The effect of obesity on intrahepatic cytokine and chemokine expression in chronic hepatitis C infection

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ABSTRACT

Background Obese subjects with chronic hepatitis C virus (HCV) infection have more rapidly progressive liver disease.

Objective In this study, we aimed to compare the hepatic cytokine and chemokine profiles in obese and lean subjects with chronic HCV infection using qRT-PCR and immunohistochemistry.

Methods Liver biopsies from 55 subjects were studied, including 20 with chronic hepatitis C, 25 with non-alcoholic fatty liver disease and 10 subjects with non-diseased liver.

Results Compared to the control groups, the liver injury in chronic hepatitis C was characterised by increased expression of several T-helper-1 cytokines including interferon-γ and tumour necrosis factor-α, and chemokines such as RANTES, IP-10 and MCP-1. In particular, in comparison with lean (BMI $\leq$25 kg/m^2) HCV infected subjects, obese (BMI$\geq$30 kg/m^2) HCV infected subjects had increased hepatic expression of interferon-γ ($p=0.004$) and tumour necrosis factor-α ($p<0.001$), as well as increased expression of IP-10 ($p=0.009$) and MCP-1 ($p<0.001$). Localisation of these inflammatory chemokines revealed that in comparison to lean-HCV subjects, HCV infected liver from obese subjects exhibited significantly increased expression of IP-10 ($p<0.001$) and MCP-1 ($p=0.02$) in the inflammatory infiltrate of the portal tracts. In parallel, there was increased CD3 infiltration in the liver of obese-HCV subjects.

Conclusions The data provide important mechanistic information on the cause of hepatic injury in obese-HCV subjects including: (1) enhanced T helper-1 cytokine response patterns—to promote hepatocellular injury; (2) increased expression of the chemokines IP-10 and MCP-1 at both the mRNA and protein levels—to enhance inflammatory cell recruitment; (3) differing localisation of these chemokines within the liver of obese-HCV versus lean-HCV subjects—implying different inducing stimuli and; (4) increased CD3 expression in the liver of obese-HCV subjects—concordant with the increased expression of T cell chemoattractants.

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide.[1] Obesity and hepatic steatosis occur frequently in subjects with chronic hepatitis C (CHC) and are associated with more rapid progression of liver disease.[2 3] Factors contributing to this pathogenic process are not clear, but may include mediators of the metabolic syndrome such as adipokines, inflammatory cytokines, increased oxidative stress, apoptosis and hepatic stellate cell activation.[4–8]

Although hepatic steatosis alone is innocuous, accumulating evidence suggests that fatty livers are more susceptible to injury induced by a secondary inflammatory stimulus, such as lipopolysaccharide or Concanavalin A administration.[9] In this setting, steatosis can exacerbate T cell mediated liver injury, partly by polarising T helper (TH) cells towards a TH-1 response, with excess production of interferon (IFN)-γ and interleukin (IL)-2.[10 11] In addition to promoting a TH-1 cytokine response, obesity and steatosis were associated with enhanced lymphocyte responsiveness to hepatic chemokines, resulting in increased lymphocyte homing to the liver in response to inflammatory insults.[12]

In CHC, the development of liver injury is largely driven by HCV-stimulated immunological and inflammatory responses. Thus, TH-1 inflammatory cells, producing IFN-γ and tumour necrosis factor (TNF)-α secretion, predominate in the liver and correlate with the degree of inflammation in chronic HCV infection.[13 14] In parallel, chemokines that attract these pro-inflammatory cells are also important in the progression of HCV-related liver disease. In particular, IP-10 (the IFN-γ-inducible protein-10, also known as CXCL10), MIG (monokine induced by IFN-γ, also known as CXCL9) and I-TAC (IFN-inducible T cell α chemoattractant, also known as CXCL11), as well as RANTES (regulated on activation normal T cell expressed and secreted, also known as CCL5), MCP-1 (monocyte chemoattractant protein-2, also known as CCL2), and MIP-1α (macrophage inflammatory protein-1α, also known as CCL3), are involved in the selective recruitment of lymphocytes to the liver. Moreover, in CHC, increased expression of IP-10 mRNA in the liver correlates with necroinflammation,[15] while high levels of MCP-1 mRNA correlate with more advanced fibrosis.[16]

Taken together, this evidence poses the question: does obesity enhance the liver injury in HCV-infected subjects by modifying intrahepatic cytokine and chemokine expression? Thus, the broad aim of this study was to examine whether obesity and the presence of steatosis in HCV-infected liver is associated with alterations in cytokine and chemokine expression patterns. For comparative purposes, we also examined the intrahepatic cytokine and chemokine profiles in the liver of obese subjects with non-alcoholic fatty liver disease (NAFLD) alone, including subjects with steatosis and those with non-alcoholic steatohepatitis (NASH).

MATERIALS AND METHODS

Study population

Liver biopsies were obtained from 55 subjects. These included 20 subjects chronically infected with HCV genotype 1 undergoing evaluation for antiviral therapy (10 obese and 10 lean). Obese subjects were defined as having a BMI≥30 kg/m^2, while lean subjects had a BMI of ≤25 kg/m^2. In addition, 25 obese subjects with NAFLD (10 with steatosis alone and 15 with NASH) who had liver biopsies obtained during bariatric surgery were studied. The final group was 10 obese subjects with non-diseased liver who also had liver biopsies obtained during bariatric surgery. Both snap frozen liver tissue (stored in liquid nitrogen at −180°C) and formalin fixed paraffin-embedded liver sections were available for all subjects.

To focus on cytokine and chemokine patterns characterising the inflammatory response alone (rather than fibrotic injury), biopsies with advanced fibrosis (stage 3 or 4) were excluded. Other exclusion criteria included co-infection with HIV or chronic hepatitis B, alcohol intake in excess of 20 g/day, and the co-existence of other forms of chronic liver disease.

