科技部補助專題研究計畫成果報告
期末報告

探索性別對三大本土慢性肝病的影響：一基於人與動物模式的聯合研究

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中 華 民 國 106 年 10 月 23 日

中 文 摘 要 ： 臨床研究證實，性別對肝臟疾病有著深遠影響。性激素，脂肪組織分佈，及性激素結合球蛋白間的 交互作用或可解釋肝臟疾病的性別差異。無論男女，性激素中的雌激素可抑制肝臟發炎，調節脂肪組織分佈，驅使全身醣類及脂肪的代謝平衡。在男性，睪丸激素經由雄激素受體作用，進而誘導肝臟儲 存膽固醇和肝癌形成。另外，性激素結合球蛋白是一種結合性激素的醣蛋白，其血清濃度經由性激素 和肝臟調節。女性停經後因雌激素濃度下降而促進肝臟纖維化，此為女性慢性肝病的一個負轉折點。 肝臟以其多重功效來調節基礎性代謝，因之慢性肝病會深刻影響性激素代謝。目前在台灣的三個主要 的慢性肝病分別是非酒精性脂肪肝，慢性B 型肝炎和慢性C 型肝炎。停經後女性非酒精性脂肪肝比率 兩倍於停經前。非酒精性脂肪肝脂肪激素的變化也男女迥異。而男性是B 型肝炎e 抗原血清陰轉後病 毒再活化，與肝硬化和肝癌的危險因子。以慢C 型肝炎而言，生育期的女性比同齡的男人病情輕微， 然則女性停經後便失去了這個優勢。相對於生理學上的“性別”，包括生理學和社會特徵的“性別”概念更應該在醫學上被推廣。而此廣義的性別醫學卻長期受到忽視，由換肝後的男性比女性更易求職 受雇可窺知一二。有關於性別影響台灣三個主要慢性肝病有些未解謎題如下：1.是否可用在停經前後性激素濃度來推測台灣女性罹患非酒精性脂肪肝，慢性B 型肝炎和慢性C 型肝 炎的嚴重度與預後(生理性)？2.男女非酒精性脂肪肝，慢性B 型肝炎和慢性C 型肝炎其脂肪激素和性激素結合球蛋白改變模式差異為何(生理性)？3.如何依性別及停經與否對酒精性脂肪肝，慢性B 型肝炎和慢性C 型肝炎患者作個人化的治療與追蹤(生理性與社會性)？吾人已成功開發出三種 C 型肝炎核心蛋白基因轉殖鼠，有著單純肝脂肪與脂肪性肝炎的不同表現 型。這可作為為C 型肝炎的動物模式。商業化的db/db 小鼠則可用作非酒精性脂肪肝的動物模式。而 小鼠尾靜脈高壓注射B 型肝炎病毒則可作為慢性B 型肝炎動物模型。此外，經由酒精性脂肪肝臨床病 例研究，吾人也已確立男女迥異的主要脂肪激素變化模式。總之，根據我們以前的研究結果，本計畫 旨在經由台灣非酒精性脂肪肝，慢性B 型肝炎和慢性C 型肝炎病人的前瞻性研究剖析關於肝內和肝外 症狀，聚焦於性激素，脂肪激素和性激素結合球蛋白改變，並以性別，經期對病人分類來釐清性別的 相關影響。同時以相對應的動物模式探索機轉。而性激素對上述三種慢性肝病的影響將進一步以雄性和雌性小鼠，正常雄性與去勢雄性小鼠的比較來闡明。此三年期計畫有望撥散臨床上的迷霧，揭示關 鍵的性別因素，並提供本土三個主要慢性肝病個人化醫療。

中 文 關 鍵 詞 ： 性別(生理性); 性別(生理性與社會性); 肝臟; 脂肪激素; 性激素結合球蛋白; 雌激素; 雄激素; 停經; 脂肪肝; 慢性B 型肝炎; 慢性C 型肝炎

英 文 摘 要 ： Currently, the three main chronic liver diseases in Taiwan are non-alcoholic fatty liver disease (NAFLD), chronic hepatitis B (CHB) and chronic hepatitis C (CHC). Using conditional transgenic mice that over-express the hepatitis C virus core in the liver, we have developed three mouse lines with phenotypes varying from simple hepatic steatosis

to steatohepatitis. This may reflect the hepatic histology of humans with CHC. Commercialized db/db mutant mice may serve as an animal model of NAFLD.While mice with tail vein hydrodynamic-injection with HBV plasmid may work as the CHB animal model. Furthermore, by using a consecutive series of NAFLD patients, we had shown that male and female NAFLD patients have distinct adipokine alteration patterns. Thus, based on our previous studies, the present proposal is designed to dissect the intriguing points regarding the impact of gender on the three main chronic liver diseases in Taiwan by conducting prospective cohorts of NAFLD, CHB and CHC with longitudinal follow-up of hepatic and extra-hepatic manifestations, focused on the sex hormones, adipokine and SHBG alteration, and stratified by sex, menstruation duration and the presence of menopause. In parallel, the associated basis will be probed by using the HCV core transgenic mice, db/db mice and hydrodynamic injected HBV mice with equivalent phenotypes. How the sex hormones affect the three chronic liver diseases will be further elucidated not only by comparing male with female mice, but also by comparing normal male with castrated male mice.

英文關鍵詞：sex; gender; liver; adipokine; SHBG; estrogen; androgen; menopause; NAFLD; CHB; CHC

關鍵詞：性別(生理性)；性別(生理性與社會性)；肝臟；脂肪激素；性激素結合球蛋白；雌激素；雄激素；停經；脂肪肝；慢性 B 型肝炎；慢性 C 型肝炎

臨床研究證實，性別對肝臟疾病有著深遠影響。性激素，脂肪組織分佈，及性激素結合球蛋白間的交互作用或可解釋肝臟疾病的性別差異。無論男女，性激素中的雌激素可抑制肝臟發炎，調節脂肪組織分佈，驅使全身醣類及脂肪的代謝平衡。在男性，睪丸激素經由雄激素受體作用，進而誘導肝臟儲存膽固醇和肝癌形成。另外，性激素結合球蛋白是一種結合性激素的醣蛋白，其血清濃度經由性激素和肝臟調節。女性停經後因雌激素濃度下降而促進肝臟纖維化，此為女性慢性肝病的一個負轉折點。肝臟以其多重功效來調節基礎性代謝，因之慢性肝病會深刻影響性激素代謝。目前在台灣的三個主要的慢性肝病分別是非酒精性脂肪肝，慢性 B 型肝炎和慢性 C 型肝炎。停經後女性非酒精性脂肪肝比率兩倍於停經前。非酒精性脂肪肝脂肪激素的變化也男女迥異。而男性是 B 型肝炎 e 抗原血清陰轉後病毒再活化，與肝硬化和肝癌的危險因子。以慢 C 型肝炎而言，生育期的女性比同齡的男人病情輕微，然則女性停經後便失去了這個優勢。相對於生理學上的“性別”，包括生理學和社會特徵的“性別”概念更應該在醫學上被推廣。而此廣義的性別醫學卻長期受到忽視，由換肝後的男性比女性更易求職受雇可窺知一二。

有關於性別影響台灣三個主要慢性肝病有些未解謎題如下：

1.是否可用在停經前後性激素濃度來推測台灣女性罹患非酒精性脂肪肝，慢性 B 型肝炎和慢性 C 型肝炎的嚴重度與預後(生理性)？

2.男女非酒精性脂肪肝，慢性 B 型肝炎和慢性 C 型肝炎其脂肪激素和性激素結合球蛋白改變模式差異為何(生理性)？

3.如何依性別及停經與否對酒精性脂肪肝，慢性 B 型肝炎和慢性 C 型肝炎患者作個人化的治療與追蹤(生理性與社會性)？

吾人已成功開發出三種 C 型肝炎核心蛋白基因轉殖鼠，有著單純肝脂肪與脂肪性肝炎的不同表現型。這可作為為 C 型肝炎的動物模式。商業化的 db/db 小鼠則可用作非酒精性脂肪肝的動物模式。而小鼠尾靜脈高壓注射 B 型肝炎病毒則可作為慢性 B 型肝炎動物模型。此外，經由酒精性脂肪肝臨床病例研究，吾人也已確立男女迥異的主要脂肪激素變化模式。總之，根據我們以前的研究結果，本計畫旨在經由台灣非酒精性脂肪肝，慢性 B 型肝炎和慢性 C 型肝炎病人的前瞻性研究剖析關於肝內和肝外症狀，聚焦於性激素，脂肪激素和性激素結合球蛋白改變，並以性別，經期對病人分類來釐清性別的相關影響。同時以相對應的動物模式探索機轉。而性激素對上述三種慢性肝病的影響將進一步以雄性和雌性小鼠，正常雄性與去勢雄性小鼠的比較來闡明。此三年期計畫有望撥散臨床上的迷霧，揭示關鍵的性別因素，並提供本土三個主要慢性肝病個人化醫療。

Keywords: sex; gender; liver; adipokine; SHBG; estrogen; androgen; menopause; NAFLD; CHB; CHC

Several clinical studies show a profound sex dimorphism in liver diseases. Interactions between sex hormones, adipose distribution and sex hormone-binding globulin (SHBG) may explain the sex-dimorphism. Among the sex hormones, estrogen improves hepatic inflammation, regulates adipose development, improves systemic glucose and lipid homeostasis in both males and females. In males, testosterone promotes hepatic cholesterol storage and hepatocellular carcinoma (HCC) formation. Moreover, SHBG is a serum glycoprotein exhibiting the unique feature of binding sex steroids. Its serum levels are regulated by sex hormone and liver. With menopause, the fall of estrogen levels can lead to fibrosis progression, and this represents a negative turning point for women with chronic liver disease. Currently, the three main chronic liver diseases in Taiwan are non-alcoholic fatty liver disease (NAFLD), chronic hepatitis B (CHB) and chronic hepatitis C (CHC). Interestingly, NAFLD is twice as common in postmenopausal women as in premenopausal women. The adipokine alteration patterns are distinct between the men and women with NAFLD. While male sex is a risk factor for reactivation of hepatitis B virus (HBV) infection after HBeAg seroconversion and for the development of cirrhosis and HCC. For CHC, women have milder disease than men during their reproductive years, although postmenopausal women lose this advantage and present with accelerated fibrosis progression in comparison with male patients of comparable ages. In contrast to " sex", the concept of "gender" includes both the biological and social characteristics, while gender-specific medicine is long-term neglected. For example, male but not female sex has favorable posttransplant employment indicates the gender- but not sex-difference in outcomes. There are some intriguing points regarding the gender-impacts on the three main chronic liver diseases: (1). Is there any cut-off level of sex hormone in determining the severity of the NAFLD, CHB and CHC in women according to pre-, peri- and post-menopausal periods (biological) ? (2).What are the specific alteration patterns of the adipokine and SHBG in patients with the NAFLD, CHB and CHC according to gender (biological) ? (3). How to treat and follow up the patients with NAFLD, CHB and CHC according to gender (biological and social) ?

