with NR or RES (data not shown).

Following multivariate analysis, variables independently associated with NR were viral genotype 1/4 (p<0.001), presence of cirrhosis on pretreatment biopsy (p = 0.025), and obesity (p = 0.010) (table 3). When comparing patients with NR with those with SVR, these factors remained independently associated with treatment non-response (data not shown).

In comparison with non-obese patients, obese subjects with viral genotype 1/4 were more likely to have steatosis (p<0.001) and had higher fasting serum insulin (p = 0.041) and C peptide (p = 0.041) levels and higher HOMA scores (0.026). In patients with viral genotype 2/3, obesity was also associated with higher grades of steatosis (p = 0.035). However, there was no significant difference in fasting serum insulin and C peptide levels or HOMA scores between obese patients and non-obese subjects (table 4) for patients with genotype 2/3.

Eleven of the 12 obese subjects with genotype 1/4 and 9 of 11 obese subjects with genotype 2/3 had steatosis, illustrating the close interrelationship between these factors. Twenty three of 54 non-obese subjects (43%) with genotype 1 and 42 of 68 non-obese subjects (63%) with genotype 2/3 also had steatosis. In these non-obese subjects, the presence of steatosis did not impair the ability to achieve RES (viral genotype 1/4, p = 0.50; viral genotype 2/3, p = 0.21).

Relationship between obesity, hepatic insulin sensitivity, and SOCS-3

To determine the effect of obesity on factors that may impair the IFN-α signalling cascade, PEPCK, TNF-α, SOCS-3, and IFN-R1 mRNA levels were determined by RT-PCR in 73 patients with chronic HCV.

In patients infected with viral genotype 1 (n = 35), obese subjects had increased hepatic expression of SOCS-3 mRNA compared with lean subjects (p = 0.047) (fig 1). By univariate analysis, SOCS-3 mRNA was associated with BMI (p = 0.047) but with no other demographic or histological feature. Following multivariate analysis adjusting for age, sex, presence of cirrhosis, steatosis, and HOMA score, the relationship between SOCS-3 mRNA expression and obesity remained significant (p = 0.023).

Similarly, obese subjects had increased hepatic expression of PEPCK mRNA compared with lean subjects (p<0.01) and this relationship remained significant after multivariate analysis adjusting for age, sex, presence of cirrhosis, steatosis, and HOMA score (p<0.001). In lean subjects, there was a significant inverse relationship between HOMA score and hepatic PEPCK mRNA levels ($r = -0.45$, p = 0.01), consistent with the role of insulin in regulation of PEPCK gene expression. In contrast, in overweight and obese subjects, there was no association between HOMA score and PEPCK mRNA levels ($r = 0.004$, p = 0.98). These results provide

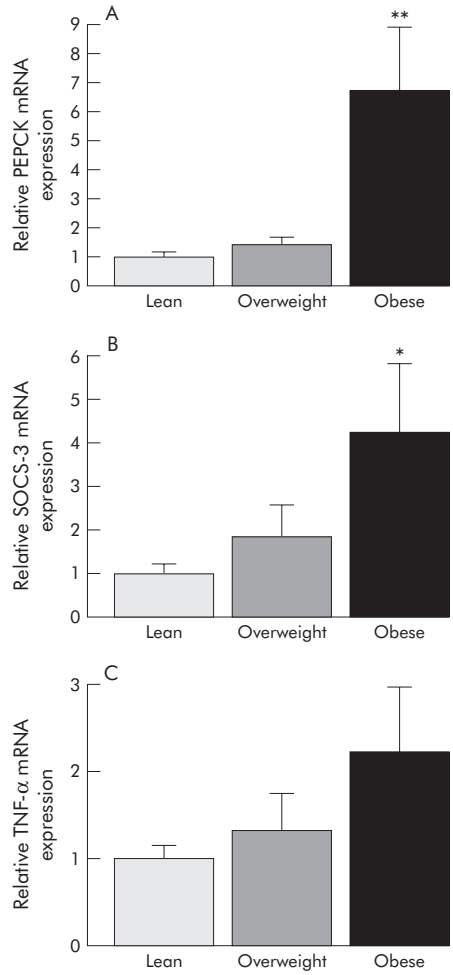

Figure 1 (A) Phosphoenolpyruvate carboxy kinase (PEPCK), (B) suppressor of cytokine signalling 3 (SOCS-3), and (C) tumour necrosis factor α (TNF-α) mRNA levels in lean (body mass index (BMI) <25 kg/m^2), overweight (BMI 25–29.9 kg/m^2), and obese (BMI ⩾30 kg/m^2) patients with chronic hepatitis C virus genotype 1. Data are presented as mean (SEM). *p<0.05, **p<0.01, obese compared with lean patients.