All patients gave written informed consent. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review board, approval number SGH06/27.

Histopathological examination

The biopsy sections were analysed by an experienced histopathologist blinded to the source of the liver biopsy, and to patient demographic and laboratory data. Liver samples from subjects with CHC were scored according to the Metavir system.[17] The degree of steatosis was semi-quantified according to the Brunt criteria[18] with slight modifications (0 is <5% of hepatocytes affected, 1 is 5–32% of hepatocytes affected, 2 is 33–66% of hepatocytes affected, and 3 is >66% of hepatocytes affected). Steatohepatitis was defined based on the findings of hepatocellular steatosis, hepatocyte ballooning, mixed inflammation and zone 3 perisinusoidal fibrosis.[18]

Real-time PCR

Primers were designed for the following genes: IFN-γ, TNF-α, IL-6, IL-10, IL-4, IL-13 RANTES, IP-10, MCP-1, MIP1-α and β-actin. Primer sequences are available on request. Primers were designed to span intron–exon boundaries to minimise the likelihood of genomic DNA amplification. All primers were purchased from Invitrogen (Paisley, UK) as desalted grade, and the identity of the amplicons was confirmed by sequencing at the Australian Genome Research Facility Ltd (Brisbane, Australia). Quantitive PCR was performed on the LightCycler 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany) as described before.[19] A melting curve program was employed in each assay to confirm the presence of a single, specific amplicon of desired size. The standard curve method was used to determine the relative concentrations of each genes of interest with the average of duplicate amplifications normalised to β-actin.

Immunohistochemistry

Formalin fixed paraffin-embedded liver biopsies were employed for immunohistochemical (IHC) studies. Staining was performed to evaluate chemokine expression as previously described.[15] The following antibodies and concentrations were employed: goat polyclonal anti-human IP-10, goat polyclonal anti-human MCP-1 and goat polyclonal anti-human RANTES (R&D Systems, Abingdon, United Kingdom) used at 7.5, 6.25 and 10 μg/ml, respectively; rabbit polyclonal anti-human CD3 antibody (DAKO Corp, California, USA) at a 1:50 dilution. Negative-control antibodies consisted of the species-matched immunoglobulin (Ig) G subclass used at the same dilution as the primary antibodies. Bound IP-10 and RANTES antibodies were detected with polyclonal rabbit anti-goat IgG biotinylated antibody (DAKO Corp) at a 1:200 dilution. Bound MCP-1 was detected with a polyclonal goat anti-mouse IgG biotinylated antibody (DAKO Corp) at a 1:200 dilution. Bound CD3 antibody was detected with the Ultravision LP Detection System AP polymer and fast red chromogen (Lab Vision Corp, Fremount, California, USA). All sections were counterstained with Dako Cytomation Mayer's Hematoxylin (DAKO Corp). All subject groups were represented in each run to ensure comparability of subjective comparisons of stain intensity between groups. Furthermore, a consistent protocol was applied to the duration of colour development. Images were captured using Nikon Codpix 4500 camera (Nikon, Japan) affixed to a LICA DM LB microscope (Leica Microsystems).

Immunohistochemistry scoring

RANTES, IP-10 and MCP-1 were independently assessed by an experienced pathologist (SO) and an H score generated, with individual scores determined for hepatocytes, inflammatory cells and bile ducts. In this method, the number of positive cells per specimen and the staining intensity expression for each of these chemokines is semiquantitatively evaluated in each different cell type as previously described.[20] CD3 expression was semiquantitated as the percentage of positive mononuclear inflammatory cells present in five consecutive high-power fields (400×; Leica DMLB; field diameter 0.5 mm). For inclusion, each field had to include both portal and lobular compartments and an average value was determined as percent of CD3 positive cells per high power field.

Statistical analysis

Continuous variables were represented as mean ± standard deviation. To determine differences between groups not normally distributed, the non-parametric Kruskal–Wallis test was used to compare groups, while the Mann–Whitney test was performed for two group comparisons. To compare the means of normally distributed variables, analysis of variance or Student t tests were performed. The degree of association between non-normally distributed variables was undertaken using Spearman's non-parametric correlation. The χ^2 test was used to determine differences in patient distribution for variables such as sex. Differences in the grade of steatosis between subjects were compared using either Mann–Whitney or the Kruskal–Wallis tests. GraphPad Prism V.3.01 (GraphPad Software Inc, San Diego, CA, USA) was used to perform all analyses.

RESULTS

Subject clinical characteristics

The clinical characteristics of all studied subjects are presented in table 1. The clinical characteristics of obese versus lean HCV subjects are presented in table 2, while the characteristics of subjects with steatosis versus NASH are shown in table 3. HCV subjects were predominantly male while patients undergoing obesity surgery were more commonly female (table 1). Not surprisingly, the BMI of subjects undergoing bariatric surgery was significantly higher than HCV-infected subjects (table 1). Similarly, fasting insulin and insulin resistance (IR), determined using the homeostasis model of assessment (HOMA)[21] were significantly elevated in the NAFLD group, who also had a higher grade of steatosis compared to other groups (table 1). Despite this, the HCV group had higher mean serum alanine amino transferase (ALT) levels and more hepatic necroinflammatory activity (table 1). Within the HCV group, obese and lean subjects were well matched for age, gender, duration of infection and viral

load (table 2). It was not possible to match obese and lean-HCV subjects for the extent of liver injury as the obese-HCV subjects had consistently higher necroinflammatory activity. Thus, in comparison to lean-HCV subjects, the obese subjects had significantly higher serum ALT levels, necroinflammatory activity and HOMA-IR scores (table 2). Similarly, all obese-HCV subjects had hepatic steatosis; two with grade 1, four with grade 2 and four with grade 3 steatosis. In contrast, in the lean HCV group, four subjects had grade 1 steatosis while six subjects had no steatosis. As for steatosis grade in subjects with NAFLD, in the steatosis group, one subject had steatosis grade 1, three had grade 2 while six subjects had grade 3. In the NASH group, steatosis grade 1 was present in one subject, four subjects had grade 2, while 10 subjects had steatosis grade 3.