Using conditional transgenic mice that over-express the hepatitis C virus core in the liver, we have developed three mouse lines with phenotypes varying from simple hepatic steatosis to steatohepatitis. This may reflect the hepatic histology of humans with CHC. Commercialized db/db mutant mice may serve as an

animal model of NAFLD.While mice with tail vein hydrodynamic-injection with HBV plasmid may work as the CHB animal model. Furthermore, by using a consecutive series of NAFLD patients, we had shown that male and female NAFLD patients have distinct adipokine alteration patterns. Thus, based on our previous studies, the present proposal is designed to dissect the intriguing points regarding the impact of gender on the three main chronic liver diseases in Taiwan by conducting prospective cohorts of NAFLD, CHB and CHC with longitudinal follow-up of hepatic and extra-hepatic manifestations, focused on the sex hormones, adipokine and SHBG alteration, and stratified by sex, menstruation duration and the presence of menopause. In parallel, the associated basis will be probed by using the HCV core transgenic mice, db/db mice and hydrodynamic injected HBV mice with equivalent phenotypes. How the sex hormones affect the three chronic liver diseases will be further elucidated not only by comparing male with female mice, but also by comparing normal male with castrated male mice. The current 3-year proposal holds promise to unveil the clinical controversies and may provide therapeutic interventions targeting crucial gender factors to control the three main chronic liver diseases in Taiwan.

報告內容:

Three papers entitled " "Association between Leptin and Complement in Hepatitis C Patients with Viral Clearance: Homeostasis of Metabolism and Immunity" and "Recovery of pan-genotypic and genotype-specific amino acid alterations in chronic hepatitis C after viral clearance: transition at the crossroad of metabolism and immunity." and "The evolving relationship between adiponectin and insulin sensitivity in hepatitis C patients during viral clearance" supported by the current grant had been published. The papers had been attached.

PLOS ONE

RESEARCH ARTICLE

Association between Leptin and Complement in Hepatitis C Patients with Viral Clearance: Homeostasis of Metabolism and Immunity

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Abstract

Background

The association between leptin and complement in hepatitis C virus (HCV) infection remains unknown.

Methods

A prospective study was conducted including 474 (250 genotype 1, 224 genotype 2) consecutive chronic hepatitis C (CHC) patients who had completed an anti-HCV therapy course and undergone pre-therapy and 24-week post-therapy assessments of interferon λ3-rs12979860 and HCV RNA/genotypes, anthropometric measurements, metabolic and liver profiles, and complement component 3 (C3), C4, and leptin levels.

Results

Of the 474 patients, 395 had a sustained virological response (SVR). Pre-therapy leptin levels did not differ between patients with and without an SVR. Univariate and multivariate analyses showed that sex (pre- and post-therapy, $p<0.001$), body mass index (BMI) (pre- and post-therapy, $p<0.001$), and C3 levels (pre-therapy, $p = 0.027$; post-therapy, $p = 0.02$) were independently associated with leptin levels with or without HCV infection. Pre-therapy BMI, total cholesterol (TC), C4 levels, and the rs12979860 genotype were independently associated with pre-therapy C3 levels in all patients. Post-therapy BMI, alanine aminotransferase, TC, C4 levels, white blood cell counts, and hepatic steatosis were independently associated with the post-therapy C3 levels of SVR patients. Compared with pre-therapy levels, SVR patients showed higher 24-week post-therapy C4 (20.32+/-7.30 vs. 21.55+/-7.07 mg/dL, $p<0.001$) and TC (171.68+/-32.67 vs. 186.97+/-36.09 mg/dL, $p<0.001$) levels; however, leptin and C3 levels remained unchanged after therapy in patients with and without an SVR.

collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions

Leptin and C3 may maintain immune and metabolic homeostasis through association with C4 and TC. Positive alterations in C4 and TC levels reflect viral clearance after therapy in CHC patients.

Introduction

Hepatitis C virus (HCV), a human pathogen responsible for acute and chronic liver disease, has variants classified into 7 major genotypes and infects an estimated 170 million individuals worldwide [1]. It affects insulin signaling, and much of its life cycle is closely associated with lipid metabolism [2]. In addition to cirrhosis and hepatocellular carcinoma, HCV is thought to cause metabolic alterations, including steatosis, dyslipidemia, insulin resistance (IR), diabetes, obesity, and cardiovascular events [2–4]. Although most HCV infections are currently curable using potent direct-acting anti-viral agents, not all HCV-associated cardiometabolic complications are reversible after viral clearance [2]. Adipose tissue has emerged as an important endocrine organ that exerts vital endocrine and immune functions via adipokines [5]. Moreover, free fatty acids and glycerol derived from visceral adipose tissue reach the liver and stimulate the biosynthesis of lipoprotein and glucose, respectively [6]. Because adipose tissues and the liver are functionally linked, elucidating the relationship between adipokine alterations and HCV infection has the potential to reveal the basis of HCV-associated cardiometabolic complications and probe the therapeutic targets.

The adipokine leptin, a product of the obese gene, is primarily expressed in adipose tissue but is also expressed in other organs, including the liver [7]. Most of the circulating leptin originates from subcutaneous, but not visceral adipose tissue, which may reduce its biological activity [5]. Leptin is crucial for maintaining total body fat and glucose homeostasis as well as regulating food intake and energy expenditure through a complex central feedback mechanism [8]. Its secretion is influenced by numerous physiological and hormonal factors. The leptin receptor is expressed in hypothalamic neurons, T cells, and hepatic stellate cells [9]. Importantly, leptin promotes IR to increase intracellular fatty acids in hepatocytes, amplifies proinflammatory responses [10], and mediates hepatic fibrogenesis during chronic liver injury [11] through the activation of hepatic stellate cells [12]. Concordantly, leptin levels are elevated in patients with a higher fibrosis index [13]. Importantly, leptin is critical for the modulation of adaptive and innate immune responses, such as regulating T-cell-mediated immune responses [14] and natural killer cell activity [15], as well as increasing complement component 3 (C3) levels [16]. Because both HCV infection and leptin are critically involved in metabolism, inflammation, and immunity [5–16], their potential relationship has attracted attention; however, no definite conclusion regarding such a relationship has been drawn [16–21]. In addition to the multifaceted functions of leptin, this uncertainty is primarily due to variability among individuals, which is difficult to completely eliminate from case-control studies, retrospective studies, or prospective studies with inadequate confounder adjustments. Indeed, although the impact of HCV infection on alterations in leptin levels is unclear, even less is known regarding whether viral genotype-specific influences on these alterations exist [21–23]. Therefore, we sought to elucidate the aforementioned relationships by conducting a prospective study to analyze the leptin levels adjusting for viral, metabolic, and immune profiles in genotype 1 (G1) and genotype 2 (G2) CHC patients who completed anti-HCV therapy.

Methods

Patients

The study group comprised subjects 18 years or older with G1 or G2 CHC, which was defined as the presence of documented HCV antibody positivity and detectable HCV RNA for >24 weeks. Subjects with heavy alcohol consumption (alcohol consumption more than 10 g/day for women and 20 g/day for men [5]), human immunodeficiency virus infection, hepatitis B infection, hemochromatosis, coronary heart disease, renal insufficiency, or malignancy and recipients of solid organ transplants were excluded.

Methods

A total of 250 G1 and 224 G2 CHC patients were consecutively recruited at a tertiary referral center between July 2010 and June 2015. All patients received anti-HCV therapy with weight-based pegylated interferon-α-2b (1.5 μg/kg/week) and ribavirin (800–1400 mg/day) for either 24 or 48 weeks according to the therapeutic response-guided protocol [1,4]. The HCV RNA levels were assessed using a COBAS Amplicor (Roche Diagnostics, Tokyo, Japan). The HCV genotype was determined using the InoLipa method (Roche Diagnostics). Single nucleotide polymorphisms of interferon λ3 (IFNL3 or interleukin-28B) rs12979860 were assessed using genomic DNA, as previously described [1,4]. The patients were evaluated for HCV RNA to examine the therapeutic response 2 weeks prior to therapy, after 4, 12, and 24 weeks of therapy, at the end of therapy, and 12 and 24 weeks after the end of therapy. At 2 weeks prior to therapy and 24 weeks after the end of therapy, after fasting, the patients were evaluated for body mass index (BMI), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), triglycerides (TGs), uric acid, homeostasis model assessment-estimated insulin resistance (HOMA-IR) [fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5], alanine aminotransferase (ALT), aspartate aminotransferase (AST)-to-platelet ratio index (APRI), white blood cell (WBC) count, platelet count, and high-sensitivity C-reactive protein (hsCRP), C3, C4, and leptin (R&D Systems, MN, USA) levels. Abdominal ultrasound studies were performed in every patient prior to therapy and every 6 months thereafter to assess the presence and severity of fatty liver and cirrhosis. IR was defined as a HOMA-IR score ≥2.5. A sustained virological response (SVR) was defined as undetectable HCV RNA levels 24 weeks after the completion of therapy.

A liver biopsy was performed in every patient before anti-HCV therapy to assess the liver histology. The liver biopsy specimens were semi-quantitatively scored by an experienced hepatopathologist blinded to the clinical data. Histological scores for steatosis and fibrosis were reported using the criteria of Kleiner et al. [24] and staged according to the Metavir scoring system [25], respectively.

Statistics

All statistical analyses were performed using the Statistical Package for Social Science software (SPSS ver. 21.0, SPSS Inc., Chicago, IL, USA). Continuous variables were analyzed using Student's t-test or the Mann-Whitney U test, and categorical variables were analyzed using the chi-square test or Fisher's exact test, as appropriate. Univariate and multivariate linear regression models were used to assess the relationships between various pre-therapy dependent and independent variables. Paired t-tests were used to compare the variables prior to and 24 weeks after anti-HCV therapy within the same individuals. The variable values were logarithmically transformed and then used for the statistical analyses where indicated. Statistical significance was defined at the 5% level based on two-tailed tests of the null hypothesis.

Informed consent

Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Chang Gung Memorial Hospital institutional review board.

Results

Pre-therapy leptin levels did not differ between patients with and without an SVR

The baseline characteristics of the 474 CHC patients are listed in Table 1. Patients with an SVR (n = 395) had a lower BMI, lower HCV RNA and HOMA-IR levels, and a lower prevalence of G1 HCV infection and severe fibrosis (F3 and F4) but a higher prevalence of the IFNL3-rs12979860 CC genotype than patients without an SVR (n = 79). No differences in pre-therapy leptin levels were noted between the patients with and without an SVR. With regard to the genotype impact on leptin, no difference in the pre-therapy leptin levels was noted between the G1 and G2 patients after sex stratification (p = 0.328 for the males and p = 0.177 for the females).