circumstantial evidence that in comparison with lean patients, our obese subjects had impaired hepatic insulin sensitivity. Although we did not find a relationship between SOCS-3 expression and markers of *systemic* insulin resistance such as HOMA, insulin, and C peptide, there was a significant association between SOCS-3 mRNA levels and PEPCK mRNA levels ($r = 0.5$, $p = 0.0092$). This may reflect a relationship between SOCS-3 expression and *hepatic* insulin resistance.

The increase in expression of TNF-α in obese subjects compared with lean subjects approached significance ($p = 0.071$) (fig 1). In patients with viral genotype 1, there was a significant correlation between TNF-α and PEPCK mRNA levels ($r = 0.34$, $p<0.001$) and between TNF-α and SOCS-3 mRNA levels ($r = 0.72$, $p<0.001$).

In patients infected with viral genotype 3, there was no significant difference in SOCS-3, PEPCK, or TNF-α mRNA expression between obese and lean subjects ($p = 0.46$, $p = 0.67$, $p = 0.59$, respectively). No relationship was seen between IFN-R1 mRNA levels and BMI or other markers of insulin resistance for all genotypes (data not shown).

Increased hepatic SOCS-3 expression in patients with treatment non-response

To address the association between SOCS-3 expression and response to antiviral therapy, immunohistochemistry for SOCS-3 was performed in liver sections from subjects infected with HCV genotype 1 or 4 who subsequently received antiviral therapy. Immunoreactive product was seen in liver biopsies from all patients who were treatment non-responders, and all patients, except one, who were responders. Staining was present predominantly in hepatocytes and was localised to the cytoplasm. Hepatocytes were positive in periportal and other areas of the lobules. Immunoreactive staining was also present in bile ducts but only infrequently in Kupffer cells and portal macrophages. (fig 2)

Compared with responders, patients who were non-responders had significantly higher mean levels of SOCS-3 immunoreactivity ($p = 0.014$) (fig 3). After correcting for age, sex, presence of cirrhosis on pretreatment biopsy, HOMA score, previous ethanol consumption, steatosis, and BMI, increased SOCS-3 protein expression was independently associated with treatment NR in patients with viral genotype 1 ($p<0.0001$).

SOCS-3 immunoreactivity was significantly higher in non-lean subjects compared with lean subjects ($p<0.013$). By univariate analysis, SOCS-3 immunoreactivity was not associated with any other demographic, metabolic, or histological feature. Following multivariate analysis adjusting for age, sex, presence of cirrhosis, steatosis, and HOMA score, the relationship between SOCS-3 immunoreactivity and obesity remained significant ($p = 0.012$).

DISCUSSION

In this single centre study, obesity was independently associated with non-response to antiviral therapy in subjects infected with HCV genotype 1. Virtually all obese subjects (87%) had steatosis, underlining the interrelationship between these variables. However, steatosis was also present in 55% of non-obese subjects. In these latter patients, the presence or severity of hepatic steatosis did not influence the ability to achieve a response to treatment. In support of these findings, a recent study by Bressler and colleagues also demonstrated that obesity but not hepatic steatosis was an independent negative predictor of response to HCV treatment.[9]

Many previous studies of both standard and pegylated interferon have identified body weight as a factor impacting on treatment response rates.[11–14 31] It remains unclear whether weight based dosing would improve the response rate or whether obesity is associated with a greater degree of resistance to antiviral therapy.[1] In this study, we demonstrated that obese subjects infected with HCV genotype 1 had increased hepatic expression of SOCS-3, a factor that has been shown to inhibit IFN-α signalling. This relationship between obesity and increased SOCS-3 expression remained significant after correction for other factors associated with non-response to treatment.