Obesity is associated with enhanced TH-1 cytokine expression in CHC

Compared to subjects with normal histology and NAFLD, all HCV subjects exhibited increased mRNA expression of the cytokines, IFN-γ ($p<0.001$), TNF-α ($p<0.001$) and IL-6 ($p<0.001$) (figure 1). There was however, no difference in the expression of the TH-2 cytokines IL-4, IL-10 and IL-13 (figure 1). Overall, TH-2 cytokines were expressed at lower levels compared to TH-1 cytokines (figure 1).

Within the HCV group, the intrahepatic mRNA expression of IFN-γ and TNF-α were significantly increased in obese-HCV subjects compared to lean-HCV subjects ($p=0.004$ and $p<0.001$ respectively) (figure 2A).

Compared to non-diseased liver, the NAFLD group as a whole had increased expression of TNF-α ($p=0.003$) (figure 1). Within the NAFLD group, only TNF-α mRNA tended to be increased in the NASH group compared to subjects with steatosis alone ($p=0.05$).

Obesity is associated with enhanced chemokine expression in CHC

All HCV subjects had increased hepatic mRNA expression of RANTES ($p<0.001$), IP-10 ($p<0.001$) and MCP-1 ($p<0.001$) (figure 3) and MIP-1α ($p=0.02$) (data not shown) compared to other groups. In comparison to lean-HCV subjects, mRNA expression of IP-10 ($p=0.009$) and MCP-1 ($p<0.001$) was significantly increased in obese-HCV subjects (figure 2B).

Compared to non-diseased liver, only the chemokine MCP-1 was increased in the NAFLD group ($p=0.01$) (figure 3). Within the NAFLD group, MCP-1 mRNA was increased in NASH subjects compared to subjects with steatosis alone ($p=0.04$) (data not shown).

Table 1 Clinical characteristics of subject groups

Variable*	HCV (n=20)	NAFLD (n=25)	Non-diseased liver (n=10)	p Value†
Age	45±6	53±12	41±14	NS
Gender (M:F)	16:4	8:17	2:8	0.001‡
BMI (kg/m^2)	28±6	42±9	41±6	<0.001
Fasting insulin (mU/l)	14±11	21±12	15±6	0.05
HOMA-IR	3.0±1.8	6.7±5.1	4.0±1.6	0.04
ALT (U/l)	84±44	38±24	28±10	0.005
Lobular inflammation	1.9±0.9	1.0±0.7	0	0.002§
Portal inflammation	1.8±0.8	0.9±0.8	0	0.001§
Steatosis grade	1.2±1.1	2.6±0.7	0	0.005§

*Results are expressed as mean±SD.
†Unless otherwise specified, Kruskal Wallis test, comparing HCV, NAFLD and non-diseased.
‡χ^2 Test.
§Mann–Whitney comparing HCV and NAFLD.
ALT, alanine amino transferase; BMI, body mass index; HCV, hepatitis C virus; HOMA-IR, homeostasis model of assessment-insulin resistance; NAFLD, non-alcoholic fatty liver disease.

Table 2 Clinical characteristics of obese and lean HCV subjects

Variable*	Obese HCV (n=10)	Lean HCV (n=10)	p Value†
Age	45±6	44±6	NS
Gender (M:F)	8:2	8:2	NS‡
BMI (kg/m^2)	33±3.3	23±3	0.005
Duration of HCV infection (yr)	21±9	20±9	NS
Fasting insulin (mU/l)	20±13	8±3	0.05
HOMA-IR	4.0±1.3	2.2±1.9	0.009
HCV viral load (IU/ml)	2.1×10^6 ($\pm2.5\times10^6$)	1.4×10^6 ($\pm1.3\times10^6$)	NS
ALT (U/l)	104±46	56±12	0.02
Lobular inflammation	2.4±1.0	1.3±0.8	0.01
Portal inflammation	2.4±0.5	1.2±0.4	0.004
Steatosis grade	2.2±1.9	0.3±0.5	0.009

*Results are expressed as mean ± SD.
†Mann–Whitney test unless otherwise specified.
‡χ^2 Test.
ALT, alanine amino transferase; BMI, body mass index; HCV, hepatitis C virus; HOMA-IR, homeostasis model of assessment-insulin resistance.

Localisation of intrahepatic chemokine expressions

To further validate the differences in mRNA expression of the chemokines, and to determine whether obesity and the presence of steatosis influence the cellular source(s) of chemokines within HCV-infected liver, the extent of protein expression and the cellular localisation was compared by IHC for RANTES, IP-10 and MCP-1 in the liver of the HCV and NAFLD groups. In the HCV and NAFLD (steatosis and NASH) livers, RANTES was primarily detected in the hepatocytes and inflammatory cells (figure 4). Consistent with the mRNA findings, HCV-infected livers from lean and obese subjects exhibited more RANTES expression compared to livers from NAFLD subjects (figure 3A–C). There were no differences between RANTES expression (immunoreactivity) in the liver of obese and lean HCV subjects (figure 4A,B). There was minimal expression of RANTES in non-diseased liver (figure 4D).