Sex, BMI, and C3 levels were independently associated with leptin levels regardless of viral presence

The results of the univariate and multivariate analyses performed to determine the factors associated with the pre-therapy (for all patients, n = 474) and 24-week post-therapy (for patients with an SVR, n = 395) leptin levels are listed in Table 2. The univariate analyses revealed that sex, pre-therapy BMI, C3 levels, and hepatic steatosis were associated with pre-therapy leptin levels, whereas the multivariate analysis showed that sex, pre-therapy BMI, and C3 levels were independently associated with the pre-therapy leptin levels. Regarding the post-therapy leptin levels, the univariate analyses showed an association with sex, post-therapy BMI, HOMA-IR, C3 levels, and hepatic steatosis, whereas the multivariate analyses showed that sex, post-therapy BMI, and C3 levels were associated factors. Regarding the pre-therapy HCV RNA levels, age [estimated β: -0.054, 95% confidence interval (CI) of β: -0.105–0.004, p = 0.035] and pre-therapy TGs (estimated β: 0.021, 95% CI of β: 0.01–0.033, $p<0.001$) were independent factors.

Because the HOMA-IR was reported to be positively correlated with the leptin serum levels in nondiabetic CHC patients [19,26], we stratified the patients by the presence of IR and performed univariate and multivariate analyses to determine the leptin levels. Pre-therapy HOMA-IR was an independent factor for pre-therapy leptin in patients without IR (estimated β: 3498, 95% CI: 1395–5600, p = 0.001) but not in patients with IR (p = 0.521).

TC and C4 levels were independently associated with C3 levels regardless of viral presence

Because sex, BMI, and C3 levels were associated with the leptin levels regardless of the presence of HCV, we investigated the factors associated with sex, BMI, and C3 using univariate and multivariate analyses (Table 3). Regarding the pre-therapy levels of all the patients, the univariate and multivariate analyses showed that uric acid and HDL-C were independently associated with sex; HDL-C, C3, and hepatic steatosis were independently associated with BMI; and BMI, TC, C4, and the IFNL3-rs12979860 CC genotype were independently associated with the C3 level. Regarding the post-therapy levels of the patients with an SVR, HDL-C, uric acid, and WBC count were independently associated with sex; HDL, HOMA-IR, uric acid, and C3 were

Table 1. Baseline characteristics of CHC patients.

	All CHC patients (n = 474)	Patients with SVR (n = 395)	Patients without SVR (n = 79)	Student's *t*-test p-values
Male, n (%)	270 (57.0)	228 (57.7)	42 (53.2)	0.456
Age (yr)	55.18+/-11.92	53.72+/-11.61	55.99+/-10.59	0.109
BMI	24.89+/-3.78	24.79+/-3.59	25.77+/-4.10	0.035*
HCV RNA (Log_{10}IU/ml)	5.97+/-1.12	5.84+/-1.18	6.45+/-0.75	<0.001*
HCV genotype [G1, n (%)]	250 (52.7)	187 (47.3)	63 (79.7)	<0.001*
AST (U/L)	75.06+/-68.55	73.06+/-61.63	73.74+/-63.90	0.933
ALT (U/L)	92.20+/-94.24	97.53+/-88.50	80.39+/-64.12	0.124
TC (mg/dL)	171.55+/-34.55	171.7+/-32.7	172.7+/-29.8	0.805
HDL (mg/dL)	42.82+/-13.81	48.22+/- 13.80	43.12+/-13.8	0.867
TG (mg/dL)	104.85+/-54.96	106.19+/-57.88	96.13+/-39.4	0.099
HOMA-IR	3.21+/-5.38	2.88+/-3.03	4.46 +/-9.42	0.039*
Uric acid (mg/dL)	5.91+/-1.56	5.94+/-1.54	5.86+/-1.50	0.709
WBC count (10^3/μL)	5.72+/-3.23	5.82+/-1.91	5.63+/-1.73	0.433
Platelets (10^3/μL)	176.3+/-64.3	181.4+/-58.1	156.0+/-58.7	0.001*
hsCRP (mg/dL)	1.87+/-3.72	1.68+/-3.13	1.56+/-1.95	0.774
C3 (mg/dL)	105.80+/-20.00	107.4+/-19.8	102.6+/-17.2	0.088
C4 (mg/dL)	20.42+/-7.89	20.56+/-7.70	19.25+/-7.28	0.238
APRI	1.65+/-2.05	1.47+/-1.62	1.75+/-1.83	0.195
Hepatic steatosis				
None, n (%)	166 (35)	150 (33)	16 (20.2)	0.395
Mild, n (%)	213 (45)	176 (44.6)	37 (46.8)	0.5
Moderate, n (%)	85 (17.9)	62 (15.7)	23 (29.1)	0.49
Severe, n (%)	10 (2.1)	7 (2.5)	3 (3.7)	0.48
Fibrosis				
F0, n (%)	76 (16)	68 (17.2)	8 (10.1)	0.095
F1, n (%)	171 (36)	151 (38.2)	20 (25.3)	0.17
F2, n (%)	166 (35)	138 (34.9)	28 (35.4)	0.317
F3, n (%)	38 (8)	25 (6.3)	13 (16.4)	0.049*
F4, n (%)	23 (5)	13 (3.3)	10 (12.6)	0.032*
Leptin (pg/ml)	9748.6+/-9968.2	9332+/-9049	12168+/-14092	0.267
Log leptin (Log_{10}pg/ml)	3.77+/-0.52	3.75+/-0.54	3.83+/-0.48	0.4
SNP rs12979860 CC, n (%)	401 (85)	348 (88.1)	53 (67.1)	0.013*

BMI: body mass index; G1: genotype 1; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TC: total cholesterol; HDL: high-density lipoprotein cholesterol; TGs: triglycerides; HOMA-IR: homeostasis model assessment-estimated insulin resistance; WBC: white blood cell; hsCRP: high-sensitivity C-reactive protein; C3: complement component 3; C4: complement component 4; APRI: AST to platelet ratio index; Log: logarithmic; SNP: single nucleotide polymorphism;

*: $p<0.05$.

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independently associated with BMI; and BMI, ALT, TC, WBC count, hepatic steatosis, and C4 were independently associated with the C3 level. The associated leptin-centered relationships between the dependent and independent factors (pre-therapy and post-therapy) are summarized in Fig 1.

After anti-HCV therapy, the lipid profile and C4 levels increased, and the C3 and leptin levels remained unchanged

Although trivial, patients with and without an SVR had a decreased BMI after anti-HCV therapy. However, only patients with an SVR had decreased AST, ALT, and APRI levels but

Table 2. Univariate and multivariate analyses of factors associated with pre- and post-therapy leptin levels.

	Pre-therapy leptin (all patients)		Post-therapy leptin (patients with SVR)	
	Univariate analysis 95% CI of β [β](p value)	**Multivariate analysis 95% CI of β [β](p value)**	**Univariate analysis 95% CI of β [β](p value)**	**Multivariate analysis 95% CI of β [β](p value)**
Sex (male)	-11325~-6839[–8857](<0.001*)	-12362.7~-8313.0[-10333.7] (<0.001*)	-13016~-7235[–10126] (<0.001*)	-39774.2~-18863.2[-12592.2] (<0.001*)
Age (yr)	-108.9~138.9[11.48] (0.851)		-88.2~204.0[57.8](0.436)	
BMI	999.7~1649.7 [1324](<0.001*)	1075.7~1679.3 [1377.5] (<0.001*)	729.3~1226.4[1210] (<0.001*)	1013.8~1813.8[1422.8] (<0.001*)
HCV RNA (Log_{10}IU/ml)	-493.8~2189.0[847.6](0.214)		NA	
HCV genotype	-2133.3~456.3[-838.4] (0.203)		NA	
AST (U/L)	-26.3~16.5[-4.81](0.653)		-227.0~109.2[-58.9](0.49)	
ALT (U/L)	-22.8~8.8[-7.0](0.384)		-94.1~115.9[10.9](0.838)	
TC (mg/dL)	-34.9~49.0[7.04](0.749)		-40.5~48.9[4.17](0.854)	
HDL (mg/dL)	-115.7~96.4[-9.6](0.858)		-15.8~233.8 [109.0](0.087)	
TG (mg/dL)	-10.1~43.3[16.6](0.223)		-21.4~25.8[2.22](0.853)	
HOMA-IR	-108.7~348.7[119.9](0.302)		32.8~935.6[484.2](0.036*)	-216.0~419.6[126.8](0.466)
Uric acid (mg/dL)	-668.1~1113.9[222.9](0.622)		-1677.9~346.3[-665.8](0.196)	
WBC count (10^3/μL)	-459.3~941.0[241.0](0.498)		-997.9~748.1[-124.9](0.778)	
Platelets (10^3/μL)	-12.1~32.8[10.37](0.364)		-45.8~11.6[-17.1](0.242)	
hsCRP (mg/dL)	-1.7~987.5[492.8](0.051)		-148.9~444.6[147.8](0.327)	
C3 (mg/dL)	72.1~207.5[139.8](<0.001*)	7.2~116.3[61.7](0.027*)	140.2~330.5[236.4](<0.001*)	16.8~185.8[101.0](0.02*)
C4 (mg/dL)	-120.1~240.2[59.1](0.521)		-36.6~435.2[199.4](0.097)	
APRI	-1183.1~348.7[-417.2](0.284)		-6173.4~4671.5[-740.8](0.788)	
Hepatic steatosis (yes)	2191.3~7591.7[4879](<0.001*)	-1527.6~2820.2[646.3] (0.558)	2275.8~8578.8[5420.5] (0.001*)	-501.9~4558.3 [2028.2](0.115)
SNP rs12979860	-2359.9~4020.2[830.1](0.608)		-2893.1~4819.8[963.4] (0.622)	

BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TC: total cholesterol; HDL: high-density lipoprotein cholesterol; TGs: triglycerides; HOMA-IR: homeostasis model assessment-estimated insulin resistance; WBC: white blood cell; hsCRP: high-sensitivity C-reactive protein; C3: complement component 3; C4: complement component 4; APRI: AST to platelet ratio index; SNP: single nucleotide polymorphism;
*: $p<0.05$.

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increased lipid profiles, uric acid, and C4 levels. The leptin and C3 levels remained unchanged regardless of the therapeutic response (Table 4).

Discussion

To the best of our knowledge, this prospective study is the first to demonstrate the relationship between leptin and complements in CHC patients. There were several compelling results. (1). No differences in pre-therapy leptin levels were noted between patients with and without an SVR or between G1 and G2 patients. (2). Leptin levels were not associated with HCV RNA levels. (3). Sex, BMI, and C3 levels were independently associated with the leptin levels regardless of the presence of HCV. (4). The IFNL3 genotype, pre-therapy BMI, TC, and C4 were associated with pre-therapy C3 levels, whereas post-therapy BMI, ALT, TC, WBC count, C4, and hepatic steatosis were associated with post-therapy C3 levels. (5). Although both the leptin and C3 levels remained unchanged after viral clearance, C4 and TC, which were independent factors for C3, were significantly increased 24 weeks post-therapy.