Importantly, patients with HCV genotype 1 who were NR had significantly higher levels of SOCS-3 protein expression compared with RES. Engagement of IFN-α with its receptor activates receptor associated tyrosine kinases that phosphorylate signal transducer and activator of transcription (STAT) factors 1 and 2.[32 33] Phosphorylated STAT proteins migrate to the nucleus and induce transcription of several antiviral target genes. This IFN-α signalling pathway is downregulated by members of the SOCS family of proteins.[18 34] SOCS 1 and 3 have been shown to inhibit the tyrosine phosphorylation and nuclear translocation of STAT 1 in response to IFN-α stimulation and this inhibition occurs at very low levels of SOCS protein expression.[35]

Several factors, including the HCV core protein,[17] liver toxins,[22] and various cytokines[36 37] have been shown to induce hepatic SOCS-3 expression. In chronic HCV, TNF-α may have a key role in this inhibitory pathway. TNF-α levels are increased in the serum,[38] liver, and peripheral blood

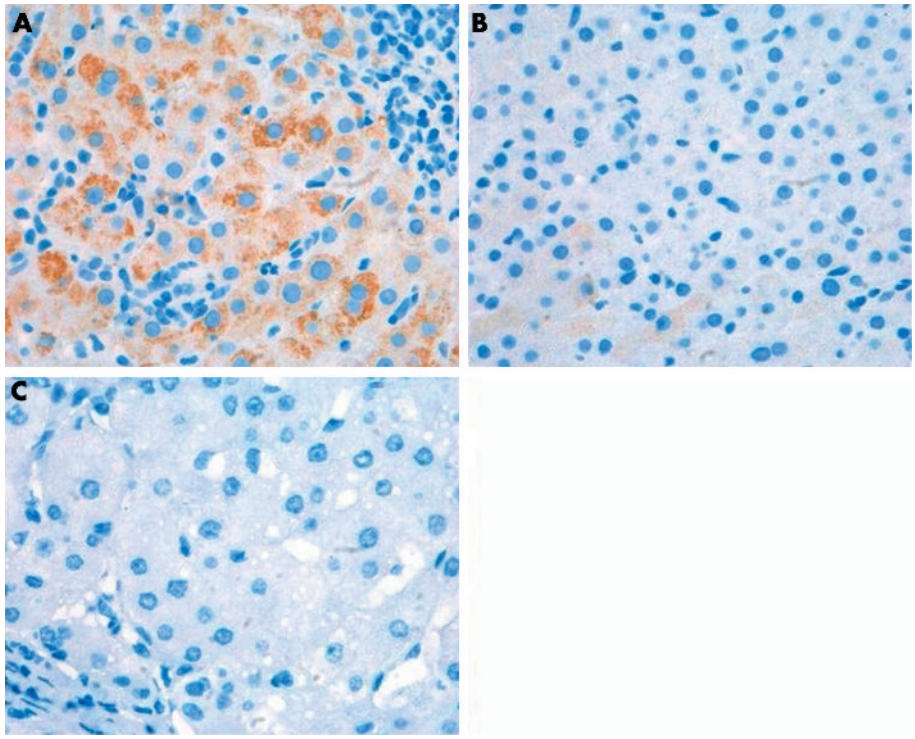

Figure 2 Immunohistochemical analysis of suppressor of cytokine signalling 3 (SOCS-3) protein expression in patients with chronic hepatitis C. (A) Obese treatment, non-responder. Strong staining is present predominantly in hepatocytes and is localised to the cytoplasm. (B) Treatment responder. (C) Negative control. Original magnification ×400.

mononuclear cells[20] of subjects with chronic HCV compared with control subjects. Higher pretreatment intrahepatic[19] and peripheral blood mononuclear cell[20] TNF-α mRNA levels were observed in patients who subsequently failed to respond to IFN-α therapy compared with those subjects who had an SVR. More recently, Hong *et al* have shown in a mouse model that injection of TNF-α markedly induced expression of SOCS-3, resulting in inhibition of IFN-α signalling in hepatic cells.[22] In our cohort of patients with chronic HCV, there was a striking correlation between TNF-α and SOCS-3 mRNA levels, consistent with a role for this cytokine in induction of SOCS-3 expression in vivo.