In HCV-infected liver samples, IP-10 was predominately expressed by hepatocytes, biliary epithelial cells and inflammatory cells in the portal tracts. In particular, the liver of obese-HCV subjects had significantly increased expression of IP-10 within the portal tracts compared to livers of lean-HCV subjects ($p<0.001$) (figure 5A,B and 7A). Moreover, except for some

Table 3 Clinical characteristics of NAFLD (steatosis versus NASH) subjects

Variable*	Steatosis (n=10)	NASH (n=15)	p Value†
Age	50±13	55±12	NS
Gender (M:F)	3:7	5:10	NS‡
BMI (kg/m^2)	41±8	42±10	NS
Fasting insulin (mU/l)	18±10	23±14	NS
HOMA-IR	5.8±3.8	7.4±6.1	NS
ALT (U/l)	31±18	45±29	NS
Steatosis grade	2.5±0.7	2.6±0.6	NS
Lobular inflammation	0.5±0.5	1.3±0.6	0.006
Portal inflammation	0.2±0.3	1.6±0.6	*<0.001*

*Results are expressed as mean±SD.
†Mann–Whitney test unless otherwise specified.
‡χ^2 Test.
ALT, alanine amino transferase; HOMA-IR, homeostasis model of assessment-insulin resistance; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis.

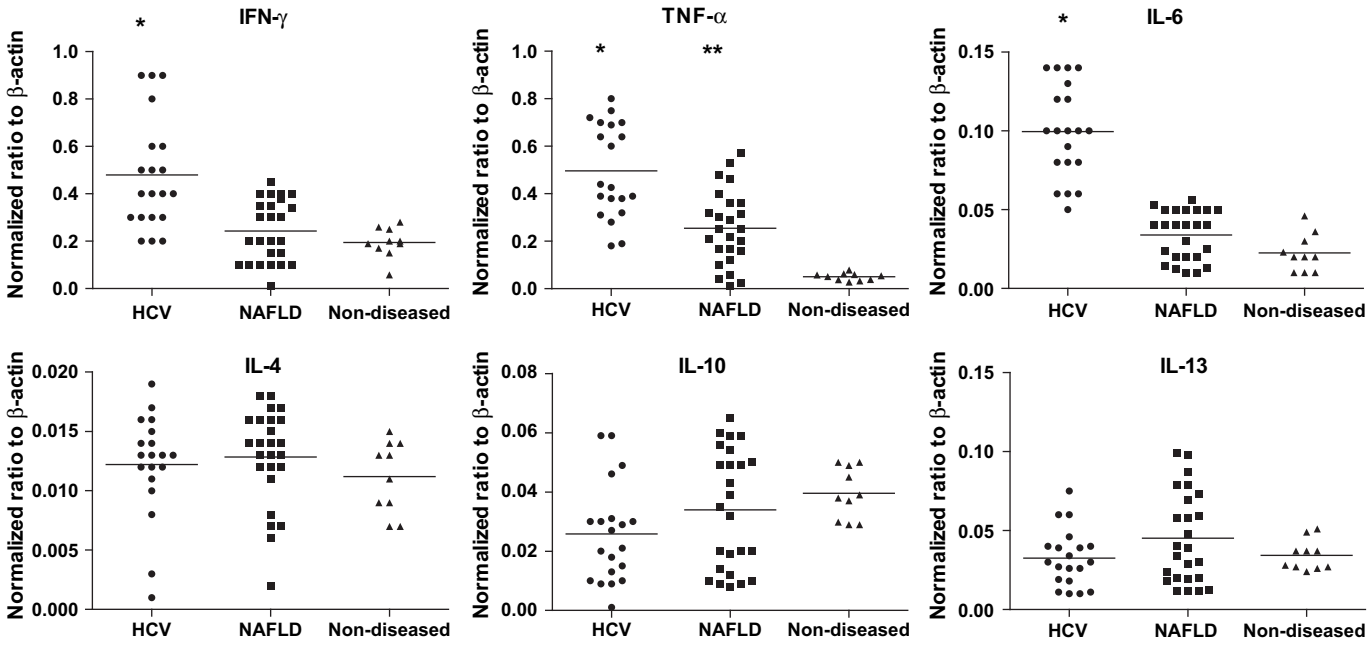

Figure 1 Hepatic cytokine mRNA expression in all studied groups. Compared to NAFLD and non-diseased liver, subjects with CHC had increased mRNA expression of the TH-1 cytokines, IFN-γ, TNF-α and IL-6 (*p<0.001 for all comparisons) but not the TH-2 cytokines, IL-4, IL-10 and IL-13. In addition, livers from subjects with NAFLD had increased mRNA expression of TNF-α (**p=0.003) in comparison to non-diseased livers.

relatively scant staining of biliary epithelial cells, there was no other positive staining of IP-10 within the portal tracts of subjects with NAFLD (steatosis or NASH) or those with non-diseased liver (figure 5C,D).

Similarly, MCP-1 expression was evident within hepatocytes, biliary epithelial cells and the inflammatory infiltrate in the portal tracts of the liver samples from HCV-infected subjects, compared to all other groups (figure 6). Again, MCP-1 expression was significantly increased in the portal tracts of obese-HCV subjects compared to lean-HCV subjects (p=0.02) (figures 6A,B and 7B).

Within the NAFLD group, hepatocyte expression of MCP-1 tended to be enhanced in NASH compared to steatosis alone (p=0.05) (figure 6C,D). Moreover, in livers from NAFLD subjects (steatosis or NASH), there appeared to be increased expression of MCP-1 in hepatocytes adjacent to steatotic regions (figure 6D). Thus, while HCV-infected livers exhibited increased expression of MCP-1 within the portal tracts, livers with NAFLD had increased MCP-1 expression in hepatocytes, particularly in steatotic regions.