Table 3. Multivariate analyses of factors associated with pre- and post-therapy sex, BMI, and C3 levels.

	Pre-therapy dependent variables (all patients)			Post-therapy dependent variables (SVR patients)		
	Sex	BMI	C3	Sex	BMI	C3
	Multivariate analysis, 95% CI of OD [OD], (p value)	Multivariate analysis, 95% CI of β [estimated β], (p value)		Multivariate analysis, 95% CI of OD [OD], (p value)	Multivariate analysis, 95% CI of β [estimated β], (p value)	
Sex (male)					-0.701~1.075 [0.187](0. 679)	
BMI			0.441~1.514 [0.978](<0.001*)	0.893 ~1.070 [0.977] (0.621)		0.35~1.318 [0.834](0. 001*)
ALT (U/L)	1.0~1.004[1.002] (0.0059)		0.0~0.06 [0.03] (0.05)	0.987~1.025 [1.006] (0.529)	-0.01~0.04 [0.015] (0.239)	0.092~0.296 [0.194] (<0.001*)
TC (mg/dL)	0.914~1.005 [1.0] (0.934)		0.046~0.184 [0.115](0.001*)			0.027~0.019 [0.073](0.002*)
HDL (mg/dL)	0.934~0.965 [0.949] (<0.001*)	-0.077~-0.018 [-0.048](0.002*)	-0.295~0.074 [-0.11](0.24)	0.903~0.952 [0.927] (<0.001*)	-0.08~-0.011 [-0.046](0.009*)	-0.18~0.099 [-0.04](0.568)
TG (mg/dL)		-0.009~0.007 [-0.001](0. 778)	-0.006~0.085 [0.039](0.087)		-0.009~0.002 [-0.003](0.271)	-0.023~0.025 [0.001](0.936)
HOMA-IR		-0.018~0.137 [0.059](0.131)	-0.344~0.385 [0.02](0.912)		0.077~0.315 [0.196](0.001*)	-0.102~0.898 [0.398](0.118)
Uric acid (mg/dL)	1.462~1.919 [1.675] (<0.001*)		-0.443~2.305 [0.931](0.183)	1.41~2.233 [1.775] (<0.001*)	0.188~0.745 [0.466](0.001*)	-1.128~1.090 [-0.019](0.973)
WBC count (10^3/μL)	0.975~1.184 [1.074] (0.149)	-0.05~0.346 [0.148](0.141)	-0.779~1.452 [0.337](0.553)	1.011~1.411 [1.194] (0.037*)	-0.156~0.33 [0.087](0.481)	0.285~2.269 [1.277](0.012*)
Platelets (10^3/μL)			-0.028~0.061 [0.017](0.462)		-0.003~0.012 [0.004](0.277)	-0.013~0.049 [0.018](0.265)
hsCRP (mg/dL)		-0.082~0.208 [0.063] (0.39)	-0.261~0.188 [0.463](0.209)		-0.041~0.114 [0.037](0.355)	-0.037~0.606 [0.285](0.082)
C3 (mg/dL)		0.028~0.073 [0.05](<0.001*)			0.019~0.075 [0.047](0.001*)	
C4 (mg/dL)	0.987~1.034 [0.558] (0.383)	-0.051~0.064 [0.007](0.815)	0.74~1.302 [1.021](<0.001*)		-0.047~0.064 [0.008](0.766)	0.512~0.943 [0.728](<0.001*)
APRI			-2.231~1.415 [-0.408](0.66)			
Hepatic steatosis (yes)		0.354~1.885 [1.12](0.004*)	-2.4~5.7 [1.654] (0.423)		-0.47~0.064 [0.298] (0.445)	0.341~6.651 [3.496](0.03*)
SNP rs12979860 CC genotype			1.233~9.747 [5.49](0.012*)			

Only the variables significant in the univariate analyses (data not shown) were included in the multivariate analyses; OR: odds ratio; BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TC: total cholesterol; HDL: high-density lipoprotein cholesterol; TGs: triglycerides; HOMA-IR: homeostasis model assessment-estimated insulin resistance; WBC: white blood cell; hsCRP: high-sensitivity C-reactive protein; C3: complement component 3; C4: complement component 4; APRI: AST to platelet ratio index; SNP: single nucleotide polymorphism

*: $p<0.05$.

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Because both fibrosis and the IFNL3 non-CC genotype are two well-documented negative factors for SVR in interferon-based therapy [2], the reliability of this prospective study of 474 CHC patients who had completed a course of anti-HCV therapy is assured by the fact that the non-SVR patients had significantly more advanced fibrosis but a lower prevalence of the IFNL3 CC genotype than the SVR patients. Whether hyperleptinemia is associated with HCV infection [17,18,23] and the pan-genotypic [27,28] or genotype-specific anti-HCV therapeutic responses [29] has remained a matter of debate. In this study, based on the lack of an association between the leptin and HCV RNA levels, and the fact that the leptin levels remained

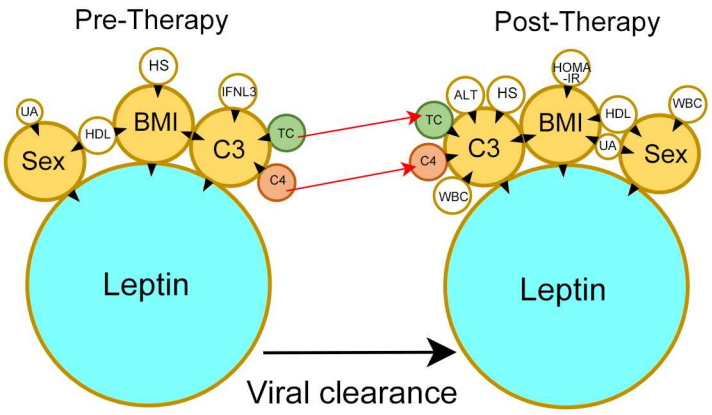

Fig 1. The leptin-centered associations between the dependent and independent factors before (pre-therapy) and 24 weeks after anti-hepatitis C therapy (post-therapy). Tips of black arrowheads: dependent factors; Bases of black arrowheads: independent factors; UA: uric acid; HDL-C: high-density lipoprotein-cholesterol; BMI: body mass index; HS: hepatic steatosis; IFNL3: interferon, λ3; TC: total cholesterol; C3: complement component 3; complement component 4: C4; WBC: white blood cell; HOMA-IR: homeostasis model assessment-estimated insulin resistance; red arrows: post-therapeutic increases in the TC and C4 levels.

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unchanged after an SVR, it is likely that any relationship between leptin alterations and HCV infections is indirect rather than direct. Furthermore, the role of leptin levels in predicting the anti-HCV therapeutic response is negligible, regardless of the HCV genotype, as the pre-therapy leptin levels between the SVR and non-SVR patients and between G1 and G2 patients were similar.

Due to the unavailability of immunoprecipitation which directly assesses the interaction between two proteins [30], it is truly difficult to elucidate the role of leptin in homeostasis upon viral clearance in clinical studies of CHC, especially as the pre- and post-therapy leptin levels were similar. Thus, we adopted the "concept" of popular software programs that organize high-throughput bioinformatic data, such as Metacore and IPA [31], to dissect the interactions between the independent and dependent factors based on the statistical results and the literature. Previous studies of 133~194 nondiabetic CHC patients [19, 26] showed a positive relationship between HOMA-IR and leptin. Consistently, we could only demonstrate this trend in the patients without IR but not in those with IR. All of the above confirmed that the connection between the HOMA-IR and leptin levels vanished with deteriorating glucose metabolism, which may be an HCV-associated sequela [2]. In contrast, sex, BMI, and C3 were independently associated with the leptin level regardless of the presence of HCV infection. With regard to sex and BMI, that female and obese patients have higher leptin levels than male and lean subjects, respectively, is a central dogma of leptin dynamics [5]. The positive association of leptin with C3 is likely due to the regulation of leptin in innate immunity [15] and the co-association of leptin with BMI [5], which was consistent with the results of previous non-HCV studies of the co-culture of adipocytes with macrophages [32] and of obese subjects [16]. The above findings seemed to indicate a non-HCV-specific phenomenon in a CHC cohort. However, the close association between leptin and C3 and the different trends in post-therapeutic changes in C3 and C4 suggest that HCV infection may affect leptin in a qualitative but not quantitative manner through leptin-associated factors. Both C3 and C4 are major proteins of the complement pathways [33], and their synthesis has been shown to be transcriptionally downregulated by the HCV core and NS5A proteins in *in vitro* studies [34,35]. However, this negative regulation is not compatible with the results of either our previous study on

Table 4. Comparison of the pre- and 24-week post-therapy variables in CHC patients stratified by the therapeutic response.

	SVR (+) (*n* = 395)		Paired *t*-test *p*-values	SVR (-) (*n* = 79)		Paired *t*-test *p*-values
	Pre-therapy value	Post-therapy value		Pre-therapy value	Post-therapy value	
BMI	24.77+/-3.65	24.36+/-3.53	<0.001*	25.84+/-4.10	24.86+/-3.60	<0.001*
AST (U/L)	73.08+/-62.01	26.21+/-11.15	<0.001*	74.93+/-64.40	73.37+/-66.06	0.841
ALT (U/L)	97.40+/-88.67	21.64+/-14.70	<0.001*	81.60+/-64.52	71.20+/-67.80	0.799
TC (mg/dL)	171.68+/-32.67	186.97+/-36.09	<0.001*	173.54+/-29.79	171.85+/-32.81	0.615
HDL (mg/dL)	48.15+/-13.80	49.85+/-13.35	<0.001*	48.83+/-14.82	49.91+/-14.77	0.314
TG (mg/dL)	101.35+/-46.74	120.73+/-75.58	<0.001*	115.97+/-66.98	104.79+/-47.70	0.069
HOMA-IR	2.88+/-4.75	2.74+/-2.98	0.374	4.50+/-5.94	4.69+/-7.81	0.703
Uric acid (mg/dL)	5.89+/-1.52	6.13+/-1.54	<0.001*	5.88+/-1.48	5.89+/-1.46	0.9
WBC count (10^3/μL)	5.85+/-1.94	5.83+/-1.81	0.801	5.56+/-1.69	5.13+/-1.26	0.032*
Platelets (10^3/μL)	182.3+/-58.78	184.53+/-56.51	0.239	154.9+/-58.73	148.9+/-54.2	0.203
hsCRP (mg/dL)	1.59+/-2.73	1.78+/-4.46	0.494	1.58+/-1.99	1.90+/-3.64	0.436
C3 (mg/dL)	107.2+/-19.74	108.6+/-17.39	0.169	101.9+/-16.66	102.6+/-17.58	0.677
C4 (mg/dL)	20.32+/-7.30	21.55+/-7.07	<0.001*	19.08+/-7.77	19.47+/-8.33	0.536
APRI	1.47+/-1.65	0.50+/-0.39	<0.001*	1.76+/-1.84	1.71+/-1.75	0.845
Hepatic steatosis (yes)	(51)	(52)	0.630	(43)	(49)	0.288
Leptin (pg/mL)	9441.4+/-8992.8	9809.4+/-9448.8	0.543	10229.7 +/-10528.5	10821.7+/-12650.4	0.763
Log leptin (Log_{10}pg/mL)	3.74+/-0.55	3.77+/-0.54	0.392	3.83+/-0.38	3.85+/-0.40	0.835

SVR: sustained virological response; Log: log transformation; BMI: body mass index; AST: aspartate aminotransferase; ALT: alanine aminotransferase; TC: total cholesterol; HDL: high-density lipoprotein cholesterol; TGs: triglycerides; HOMA-IR: homeostasis model assessment-estimated insulin resistance; WBC: white blood cell; hsCRP: high-sensitivity C-reactive protein; C3: complement component 3; C4: complement component 4; APRI: AST to platelet ratio index; SNP: single nucleotide polymorphism

*: $p<0.05$.