Importantly, expression of SOCS-3 in the liver is induced by cytokines and hormones that are associated with obesity dependent insulin resistance.[39] Upregulation of hepatic SOCS-1 and -3 mRNA and proteins has been observed in various insulin resistant animal models.[26] A marked decrease in SOCS-3 expression was found in obese mice lacking TNF-α signalling, supporting the premise that elevated levels of SOCS-3 in obesity may be related to increased TNF-α expression.[40] In our study, obese subjects with viral genotype 1 had increased hepatic expression of PEPCK compared with non-obese subjects, consistent with impaired hepatocyte insulin sensitivity. PEPCK catalyses a key step in gluconeogenesis and insulin decreases the transcription of its gene.[41] Insulin resistance is associated with failure of insulin to suppress the activity of enzymes involved in gluconeogenesis leading to increased hepatic glucose production.[42] In our cohort of subjects with chronic HCV, highly significant correlations were seen between hepatic PEPCK and both TNF-α and SOCS-3 mRNA levels. Similar to their inhibitory role in IFN-α signalling, TNF-α and SOCS-3 may act as negative regulators in insulin signalling.[25]

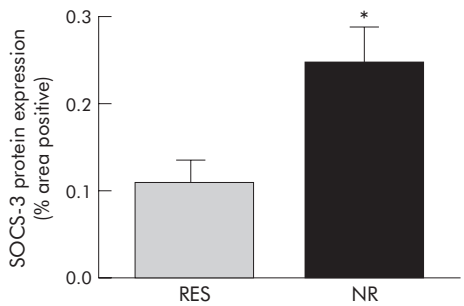

Figure 3 Suppressor of cytokine signalling 3 (SOCS-3) protein immunoreactivity (percentage area positive) in liver sections from patients infected with chronic hepatitis C virus viral genotype 1/4 who were non-responders (NR) or responders (RES) to antiviral treatment. Data are represented as mean (SEM). *$p<0.05$.

In our patients infected with viral genotype 3, there was no statistically significant difference in markers of insulin resistance or hepatic gene expression of PEPCK, SOCS-3, or TNF-α between obese and non-obese patients. It remains unclear why the effect of obesity on hepatic insulin resistance and expression of SOCS-3 was seen in subjects with viral genotype 1 but not genotype 3. The prevalence of cirrhosis was higher in the cohort of subjects infected with viral genotypes 1 or 4, and this may have contributed to an overall increase in insulin resistance in this population. However, the relationships between obesity and expression of PEPCK, SOCS-3, or TNF-α were found to be independent of the presence of cirrhosis. Interestingly, a previous study has also shown that subjects with genotype 3 have lower levels of insulin resistance compared with other viral genotypes.[43] In addition, the increased risk for the development of type 2 diabetes in chronic HCV appears to be largely among non-genotype 3 infected subjects.[43–45] In contrast with the effect of obesity on hepatic gene expression, no differences in mRNA levels studied were seen between subjects with or without hepatic steatosis.

A number of earlier studies demonstrated that a sustained response to antiviral therapy is dependent on high levels of expression of the IFN-α receptor.[46 47] Information regarding the regulation of expression of the interferon receptor in chronic HCV remains limited. In our study, we found no difference in expression of the IFN-R1 between obese and

non-obese subjects. Although a previous study demonstrated downregulation of IFN-R1 mRNA in fibrotic livers,[48] we did not observe this finding in our subjects with chronic HCV.

In summary, in our cohort of patients with chronic HCV, BMI $\geqslant$ 30 kg/m^2 was independently associated with non-response to antiviral therapy. In subjects with viral genotype 1, obesity was associated with increased PEPCK mRNA levels, consistent with impaired hepatocyte insulin sensitivity, and with increased expression of TNF-α and SOCS-3, factors that may reduce interferon signalling. Induction of hepatic SOCS-3 expression may be one mechanism by which obesity reduces the biological response to IFN-α in patients infected with viral genotype 1.