Obesity is associated with increased CD3 cells expression in HCV infected livers

As the chemokine profile detected in obese-HCV subjects may be anticipated to promote T cell recruitment, we sought to determine by IHC the expression of CD3 (a pan T cell marker) in

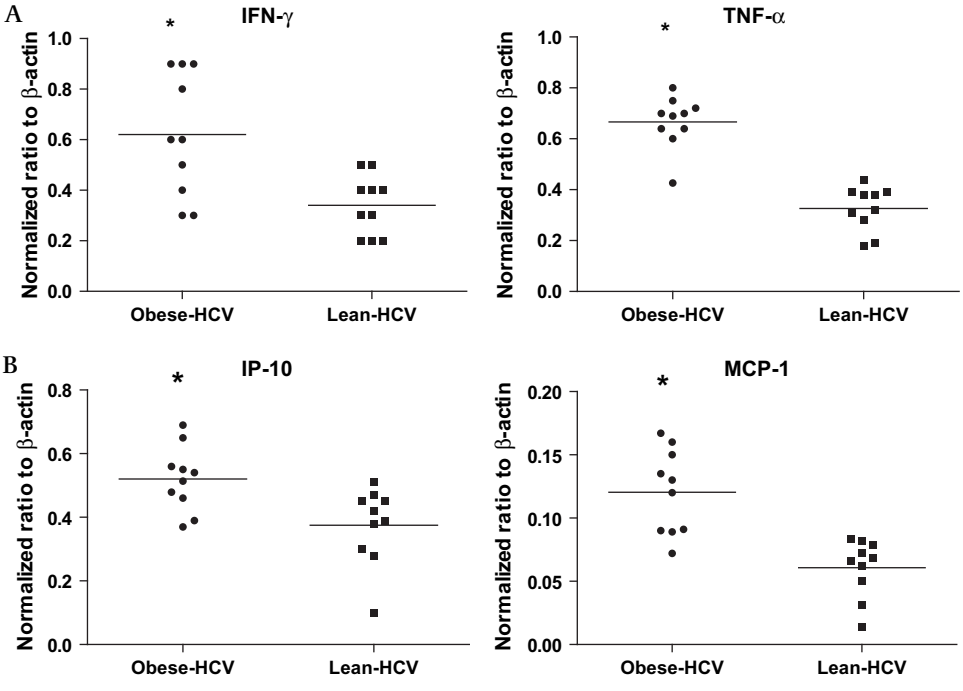

Figure 2 Cytokine and chemokine mRNA expression in obese-HCV versus lean-HCV subjects. Increased mRNA expression of: (A) the cytokines, IFN-γ (*p=0.004) and TNF-α (*p<0.001) and; (B) the chemokines, IP-10 (*p=0.009) and MCP-1 (*p<0.001) was detected in obese- versus lean-HCV subjects.

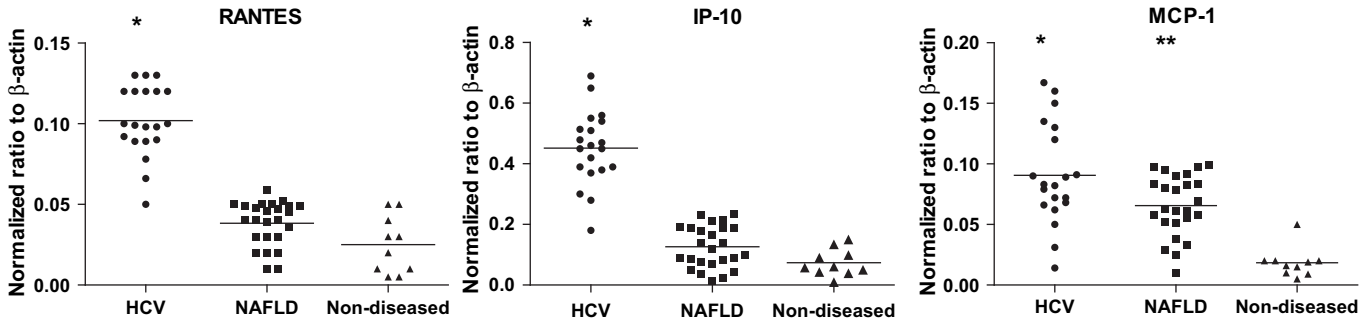

Figure 3 Hepatic chemokine mRNA expression in all studied groups. Compared to NAFLD and non-diseased liver, subjects with CHC had increased mRNA expression of the chemokines, RANTES, IP-10 and MCP-1 (*$p<0.001$ for all comparisons). In addition, livers from subjects with NAFLD had increased mRNA expression of MCP-1 (**$p=0.01$) in comparison to non-diseased livers.

obese-HCV and lean-HCV subjects. There was a significant increase in the relative expression of CD3 cells in regions of inflammatory cell infiltration within both the portal tracts and lobules of obese-HCV subjects ($p=0.02$), compared to lean-HCV subjects, including subjects with comparable inflammatory scores (figures 8A,B).

Relationship of cytokine and chemokine profiles with types of liver injury

In the HCV group, the degree of hepatic necroinflammation by the Metavir scoring system correlated with mRNA expression of TNF-α ($r=0.51$, $p=0.03$), IFN-γ($r=0.73$, $p=0.003$) and IP-10 ($r=0.8$, $p=0.002$). MCP-1 mRNA expression tended to correlate with the NAS score in the NAFLD group ($r=0.31$, $p=0.08$).