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conditional HCV core-expressing mice, which demonstrated C3 up-regulation in inflamed liver samples via microarray analyses [36], or with studies of CHC patients who had higher C3 and C4 levels than the controls [37]. The discrepancy may result from the fundamental differences between *in vitro* and *in vivo* immunological studies. In this clinical prospective study, we would like to stress that the positive associations between leptin and C3 and among C3, C4, and TC were consistent regardless of HCV infection (Fig 1). Interestingly, only C4 and TC but not C3 or leptin levels increased after SVR. Additionally, the pre-therapy IFNL3 genotype, a strong determinant of SVR [1,2,4], was associated with the pre-therapy C3 level, although this association diminished after anti-HCV therapy. Instead, the post-therapy C3 levels were affected by the post-therapy WBC count and ALT after viral clearance (Fig 1). This evolution suggests that C3 probably plays a role in HCV clearance. However, the role becomes non-HCV-specific after viral clearance. In contrast to leptin, which is primarily expressed in subcutaneous adipose tissue [5], higher expression levels of C3 and C4 have been reported in visceral than in subcutaneous adipose tissue [38]. The close associations between leptin, C3, and C4 suggest the presence of a strong collaboration between visceral and subcutaneous adipose tissues, which may be essential for maintaining whole-body homeostasis. Collectively, these findings highlight the general importance of leptin in homeostasis, as it needs to remain stable during viral infection but may modulate the immune response through C3, whose levels also

remain stable but seem to facilitate immunity and metabolism in conjunction with C4 and TC, respectively. After anti-HCV therapy, only SVR patients had decreased levels of transaminase and APRI but increased lipid profile levels, It is subsequent to the reversal of HCV-associated hepatic injury and hypolipidemia after viral clearance [2,4]. Similarly, the increase in C4 after viral clearance indicated the reversal of the HCV-associated down-regulation of the complement system [34,35].

Because adipose tissue is the major source of leptin [4], the main limitation of this study is the lack of a pathological study of adipose tissue. Moreover, making conclusions based on analyzing the associated factors is an imperfect way to build a complete picture of the leptin-associated pathways. Future studies of leptin in CHC patients with adipose tissue pathology surveys and associated fundamental cellular or animal models studies such as immunoprecipitation [30] may be required to elucidate the genuine connection and molecular basis between leptin and C3.

Together, our results demonstrate that sex, BMI, and C3 levels are independently associated with leptin levels and that TC and C4 levels are independently associated with C3 levels regardless of the presence of HCV. Compared with the pre-therapy levels, leptin and C3 remained unchanged 24 weeks post-therapy regardless of the therapeutic response, whereas the levels of C4 and TC increased among patients with an SVR. During HCV infection, leptin and C3 may maintain the homeostasis of metabolism and immunity based on associations with C4 and TC, the positive post-therapy alterations of which reflect viral clearance. These findings will facilitate the development of agents or strategies to probe viral-related metabolic and immunity alterations.

Supporting Information

S1 Dataset. The primary data of the current study are attached as supporting information.
(XLS)

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Author Contributions

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Formal analysis: HCH.

Funding acquisition: MLC.

Investigation: YYC.

Methodology: MLC.

Project administration: MLC.

Resources: CTC.

Software: CJK.

Supervision: MLC.

Visualization: CTC.

Writing – original draft: MLC.

Writing – review & editing: MLC.

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ORIGINAL ARTICLE

Recovery of pan-genotypic and genotype-specific amino acid alterations in chronic hepatitis C after viral clearance: transition at the crossroad of metabolism and immunity

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Abstract Recovery of amino acid (AA) metabolism and the associated clinical implications in chronic hepatitis C (CHC) patients with sustained virological response (SVR) following anti-hepatitis C virus (HCV) therapy remains elusive. A prospective cohort study was conducted on 222 CHC patients with SVR. Eighty-two age-matched male genotype 1 (G1) and G2 patients underwent paired serum metabolomics analyses with liquid chromatography–tandem mass spectrometry to examine AAs before and 24 weeks after anti-HCV therapy. Before anti-HCV therapy, G1 patients had a higher HCV RNA level than G2 patients. Twenty-four weeks post-therapy versus pre-therapy, repeated-measures ANOVA showed that the levels of alanine aminotransferase and most AAs decreased while those of lipids, glutamine and putrescine increased in CHC patients. The methionine sulfoxide/methionine ratio decreased, while the asymmetric dimethylarginine/arginine, glutamine/glutamate, citrulline/arginine, ornithine/arginine, kynurenine/tryptophan, tyrosine/phenylalanine and Fisher's ratios increased. Genotype-specific subgroup analyses showed that valine and serotonin/tyrosine increased in G1 and that kynurenine and tyrosine/phenylalanine increased and sarcosine decreased in G2 patients. Viral clearance in CHC patients pan-genotypically restored fuel utilization by decelerating the tricarboxylic acid cycle. Following improvement in liver function, the urea, nitric oxide, methionine, and polyamine cycles were accelerated. The cardiometabolic risk attenuated, but the augmented kynurenine pathway activity could increase the oncogenesis risk. The trends in neurotransmitter formation differed between G1 and G2 patients after SVR. Moreover, the HCV-suppressing effect of valine was evident in G1 patients; with the exception of prostate cancer, the oncogenesis risk increased, particularly in G2 patients, at least within 24 weeks post-anti-HCV therapy.

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Introduction

Hepatitis C virus (HCV), a major human pathogen responsible for liver disease, includes variants classified into seven genotypes and infects an estimated 170 million individuals worldwide (Chang et al. 2016). It is thought to cause liver steatosis, hypolipidemia, insulin resistance, diabetes, obesity, and cardiovascular events (Chang 2016; Hu et al. 2016), in addition to cirrhosis and hepatocellular carcinoma (HCC). In particular, much of the HCV life cycle is closely associated with lipid metabolism. Experimental data suggest that HCV directly interferes with the insulin cascade via proteasomal degradation of the insulin receptor substrate (Chang 2016). Although most HCV infections are currently curable using potent direct-acting anti-viral agents, HCV-associated cardiometabolic complications are not all reversible (Chang 2016), and HCV-associated HCC is not always completely eradicable after viral clearance (Toyoda et al. 2015). Clarifying the reversibility and associated basis of metabolic alterations in chronic hepatitis C (CHC) patients who have achieved sustained virological response (SVR) after anti-HCV therapy may allow for identification of therapeutic targets for HCV-associated complications. Moreover, the findings may be applicable to other viral infections, such as human immunodeficiency virus (HIV) infections, which are not currently eradicable but are critically involved in host metabolic alterations (Hu et al. 2016).

Previous studies have comprehensively demonstrated the manner by which HCV influences host lipid metabolism (Chang et al. 2014; Chang 2016; Hu et al. 2016). In contrast, although the liver is the primary site of amino acid (AA) metabolism (Fitian et al. 2014), where AAs are used as the major fuel source via the tricarboxylic acid (TCA) cycle (Jungas et al. 1992), nitrogen homeostasis is regulated via the urea and nitric oxide (NO) cycles (Breuillard et al. 2015), sulfur-containing AAs are metabolized via the methionine cycle (Jung 2015), and immunity and inflammation are regulated via the kynurenine (Kyn) and phenylalanine (Phe) pathways (Flydal and Martinez 2013), information regarding HCV-associated AA alterations is relatively scarce and limited to cross-sectional (Baniasadi et al. 2013; Bladowska et al. 2013) and case report (Babudieri et al. 2013) studies, for which it is difficult to completely eliminate individual bias. Indeed, although the impact of HCV infection on AA metabolic alterations is unclear, even less is known regarding whether genotype-specific alterations are present.

Metabolomics is a powerful technology for assessing global low-molecular-weight metabolites in a biological system (Dumas et al. 2014). In CHC patients, gas chromatography/mass spectrometry (GC–MS) and liquid chromatography (LC)–MS have been used to delineate the cholesterol synthesis pathway and to predict anti-HCV therapeutic responses, respectively, (Clark et al. 2012; Saito et al. 2013). To elucidate the characteristics and key mechanisms underlying the restoration of AA metabolism after viral clearance, we used LC–tandem mass spectrometry (LC–MS/MS) to identify the virus-associated pan-genotypic and genotype-specific AA alterations by conducting a prospective study of genotype 1 (G1) and genotype 2 (G2) CHC patients demonstrating viral clearance.

Materials and methods

Patients

As shown in Fig. 1, a total of 435 consecutive patients ≥18 years of age were confirmed to have CHC at a tertiary referral center between July 2009 and August 2014. CHC was defined as positivity for HCV antibodies and detectable HCV RNA for ≥24 weeks. Among the 435 patients, 377 had completed a course of anti-HCV therapy with peginterferon α-2b (1.5 μg/kg/week) and ribavirin (800–1400 mg/day) for up to either 24 or 48 weeks according to the response-guided therapy protocol (Chang et al. 2014, 2016). Abdominal ultrasound examinations were performed for each patient prior to the start of therapy and at 24 weeks post-therapy to assess the presence of fatty liver and cirrhosis. SVR was defined as an undetectable HCV RNA level at 24 weeks after completion of therapy. Patients with HIV, hepatitis B infection, hemochromatosis, renal insufficiency, coronary heart disease or malignancy and recipients of solid organ transplants were excluded. Moreover, female patients were excluded, as the menstruation status profoundly affects metabolism (Hu, et al. 2016). Finally, 222 male CHC patients with SVR after anti-HCV therapy were recruited. Among these 222 patients, 82 (41 G1 and 41 G2, age-matched) patients underwent paired serum targeted metabolomic studies.