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REFERENCES

1 **Di Bisceglie AM**, Hoofnagle JH. Optimal therapy of hepatitis C. *Hepatology* 2002;**36**:S121–7.
2 **Martinot-Peignoux M**, Marcellin P, Pouteau M, *et al*. Pre-treatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alfa therapy in chronic hepatitis C. *Hepatology* 1995;**22**:1050–6.
3 **Gao B**, Hong F, Radaeva S. Host factors and failure of interferon-alpha treatment in hepatitis C virus. *Hepatology* 2004;**39**:880–90.
4 **Banner BF**, Barton AL, Cable EE, *et al*. A detailed analysis of the Knodell score and other histologic parameters as predictors of response to interferon therapy in chronic hepatitis C. *Mod Pathol* 1995;**8**:232–8.
5 **Kaserer K**, Fiedler R, Steindl P, *et al*. Liver biopsy is a useful predictor of response to interferon therapy in chronic hepatitis C. *Histopathology* 1998;**32**:454–61.
6 **Poynard T**, Ratziu V, McHutchison J, *et al*. Effect of treatment with peg-interferon or interferon alfa-2b and ribavirin on steatosis in patients infected with hepatitis C. *Hepatology* 2003;**38**:75–85.
7 **Akuta N**, Suzuki F, Tsubota A, *et al*. Efficacy of interferon monotherapy to 394 consecutive naive cases infected with hepatitis C virus genotype 2a in Japan: therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. *J Hepatol* 2002;**37**:831–6.
8 **Bjoro K**, Bell H, Hellum KB, *et al*. Effect of combined interferon-alpha induction therapy and ribavirin on chronic hepatitis C virus infection: a randomized multicentre study. *Scand J Gastroenterol* 2002;**37**:226–32.
9 **Bressler BL**, Guindi M, Tomlinson G, *et al*. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. *Hepatology* 2003;**38**:639–44.
10 **McCullough AJ**. Obesity and its nurturing effect on hepatitis C. *Hepatology* 2003;**38**:557–9.
11 **Fried MW**, Shiffman ML, Reddy KR, *et al*. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;**347**:975–82.
12 **Manns MP**, McHutchison JG, Gordon SC, *et al*. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001;**358**:958–65.
13 **Camps J**, Crisostomo S, Garcia-Granero M, *et al*. Prediction of the response of chronic hepatitis C to interferon alfa: a statistical analysis of pre-treatment variables. *Gut* 1993;**34**:1714–17.
14 **Zeuzem S**, Feinman SV, Rasenack J, *et al*. Peginterferon alfa-2a in patients with chronic hepatitis C. *N Engl J Med*, 2000 Dec, **343**:1666–72.
15 **Lam NP**, Pitrak D, Speralakis R, *et al*. Effect of obesity on pharmacokinetics and biologic effect of interferon-alpha in hepatitis C. *Dig Dis Sci* 1997;**42**:178–85.
16 **Mbow ML**, Sarisky RT. What is disrupting IFN-alpha's antiviral activity? *Trends Biotechnol* 2004;**22**:395–9.
17 **Bode JG**, Ludwig S, Ehrhardt C, *et al*. IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3. *FASEB J* 2003;**17**:488–90.
18 **Vlotides G**, Sorensen AS, Kopp F, *et al*. SOCS-1 and SOCS-3 inhibit IFN-alpha-induced expression of the antiviral proteins 2,5-OAS and MxA. *Biochem Biophys Res Commun* 2004;**320**:1007–14.
19 **Dumoulin FL**, Wennrich U, Nischalke HD, *et al*. Intrahepatic mRNA levels of interferon gamma and tumor necrosis factor alpha and response to antiviral treatment of chronic hepatitis C. *J Hum Virol* 2001;**4**:195–9.
20 **Larrea E**, Garcia N, Qian C, *et al*. Tumor necrosis factor alpha gene expression and the response to interferon in chronic hepatitis C. *Hepatology* 1996;**23**:210–17.
21 **Neuman MG**, Benhamou JP, Malkiewicz IM, *et al*. Cytokines as predictors for sustained response and as markers for immunomodulation in patients with chronic hepatitis C. *Clin Biochem* 2001;**34**:173–82.
22 **Hong F**, Nguyen VA, Gao B. Tumor necrosis factor alpha attenuates interferon alpha signaling in the liver: involvement of SOCS3 and SHP2 and implication in resistance to interferon therapy. *FASEB J* 2001;**15**:1595–7.
23 **Peraldi P**, Spiegelman B. TNF-alpha and insulin resistance: summary and future prospects. *Mol Cell Biochem* 199, **182**:169–75.
24 **Hotamisligil GS**, Spiegelman BM. Tumor necrosis factor alpha: a key component of the obesity-diabetes link. *Diabetes* 1994;**43**:1271–8.