DISCUSSION

In chronic hepatitis C infection, the presence of obesity and steatosis appear to be important co-factors in increasing hepatic inflammatory activity and in accelerating the development of fibrosis.[3] The association between hepatic steatosis and the development of fibrosis is dependent upon the extent of necroinflammation.[3] As pro-inflammatory cytokines and chemokines are key mediators of necroinflammation in CHC, we sought to determine whether distinct patterns of expression of these mediators drive inflammation in obese subjects with CHC in comparison to lean subjects with CHC. To focus on cytokine and chemokine expression in association with hepatic inflammation, we studied subjects with HCV genotype 1 infection, but excluded biopsies with advanced fibrosis. Thus, by comparing the intrahepatic cytokine and chemokine profiles in obese-HCV and lean-HCV infected subjects and those with NAFLD alone, we were able to make several relevant observations. Firstly, compared to NAFLD and non-diseased liver, the liver injury in CHC is characterised by increased expression of TNF-α, IFN-γ, IP-10 and MCP-1. Secondly, obese subjects with HCV exhibited an exaggerated TH-1 cytokine profile with further increases in TNF-α and IFN-γ expression. Thirdly, in obese-HCV subjects, there was enhanced intrahepatic expression of the chemokines, IP-10 and MCP-1. Immunohistochemistry data substantiated the mRNA analysis and showed that in HCV-infected livers these chemokines were localised in areas of inflammation within hepatocytes and portal tracts. In parallel with increased expression of T cell chemoattractants, there was increased expression of CD3 cells in the livers of obese-HCV subjects. Finally, within the NAFLD group, compared to steatosis alone, subjects with NASH had increased hepatic expression of the cytokine TNF-α and the chemokine MCP-1.

Despite the more significant metabolic disturbance evident in the NAFLD group (characterised by higher BMI, fasting insulin levels, HOMA-IR and increased steatosis), subjects with CHC had more hepatic inflammation. Furthermore, there was

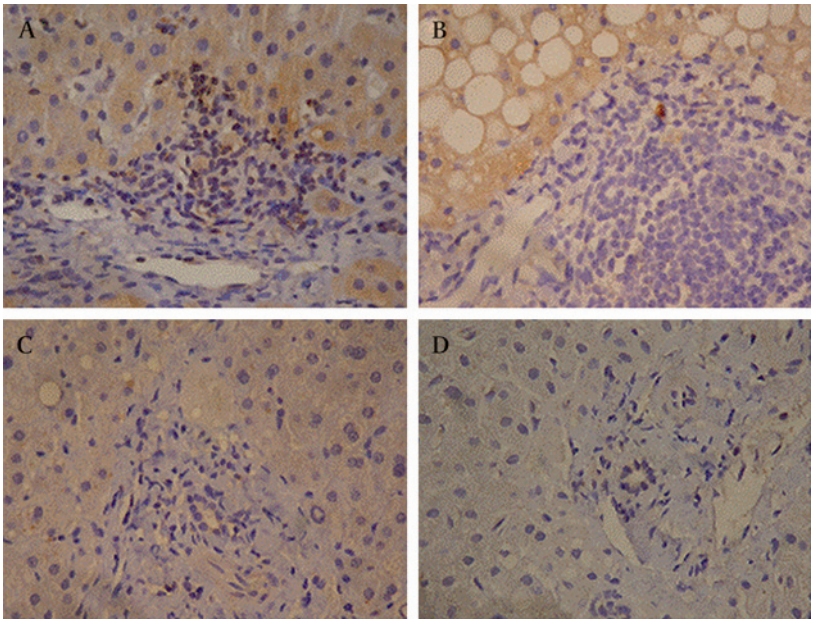

Figure 4 Immunohistochemical analysis of RANTES protein expression in studied groups. RANTES expression was primarily detected on hepatocytes and inflammatory cells. The expression of RANTES in the liver of lean-HCV subjects (A) was not increased when compared to obese-HCV subjects (B). Liver from subjects with NAFLD (C) and non-diseased liver (D) exhibited low expression of RANTES. There was no difference in the expression of RANTES between subjects with steatosis and NASH (data not shown). Original magnification ×400.

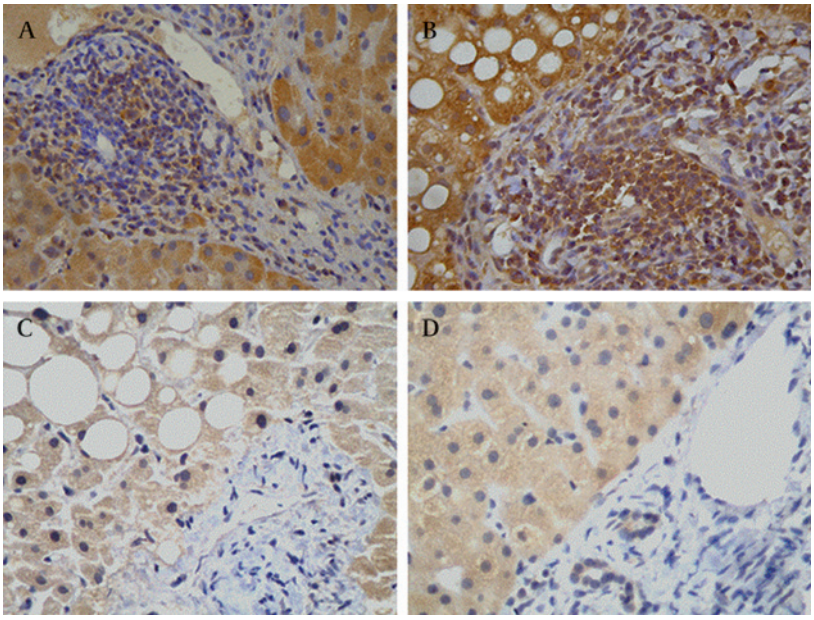

Figure 5 Immunohistochemical analysis of IP-10 protein expression in studied groups. IP-10 expression was evident in the inflammatory cells in the portal tracts and hepatocytes as well as biliary epithelial cells. Compared to lean-HCV subjects (A), there was significant expression of IP-10 within the portal tracts of obese-HCV subjects (B) ($p<0.001$). The increased expression of IP-10 in the portal tracts of obese-HCV versus lean-HCV subjects was also evident in livers with comparable degrees of portal inflammation (A & B). In NAFLD (C) and non-diseased liver (D), IP-10 was weakly expressed in the hepatocytes. There was no difference in the expression of IP-10 between subjects with steatosis and NASH (data not shown). Original magnification ×400.

increased hepatic necroinflammation in obese-HCV compared to lean-HCV infected subjects. Therefore, the data from the present study clearly emphasise the detrimental impact of the co-existence of obesity (and its related metabolic disturbances) and HCV on liver injury. Notably, the necroinflammatory liver injury due to either, HCV alone or, NAFLD alone is less aggressive than when both diseases co-exist.