Biochemistry and targeted metabolomics

At 2 weeks before the start of therapy and at 24 weeks after the end of therapy, the body mass index (BMI), HCV RNA, uric acid (UA), lipid [triglycerides (TGs), high-density lipoprotein cholesterol (HDL-C) and total cholesterol (TC)], fasting glucose, alanine aminotransferase (ALT), and bilirubin levels, homeostasis model assessment-estimated insulin resistance (HOMA-IR) [fasting insulin (μU/mL) × fasting glucose (mmol/L)/22.5], and metabolomic profiles, as well as the prevalences of liver cirrhosis and fatty liver,

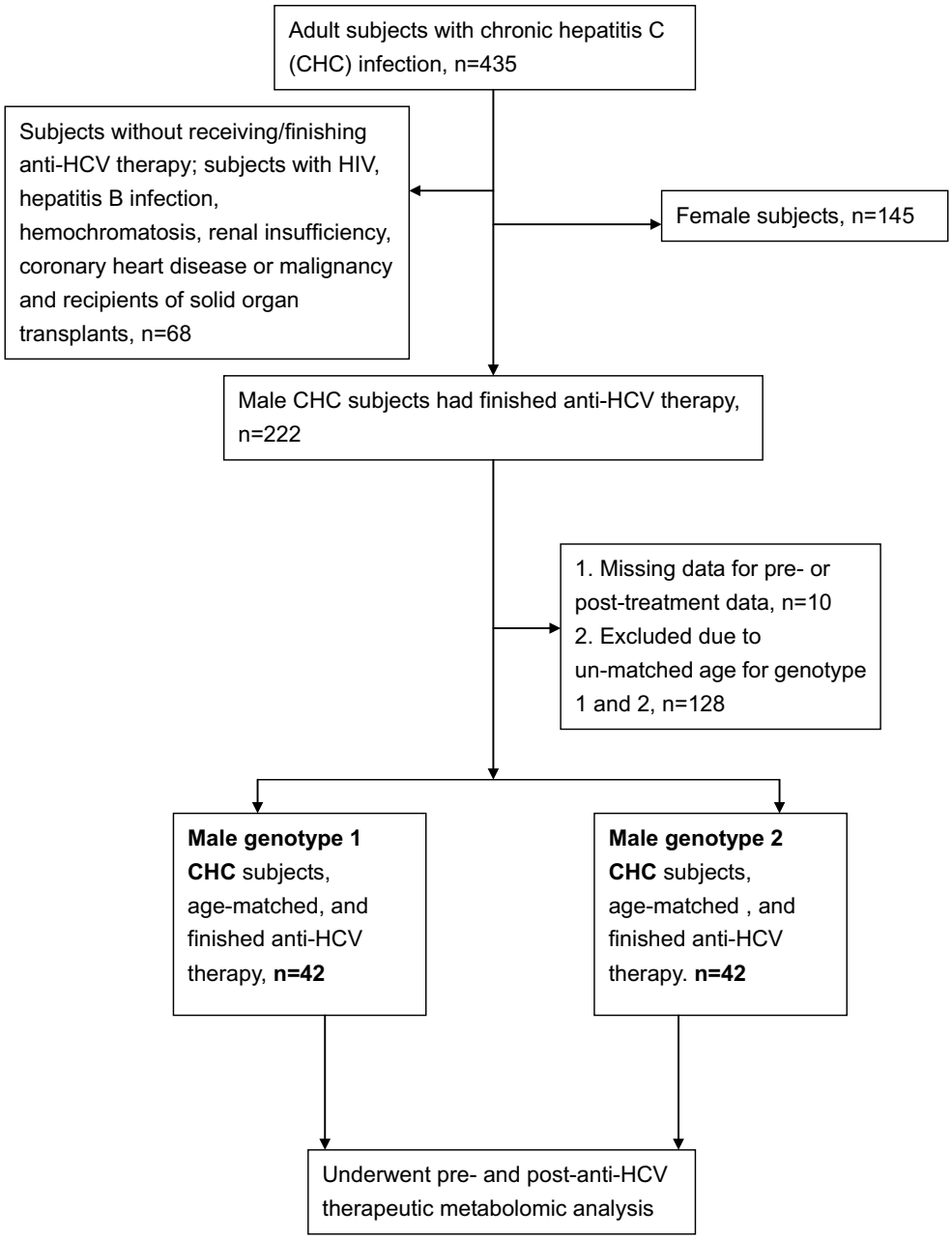


Fig. 1 Flowchart of the enrollment of chronic hepatitis C patients

were evaluated in the CHC patients. HCV RNA levels were determined using a COBAS Amplicor Kit (Roche Diagnostics, Tokyo, Japan). HCV genotypes were determined using the InoLipa method (Roche Diagnostics). Interferon-λ3 (IFNL3) or interleukin-28B rs12979860 single-nucleotide polymorphisms were assessed as described previously (Chang et al. 2014, 2016). Measurements of serum biochemical parameters and HCV RNA levels were performed in the clinical pathology or liver research laboratory in the hospital using routine automated techniques. Serum metabolome analyses were conducted with an AbsoluteIDQ® p180 Kit (Biocrates Life Science AG, Innsbruck, Austria) at the Metabolomics Core Laboratory, Healthy Aging Research Center, Chang Gung University, to identify and quantify 19 biogenic amines and 19 AAs. A 10-μL aliquot of each plasma sample was mixed with isotopically labeled internal standards in a multitier plate and dried under nitrogen. AAs and biogenic amines were derivatized with 5% phenyl isothiocyanate (PITC) for 20 min and subsequently dried under nitrogen. Three-hundred microliters of extraction solvent (5 mM ammonium acetate in methanol) was then added to each sample, and after 30 min of incubation,

Table 1 Characteristics of the 82 CHC patients with SVR received paired-metabolomic analysis

Variables	Pre-therapy		Student's *t* tests	24 weeks post-therapy		Paired *t* tests
	G1 ($n = 41$)	G2 ($n = 41$)	*p* values (G1 vs. G2)	G1 ($n = 41$)	G2 ($n = 41$)	*p* values (G1 vs. G2)
Age (years)	50.6 ± 12.0	52.4 ± 12.3	0.539			
BMI	25.4 ± 2.6	25.4 ± 3.9	0.943	25.0 ± 2.3	25.2 ± 3.9	0.943
HCV RNA (log IU/ml)	6.02 ± 1.23	5.56 ± 1.18	0.026*	0	0	
ALT (U/L)	102.5 ± 90.4	119.0 ± 106.2	0.452	22.6 ± 9.5	20.1 ± 8.9	0.233
Bilirubin (mg %)	0.92 ± 0.36	0.80 ± 0.27	0.108	0.86 ± 0.51	0.71 ± 0.23	0.093
Liver cirrhosis[a], *n* (%)	6 (14.6)	8 (19.5)	0.563	6 (14.6)	8 (19.5)	0.563
Fatty liver[a], *n* (%)	21 (51.2)	20 (48.8)	0.828	20 (48.8)	21 (51.2)	0.828
Uric acid (mg/dl)	6.85 ± 1.73	6.46 ± 1.26	0.251	6.97 ± 1.73	6.80 ± 1.54	0.629
TGs (mg/dL)	103.8 ± 40.1	99.6 ± 33.5	0.590	131.8 ± 67.3	118.3 ± 73.9	0.390
TC (mg/dL)	174.2 ± 33.0	170.6 ± 33.8	0.648	189.6 ± 39.5	184.2 ± 36.9	0.524
HDL-C (mg/dL)	42.0 ± 11.5	42.3 ± 10.7	0.731	43.4 ± 10.8	43.4 ± 8.6	0.991
HOMA-IR	2.94 ± 1.85	3.19 ± 4.51	0.754	3.2 ± 2.1	2.5 ± 1.3	0.085
IFNL3 CC[a] genotype, *n* (%)	37 (90.2)	35 (85.3)	0.110			

CHC chronic hepatitis C, *G1* genotype 1, *G2* genotype 2, *SVR* sustained virological response, *NA* not accessible, *BMI* body mass index, *IFN-λ3* interferon-λ3, *ALT* alanine aminotransferase, *TGs* triglycerides, *TC* total cholesterol, *HDL-C* high-density lipoprotein cholesterol, *HOMA-IR* homeostasis model assessment-estimated insulin resistance, *IFNL3*: interferon-λ3

* $p < 0.05$

[a] Chi-squared test

they were centrifuged for 2 min at 100×*g*. Subsequently, 150 μL of each filtrate was transferred to a microtiter plate and diluted with 150 μL of water for analysis of AAs and biogenic amines by LC–MS/MS. Identification and quantification were achieved by multiple reaction monitoring (MRM). It was standardized by spiking in isotopically labeled standards. LC–MS analysis was performed with a Waters Xevo TQ mass spectrometer coupled to an ultra-performance liquid chromatograph (Waters Corp., Milford, MA, USA). Metabolites were separated on a reverse-phase column (2.1 × 50 mm, BEH C18, Waters Corp., Milford, MA, USA) using a mobile phase composed of a gradient mixture of solvent A (0.2% formic acid in water) and solvent B (0.2% formic acid in acetonitrile) (0 min 0% B, 3.5 min 60% B, 3.8 min 0% B, and 3.9 min 0% B). Elution was performed at a flow rate of 900 μL/min. The column temperature was maintained at 50 °C. The corresponding MS settings were as follows: dwell time of 0.019–0.025 s; capillary voltage of 3.92 kV for the positive mode; capillary voltage of 1.5 kV for the negative mode; nitrogen as the collision gas medium; and a source temperature of 150 °C.

Statistics

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS, version 21, SPSS Inc., Chicago, IL, USA), TArgetLynx (Waters, MA, USA), integrated MetIDQ (Biocrates, Innsbruck, Austria) or SIMCA-P (version 13.0, Umetrics AB, Umea, Sweden) softwares. For the characteristics of the 82 CHC patients with SVR, continuous variables were summarized as the mean ± standard deviation (SD), and categorical variables were summarized as frequencies and percentages. To compare the variables between groups, continuous variables were analyzed using Student's *t* test, whereas categorical variables were analyzed using the Chi-squared test or Fisher's exact test as appropriate. The paired *t* test was used to compare variables before and at 24 weeks after anti-HCV therapy within individuals. To maximize the identification of differences in the metabolic profiles between two groups, unsupervised principal components analysis (PCA) and the orthogonal projection to latent structure-discriminant analysis (OPLS-DA) models were applied. The variable importance in the projection (VIP) value of each variable in the model was calculated to determine its contribution to the classification. VIP values of greater than 1.0 indicated significant differences. Changes in AA levels and the ratios or sums of AA levels before and at 24 weeks after anti-HCV therapy were evaluated using repeated-measures analysis of variance (ANOVA), considering time as a within-subject effect.

Informed consent

Written informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected a priori. The study was approved by the local institutional review board.

Results

Pre-therapy and 24 weeks post-therapy characteristics of the CHC patients

The characteristics of the 82 CHC patients who were assessed by paired metabolomics analyses before and at 24 weeks after anti-HCV therapy are listed in Table 1. The G1 patients had a higher pre-therapy HCV RNA level than the G2 patients. At 24 weeks post-therapy, no differences in any of the comparable variables were noted between the G1 and G2 patients; however, all of the patients with SVR had a decreased ALT level ($p < 0.001$) and increased TC ($p < 0.001$) and TG levels ($p = 0.001$) compared with the pre-therapy levels.