25 **Ueki K**, Kondo T, Kahn CR. Suppressor of cytokine signaling 1 (SOCS-1) and SOCS-3 cause insulin resistance through inhibition of tyrosine phosphorylation of insulin receptor substrate proteins by discrete mechanisms. *Mol Cell Biol* 2004;**24**:5434–46.
26 **Ueki K**, Kondo T, Tseng YH, *et al*. Central role of suppressors of cytokine signaling proteins in hepatic steatosis, insulin resistance, and the metabolic syndrome in the mouse. *Proc Natl Acad Sci U S A* 2004;**101**:10422–7.
27 **Knodell RG**, Ishak KG, Black WC, *et al*. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981;**1**:431–5.
28 **Scheuer PJ**. Classification of chronic viral-hepatitis—a need for reassessment. *J Hepatol* 1991;**13**:372–4.
29 **Matthews DR**, Hosker JP, Rudenski AS, *et al*. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985;**28**:412–19.
30 **Walsh MJ**, Vanags DM, Clouston AD, *et al*. Steatosis and liver cell apoptosis in chronic hepatitis C: a mechanism for increased liver injury. *Hepatology* 2004;**39**:1230–8.
31 **Lam NP**, DeGuzman LJ, Pitrak D, *et al*. Clinical and histologic predictors of response to interferon-alpha in patients with chronic hepatitis C viral infection. *Dig Dis Sci* 1994;**39**:2660–4.
32 **Darnell JE Jr**. STATs and gene regulation. *Science* 1997;**277**:1630–5.
33 **Radaeva S**, Jaruga B, Hong F, *et al*. Interferon-alpha activates multiple STAT signals and down-regulates c-Met in primary human hepatocytes. *Gastroenterology* 2002;**122**:1020–34.
34 **Krebs DL**, Hilton DJ. SOCS: physiological suppressors of cytokine signaling. *J Cell Sci* 2000;**113**:2813–19.
35 **Song MM**, Shuai K. The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. *J Biol Chem* 1998;**273**:35056–62.
36 **Paul C**, Seiliez I, Thissen JP, *et al*. Regulation of expression of the rat SOCS-3 gene in hepatocytes by growth hormone, interleukin-6 and glucocorticoids mRNA analysis and promoter characterization. *Eur J Biochem* 2000;**267**:5849–57.
37 **Shen X**, Hong F, Nguyen VA, *et al*. IL-10 attenuates IFN-alpha-activated STAT1 in the liver: involvement of SOCS2 and SOCS3. *FEBS Lett* 2000;**480**:132–6.
38 **Nelson DR**, Lim HL, Marousis CG, *et al*. Activation of tumor necrosis factor-alpha system in chronic hepatitis C virus infection. *Dig Dis Sci* 1997;**42**:2487–94.
39 **Senn JJ**, Klover PJ, Nowak IA, *et al*. Suppressor of cytokine signaling-3 (SOCS-3), a potential mediator of interleukin-6-dependent insulin resistance in hepatocytes. *J Biol Chem* 2003;**278**:13740–6.
40 **Emanuelli B**, Peraldi P, Filloux C, *et al*. SOCS-3 inhibits insulin signaling and is up-regulated in response to tumor necrosis factor-alpha in the adipose tissue of obese mice. *J Biol Chem* 2001;**276**:47944–9.
41 **O'Brien RM**, Lucas PC, Forest CD, *et al*. Identification of a sequence in the PEPCK gene that mediates a negative effect of insulin on transcription. *Science* 1990;**249**:533–7.
42 **Barthel A**, Schmoll D. Novel concepts in insulin regulation of hepatic gluconeogenesis. *Am J Physiol Endocrinol Metab* 2003;**285**:E685–92.
43 **Hui JM**, Sud A, Farrell GC, *et al*. Insulin resistance is associated with chronic hepatitis C virus infection and fibrosis progression. *Gastroenterology* 2003;**125**:1695–704.
44 **Knobler H**, Schihmanter R, Zifroni A, *et al*. Increased risk of type 2 diabetes in noncirrhotic patients with chronic hepatitis C virus infection. *Mayo Clin Proc* 2000;**75**:355–9.
45 **Mason AL**, Lau JY, Hoang N, *et al*. Association of diabetes mellitus and chronic hepatitis C virus infection. *Hepatology* 1999;**29**:328–33.
46 **Mathai J**, Shimoda K, Banner BF, *et al*. IFN-alpha receptor mRNA expression in a United States sample with predominantly genotype 1a/1 chronic hepatitis C liver biopsies correlates with response to IFN therapy. *J Interferon Cytokine Res* 1999;**19**:1011–18.
47 **Morita K**, Tanaka K, Saito S, *et al*. Expression of interferon receptor genes in the liver as a predictor of interferon response in patients with chronic hepatitis C. *J Med Virol* 1999;**58**:359–65.
48 **Ishimura N**, Fukuda R, Fukumoto S. Relationship between the intrahepatic expression of interferon-alpha receptor mRNA and the histological progress of hepatitis C virus-associated chronic liver diseases. *J Gastroenterol Hepatol* 1996;**11**:712–17.