Several of the pro-inflammatory cytokines and chemokines examined were differentially expressed in HCV-infected livers compared to NAFLD and control groups. However, in obese subjects with CHC infection, the expression of the TH-1 cytokines, TNF-α and IFN-γ were specifically increased when compared to lean-HCV infected subjects. In concordance with previous reports, the extent of the intrahepatic expression of these cytokines correlated with the degree of necroinflammation. Since necroinflammation is the main determinant of liver disease progression and fibrosis,[3] it is likely that the exaggerated liver injury observed in obese-HCV subjects is, in part, driven by this pro-inflammatory cytokine profile. In addition, TNF-α plays a crucial role in glucose and lipid homeostasis. By antagonising adiponectin, TNF-α promotes steatosis by enhancing fatty acid uptake, inhibiting fatty acid oxidation and reducing lipid export.[22] Similarly, increased expression of TNF-α in the liver of obese-HCV infected subjects correlates with reduced insulin sensitivity. Taken together, excess fatty acids and increased insulin resistance can therefore perpetuate an inflammatory response with the generation of further mediators including TNF-α and IFN-γ.

In response to HCV infection, there is an increased intrahepatic expression of chemokines which are responsible for lymphocyte recruitment. In particular, we found the expression of both IP-10 and MCP-1 to be significantly enhanced in the liver of obese-HCV infected subjects. The expression of IP-10 is known to be increased in both the serum and liver of patients

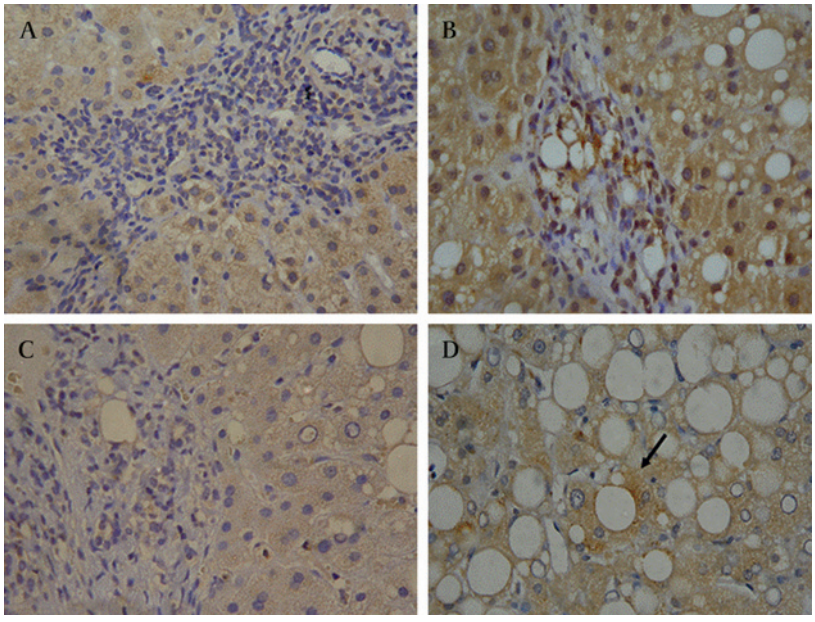

Figure 6 Immunohistochemical analysis of MCP-1 protein in studied groups. Compared to lean-HCV subjects (A), there was increased expression of MCP-1 in the portal tract inflammatory cells of obese-HCV subjects ($p=0.02$) (B). Compared to subjects with steatosis alone (C), there appeared to be increased hepatic expression of MCP-1 in subjects with NASH ($p=0.05$) (D). Staining of MCP-1 appeared accentuated in hepatocytes adjacent to fat cells (black arrow) (D). Non-diseased liver showed weak expression of MCP-1 in hepatocytes and biliary epithelium (data not shown). Original magnification ×400.

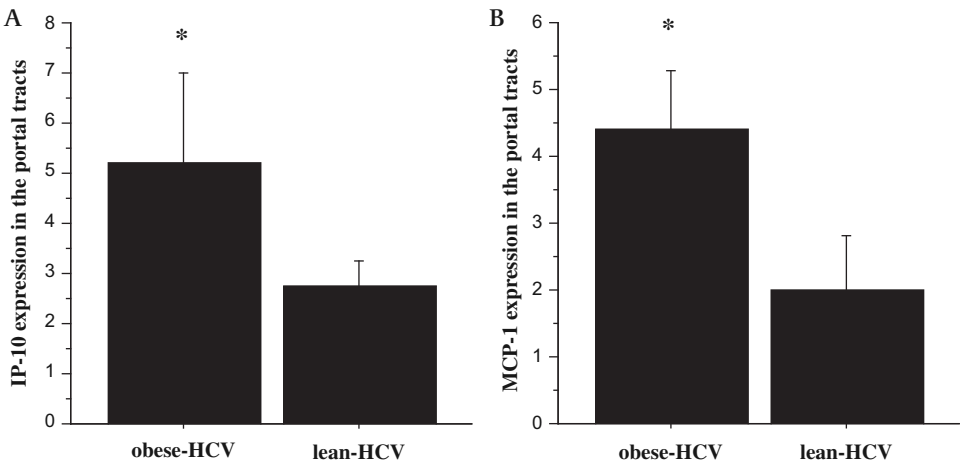

Figure 7 IP-10 (*p<0.001) (A) and MCP-1 (*p=0.02) (B) protein immunoreactivity in portal tracts of obese- versus lean-HCV subjects.