Targeted metabolomics

Although the PCA plot did not successfully discriminate between the AA metabolites altered pre-therapy versus 24 weeks post-therapy, the OPLS-DA score plot clearly differentiated between the metabolites altered at these time points (Fig. 2). The major findings are itemized below.

Characteristic AAs with decreased levels after SVR

As shown in Table 2, at 24 weeks post-therapy, the levels of many AAs and amines, including arginine (Arg), aspartic acid (Asp), glutamate (Glu), glycine (Gly), histidine (His), leucine (Leu), Phe, serine (Ser), threonine (Thr), tyrosine (Tyr), acetylornithine (Ac-Orn), asymmetric dimethylarginine (ADMA), and methionine sulfoxide (Met-SO), were decreased compared with the pre-therapy levels, while the levels of glutamine (Gln), Kyn, putrescine and taurine were increased in the CHC patients.

AA and amines ratios altered after SVR

As shown in Table 3, at 24 weeks post-therapy, the Met-SO/Met ratio was decreased, but the ADMA/Arg, Gln/Glu, citrulline (Cit)/Arg, Kyn/tryptophan (Trp), ornithine (Orn)/Arg, Tyr/Phe and Fisher's ratios [sum of branched-chain AAs (BCAAs, i.e. Val + Leu + Ile)/sum of aromatic AAs (AAAs, i.e. Tyr + Try + Phe)] were increased, compared with the pre-therapy ratios in the CHC patients.

Genotype-specific AA and amine alterations after SVR

In addition to the common alterations in the levels of the AAs and amines listed above, subgroup analyses showed that the levels of Arg, Ile, Leu and Met were decreased and that the level of Val was increased, particularly in the G1 CHC patients, at 24 weeks post-therapy compared with the pre-therapy-levels; in addition, the levels of Asp, ADMA and sarcosine were decreased, while those of Kyn and taurine were increased, particularly in the G2 patients, at 24 weeks post-therapy (Table 4).

Genotype-specific alterations in AA ratios after SVR

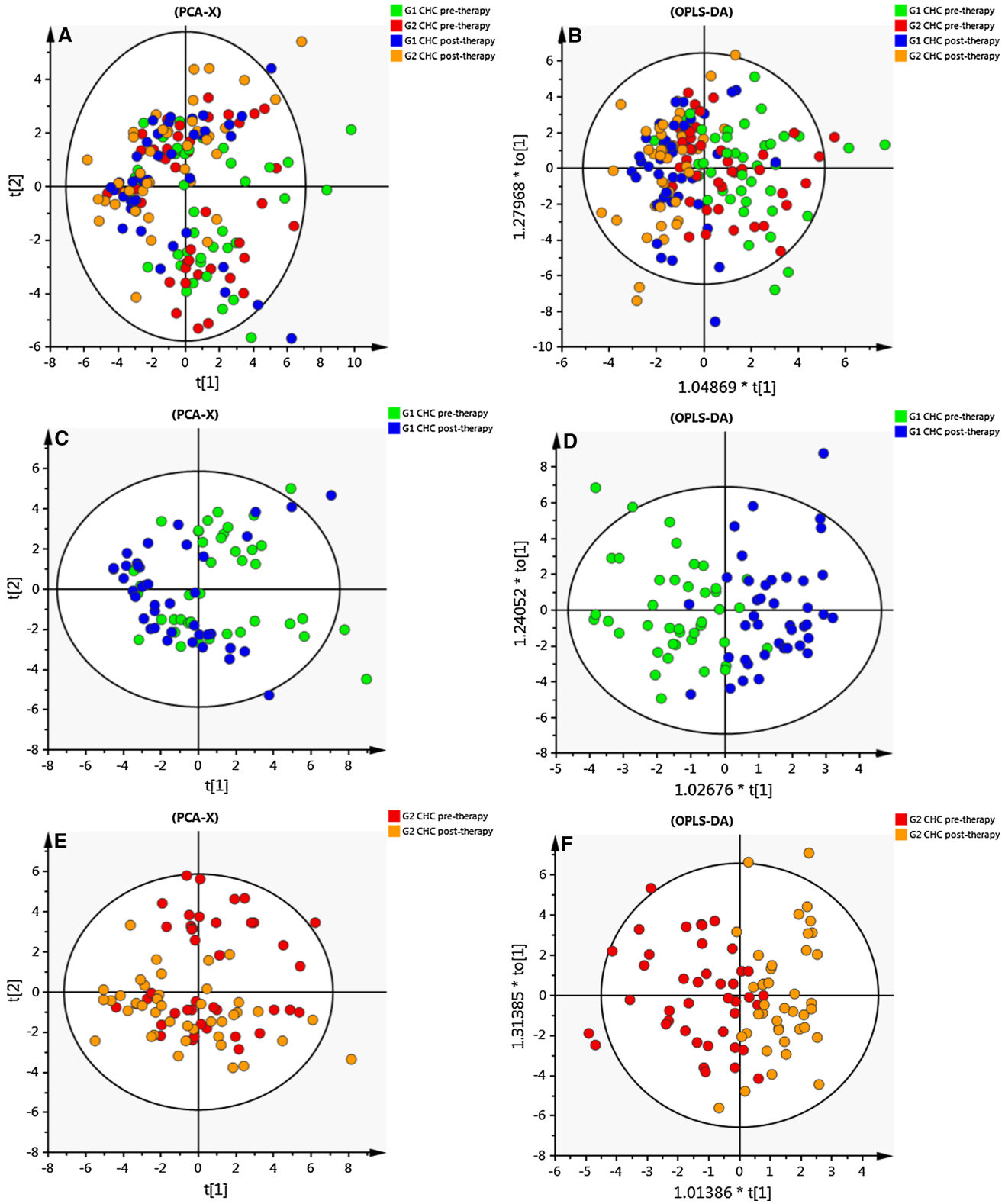
In addition to the common alterations in the ratios listed above, subgroup analyses showed that the serotonin/Trp and Tyr/Phe ratios were increased in the G1 and G2 patients, respectively, at 24 weeks post-therapy compared with the pre-therapy ratios (Table 4).

A comprehensive summary of all involved pan-genotypic and genotype-specific AA and amine alterations and the proposed associated pathways in the CHC patients after SVR is presented in Fig. 3.

Discussion

To the best of our knowledge, this metabolomics-based prospective study is the first to comprehensively examine the pan-genotypic and genotype-specific AA and amine alterations upon viral clearance in HCV. The most compelling results are as follows: (1) the G1 patients had a higher pre-therapy HCV RNA level than the age-matched G2 patients. After SVR, the decreased ALT and increased lipid levels were noted in all of the CHC patients. (2) At 24 weeks post-therapy, the levels of most AAs were decreased, while those of Gln, Kyn, putrescine and taurine were increased compared with the pre-therapy levels in the CHC patients with SVR. (3) A decreased Met-SO/Met ratio and increased ADMA/Arg, Gln/Glu, Cit/Arg, Kyn/Trp, Orn/Arg, Tyr/Phe and Fisher's ratios were detected in the CHC patients with SVR. (4) Subgroup analyses of genotype-specific changes revealed that alterations in the BCAA levels and serotonin/Trp ratio were predominant in the G1 patients, while changes in the ADMA, Kyn, sarcosine and taurine levels and Tyr/Phe ratio were more frequent in the G2 patients.

HCV has been reported to activate glycolysis (Ramière et al. 2014), leading to entry of glycolytic carbon into the TCA cycle to provide additional energy and metabolic precursors of AAs and fatty acids (Jungas et al. 1992). Our data demonstrated that the levels of most AAs, including both essential and non-essential AAs, were decreased after SVR. The levels of most AAs have been reported to be elevated in a persistently HCV-infected cell line (Sugiyama et al. 2014), and downregulation of enzymes involved in AA metabolism was reported in a CHC patient who achieved SVR (Babudieri et al. 2013). Thus, after viral clearance, decreased levels of essential AAs indicate the decreased uptake of essential AAs from the diet by host cells, i.e., attenuation of the hypermetabolic status, while



decreased levels of non-essential AAs indicate deceleration of the TCA cycle (Sugiyama et al. 2014). On the other hand, decreased levels of AAs might also reflect enhanced protein synthesis, as decomposition of the host body's own tissues to produce AAs for generation of energy is not necessary after SVR (Holm et al. 1999). In the presence of impaired liver function, decreased BCAA levels and increased AAA levels lead to a decrease in Fisher's ratio,

◂**Fig. 2** The principal components analysis (PCA) and orthogonal partial least squares-discriminant analysis (OPLS-DA) score plots for the paired metabolomics analyses of the chronic hepatitis C (CHC) patients. *G1 CHC* genotype 1 chronic hepatitis C patients, *G2 CHC* genotype 2 chronic hepatitis C patients, *pre-therapy* before anti-HCV therapy, *post-therapy* 24 weeks after anti-HCV therapy. Plasma global metabolite analysis by liquid chromatography/tandem mass spectrometry. **a**, **c** and **e** PCA of serum samples from 41 pre-therapy (*green dots*) and post-therapy (*blue dots*) G1 patients and 41 pre-therapy (*red dots*) and post-therapy (*orange dots*) G2 patients. The *ellipse* shown in the model represents the Hotelling T2 values with 95% confidence. **b**, **d** and **e** OPLS-DA of serum samples from 41 pre-therapy (*green dots*) and post-therapy (*blue dots*) G1 patients and 41 pre-therapy (*red dots*) and post-therapy (*orange dots*) G2 patients. The *ellipse* shown in the model represents the Hotelling T2 with 95% confidence