with CHC,[23] with expression correlating with both the degree of hepatic injury[23] and the response to antiviral therapy.[24–26] Furthermore in CHC, intrahepatic TH-1 cytokines and, in particular, IFN-γ, drive the increased expression of IP-10 and thereby promote the recruitment of lymphocytes to the liver. This is relevant in view of our observation of an increased TH-1 cytokine response in obese-HCV infected livers. Others have observed increased serum levels of IP-10 in overweight-HCV infected subjects with a BMI over 25 kg/m2 compared to those with a BMI below 25 kg/m2.[24] These elevated serum IP-10 levels were associated with a higher steatosis grade.[24] The reasons why IP-10 in particular is increased in conjunction with steatosis and HCV is not entirely clear. It has been shown however that saturated free fatty acids can directly augment the production of IP-10, and enhance migration of lymphocytes to inflammatory sites.[27] However, in liver samples with steatosis alone, there was no apparent increase in IP-10 expression. It is likely therefore that the increase in IP-10 mRNA expression in the liver of obese-HCV infected subjects is produced by the cumulative effects of fatty acid accumulation and HCV infection.

MCP-1 is important in monocyte and T cell recruitment, is induced by HCV and also has an emerging role in the pathogenesis of the metabolic syndrome.[28–30] In concordance with our data, during chronic hepatitis, increased expression of MCP-1 has been shown to be evident (by immunostaining and gene expression) in portal tract inflammatory infiltrates.[31] Further, increased expression of serum and hepatic MCP-1 was detected in subjects with NASH compared to those with steatosis alone.[30] Expression of MCP-1 is specifically increased in atherosclerotic lesions,[32 33] and inhibition of MCP-1 expression or that of its receptor CC chemokine receptor 2 reduces the extent of atheroma formation.[34] In obese mice, MCP-1 contributes to the development of hepatic steatosis, by inducing the expression of SREBP-1c—an important transcription factor for lipid synthesis.[29] Therefore taken together, in the setting of HCV and steatosis, MCP-1 is likely to play an important role in liver injury by promoting both hepatic inflammation and the further development of steatosis.

Increased hepatic chemokine expression during chronic inflammation would be expected to promote recruitment and retention of inflammatory cells at the site of injury.[35–37] In support of this paradigm, we detected increased T cell infiltration in the parenchyma and portal tracts of HCV-infected liver with steatosis. Of relevance to our observation, recent data in mouse models of obesity indicate that fatty liver has a significantly higher propensity to recruit circulating lymphocytes (CD4 and CD8 T cells as well as B cells), compared to non-fatty liver.[12] This property of fatty liver to recruit lymphocytes is further amplified in the presence of an additional inflammatory stimulus such as lipopolysaccharide.[12] Preferential homing of lymphocytes to mouse fatty liver was further shown to be due to increased lymphocyte sensitivity to chemokines, a response not observed in lean animals.[12] Thus, restoration of normal weight in obese mice abolished lymphocyte sensitivity to liver chemokines.[12]

Apparent low level expression of several of the cytokines and chemokines studied was found both in liver samples with normal histology and in those with steatosis alone (both by mRNA and protein expression techniques). This finding may support the concept that these mediators represent part of the physiological immune surveillance of the liver.[36] Alternatively, as these biopsy specimens were obtained from obese subjects, the low level expression of these cytokines and chemokines may relate to obesity or the metabolic syndrome. In this regard, it may be pertinent that the expression of TNF-α tended to be increased in the NASH group compared to both control subjects and those with steatosis alone. Similarly, in agreement with others in

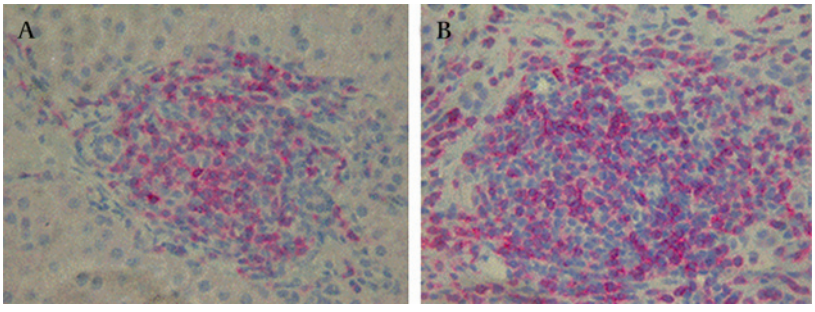

Figure 8 CD3 (T cell) protein expression in obese- and lean-HCV subjects. Compared to lean-HCV subjects (A), obese-HCV subjects (B) had increased T cell infiltration in areas of hepatic inflammation (p=0.02). Original magnification ×200.

NASH subjects, we found increased MCP-1 expression suggesting that these inflammatory mediators are also important in the pathogenesis of inflammation in NASH.

In summary it appears that both host and viral-related factors play a key role in driving the liver injury observed in subjects with CHC. In particular in obese-HCV subjects, there is upregulation of cytokines (TNF-α and IFN-γ) and chemokines (IP-10 and MCP-1) that are important in inducing both hepatic inflammation and lipid deposition thereby perpetuating further inflammation. The net effect of this mileu is an environment of chronic inflammation and progressive liver damage. This data is relevant to clinical practice as interrupting the forward loop of inflammation by viral eradication and or weight loss may be expected to impact favourably on liver injury.

Competing interests None.

Ethics approval This study was conducted with the approval of the St George Hospital in Sydney, Australia.

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