which is the ratio of BCAAs to AAAs (Kawaguchi et al. 2011). These changes are caused by increased BCAA catabolism in muscles and decreased AAA breakdown in the diseased liver (Holecek 2015). Consistent with the decreased ALT level and increased lipid levels (Chang et al. 2014; Chang 2016), the increased Fisher's ratio in the CHC patients after SVR reflected improved liver function, resulting in a cascade of accelerating associated reactions including NO, urea, Met, polyamine, and Kyn cycles (Fig. 3). Notably, Gln plays a crucial role in the inter-organ transport of carbon, nitrogen, and energy. In the liver, it is a substrate for ureagenesis, as its hydrolysis provides Glu for urea synthesis and gluconeogenesis (Xiao et al. 2016). Deamination of Gln and Glu can account for approximately 35% of the increase in ammonia production. In addition, Gln generates the antioxidant glutathione to remove reactive oxygen species (ROSs) and regulates critical cell signaling pathways (Burgess 2013). The Gln/Glu ratios are negatively associated with the diabetes risk in the Framingham Heart cohort (Cheng et al. 2012). Thus, the increased Gln level, decreased Glu level, and increased Gln/Glu ratio in the CHC patients after SVR indicate reduced risks of cardiometabolic complications subsequent to attenuation of oxidative stress. Accordingly, anti-HCV therapy has been shown to improve the cardiovascular outcomes of diabetic CHC patients (Hsu et al. 2014). With regard to the NO and urea cycles, Arg plays a crucial role in the synthesis of NO, the most potent endogenous vasodilator (Papageorgiou et al. 2015), through the conversion of Arg to Cit via the enzyme NO synthase (NOS). Arg can also be metabolized by arginase to generate urea and Orn. Orn can be further converted to Cit in the urea cycle. In the CHC patients after SVR, the increased Cit/Arg and Orn/Arg ratios, thus, indicated the presence of increased NOS for NO synthesis and increased arginase for transforming ammonia into urea, respectively (Lin et al. 2016). Moreover, Arg is a precursor for methylated metabolites, including ADMA, an intrinsic competitive endogenous feedback inhibitor of the catalytic activity of NOS (Fultang et al. 2016). The ADMA level is increased in conditions associated with atherosclerosis, liver and renal diseases. The plasma ADMA/Arg ratio is considered a stronger prognostic marker of atherosclerotic risk than ADMA alone (van Dyk et al. 2015). Thus, in the CHC patients after SVR, the decreased cardiovascular risks, as indicated by the increased Gln/Glu, Cit/Arg and Orn/Arg ratios, must be weighted by the increased atherosclerotic risk, as indicated by the increased ADMA/Arg ratio; while the basis of the increased ADMA/Arg ratio requires further study. Met is an essential sulfur-containing AA metabolized mainly in the liver that is converted to S-adenosylmethionine (SAM), which participates in polyamine synthesis and transsulfuration to transfer sulfur from Met to Ser, forming Cys and then taurine (Jung 2015). The hepatic polyamine synthesis and transsulfuration pathway activities are impaired in hepatic injury. Taurine is a sulfur-containing AA and organic osmolyte that is involved in a variety of physiological activities, including anti-oxidative, anti-metabolic syndrome, and anti-inflammatory activities (Imae et al. 2014). Consistently, both human and animal studies have shown the perturbation of taurine metabolism during HCV infection (Zhang et al. 2013; Sun et al. 2013). The increased taurine level after SVR, thus, reflected the acceleration of transsulfuration following the improvement in liver function. On the other hand, putrescine is a polyamine involved in transcription, translation, autophagy and stress resistance. It is predominantly derived from Orn and Met, while Arg and lysine serve as alternative, secondary sources of these metabolites (Miller-Fleming et al. 2015). Along with the decreased Met and Arg levels, the increased putrescine level after SVR was consistent with the accelerating polyamine biosynthesis subsequent to the improvement in liver function. Methylthioadenosine (MTA) is a sulfur-containing adenine nucleoside produced from SAM during the synthesis of polyamines, including spermine and spermidine. MTA has an inhibitory effect on the synthesis of spermine from putrescine. A significant decrease in the MTA level has been observed during the late stage of HCV infection in cells (Roe et al. 2011). After SVR, reversal of the HCV-associated decrease in the MTA level might also account for the increased putrescine level. Moreover, Met is particularly susceptible to elevated ROS levels. Upon reacting to ROSs, protein-bound Met is readily oxidized to form Met-SO (Jung 2015). Thus, in CHC patients after SVR, a decreased Met-SO level and Met-SO/Met ratio indicate decreased oxidative stress subsequent to improved liver function. Similar to what occurs in the Kyn cycle, 95% of Trp is metabolized in the liver by indoleamine-2,3-dioxygenase (IDO), leading to the biosynthesis of Kyn (Flydal and Martinez 2013), which is an immunosuppressive derivative of Trp, through induction of T-cell exhaustion and Treg expansion. IDO induces inflammation but also controls infection. Both HCV infection and

Table 2 Comparison of pre-therapy and post-therapy levels of amino acids (or amines) for CHC patients with SVR

AA (M)	Pre-therapy levels Median/mean (SD)	Post-therapy levels	Repeated-measures ANOVA F test *p* values Within-subject effect
	$N = 82$	$N = 82$	Time
Arg	157.5/162.9 (34.33)	122.0/133.1 (60.19)	0.0001
Asp	20.30/22.2 (12.38)	14.85/18.25 (11.44)	0.0147
Gln	605.5/575.9 (182.34)	680.0/672.9 (120.95)	1.57×10^{-6}
Glu	149.5/183.1 (146.6)	63.0/80.7 (61.7)	9.20×10^{-9}
Gly	311.0/319.1 (84.13)	274.5/280.8 (62.65)	0.0003
His	104.5/104.1 (17.03)	95.3/96.6 (15.33)	0.0012
Leu	179.0/183.4 (36.02)	168.5/169.4 (36.69)	0.0014
Met	29.6/31.5 (8.45)	27.1/28.0 (5.77)	0.0008
Phe	135.0136.1 (46.6)	88.4/100.3 (35.88)	2.74×10^{-8}
Ser	189.5/186.4 (38.49)	147.5/150.2 (32.53)	2.61×10^{-10}
Thr	130.5/134.0 (25.85)	117.0/118.6 (22.58)	2.18×10^{-7}
Tyr	86.8/90.0 (20.67)	72.6/75.9 (18.77)	2.13×10^{-11}
Ac-Orn	4/4.8 (3.67)	3.05/4.3 (3.16)	0.0007
ADMA	0.50/0.47 (0.12)	0.40/0.42 (0.12)	0.0008
Kyn	2.40/2.43 (0.76)	2.50/2.66 (0.91)	0.0125
Met-SO	1.05/1.15 (0.67)	0.30/0.41 (0.68)	2.65×10^{-12}
Putrescine	0.10/0.08 (0.04)	0.10/0.13 (0.04)	0.0002
Taurine	59.9/67.005 (25.01)	68.3/78.1 (33.29)	0.0075

Only metabolites with *p* values <0.05 are listed

ANOVA analysis of variance, *SD* standard deviation, *AA* amino acid, *CHC* chronic hepatitis C, *SVR* sustained virological response, *Arg* arginine, *Asp* Aspartic acid, *Gln* glutamine, *Glu* glutamate, *Gly* glycine, *His* histidine, *Leu* leucine, *Met* methionine, *Phe* phenylalanine, *Ser* serine, *Thr* threonine, *Tyr* tyrosine, *Ac-Orn* acetylornithine, *ADMA* asymmetric dimethylarginine, *Kyn* kynurenine, *Met-SO* methionine sulfoxide

Table 3 Comparison of pre-therapy and post-therapy ratios of amino acids (or amines) for CHC patients with SVR

AA	Median/mean (SD)		Within-subject effect
	Pre-therapy levels	Post-therapy levels	Repeated-measures ANOVA F test *p* values
	$N = 82$	$N = 82$	Time
ADMA/Arg	0.003/0.0030 (0.0011)	0.003/0.0034 (0.0014)	0.0137
Gln/Glu	4.19/6.52 (5.96)	9.79/11.87 (7.47)	2.82×10^{-9}
Cit/Arg	0.22/0.22 (0.06)	0.27/0.27 (0.08)	3.52×10^{-9}
Fisher ratio	1.72/1.74 (0.34)	1.99/2.04 (0.43)	6.7×10^{-9}
Kyn/Trp	0.03/0.03 (0.01)	0.04/0.04 (0.01)	0.0075
Met-SO/Met	0.04/0.04 (0.02)	0.01/0.01 (0.02)	1.87×10^{-11}
Orn/Arg	0.62/0.65 (0.22)	0.78/0.80 (0.31)	0.0001
Tyr/Phe	0.66/0.72 (0.24)	0.79/0.82 (0.26)	0.0047

Only metabolites with *p* values <0.05 are listed

ANOVA analysis of variance, *SD* standard deviation, *ADMA/Arg* asymmetric dimethylarginine/arginine, *Gln/Glu* glutamine/glutamate, *Cit/Arg* citrulline/arginine, *Fisher ratio* ratio of sum of branched amino acids/sum of aromatic amino acids, *Kyn/Trp* kynurenine/tryptophan, *Orn/Arg* ornithine/arginine, *Tyr/Phe* tyrosine/phenylalanine

interferon-α therapy are thought to stimulate IDO expression (Hoyo-Becerra et al. 2014). An increased Kyn/Trp ratio, an estimate of IDO enzymatic activity, represents an independent marker of progression of diseases including obesity, HIV infection and HCV infection (Hoyo-Becerra et al. 2014). However, NO inhibits the expression and function of IDO. Although NO cycle activity was promoted as mentioned above, paradoxically, the Kyn level and Kyn/Trp ratio were unexpectedly increased in the CHC patients after SVR. As a previous study has shown that the Kyn level

Table 4 Significant genotype-specific changes of amino acids or amines

Genotype	Median/mean (SD)		Within-subject effect
	Pre-therapy levels	Post-therapy levels	Repeated-measures ANOVA F test *p* values
	$N = 41$	$N = 41$	Time
1			
Arg (M)	161.0/166.908 (35.73)	122.0/131.408 (31.71)	7.20×10^{-6}
Ile (M)	91.0/92.704 (18.8)	88.0/87.009 (17.5)	0.0202
Leu (M)	186.0/188.4 (41.19)	174.0/171.805 (36.52)	0.0097
Val (M)	231.0/228.601 (33.4)	238.0/246.1 (37.1)	0.0327
Met (M)	30.4/31.904 (7.39)	26.9/28.0 (5.54)	0.0019
Serotonin/Trp	0.010/0.007 (0.0042)	0.010/0.0085 (0.0047)	0.0394
2			
Asp (M)	19.20/21.29 (13.63)	14.50/15.92 (8.22)	0.0283
ADMA (M)	0.50/0.49 (0.12)	0.40/0.43 (0.12)	0.0048
Kynurenine (M)	2.30/2.39 (0.86)	2.50/2.72 (1.02)	0.0434
Sarcosine (M)	4.10/12.45 (10.58)	3.40/11.06 (9.59)	0.0214
Taurine (M)	57.7/64.506 (22.72)	65.2/76.801 (33.81)	0.0286
Tyr/Phe	0.66/0.69 (0.21)	0.81/0.84 (0.26)	0.0020

Only metabolites with p values <0.05 are listed

ANOVA analysis of variance, *SD* standard deviation, *Arg* arginine, *Ile* isoleucine, *Val* valine, *Met* methionine, *Trp* tryptophan, *Asp* asparagine, *ADMA* asymmetric dimethylarginine, *Tyr/Phe* tyrosine/phenylalanine

increased in patients after completion of interferon-based therapy regardless of the therapeutic response (Saito et al. 2013), the increases in the Kyn level and Kyn/Trp ratio may be short-term phenomena subsequent to interferon therapy, and follow-up for longer than 24 weeks may be warranted to observe the precise long-term Kyn homeostasis in CHC patients with SVR. Furthermore, due to the immune escape characteristics of IDO, close monitoring for the development of cancer is still required for CHC patients, even after viral clearance. The increased IDO level might also account for the failure to eradicate some types of HCC and other types of cancer after SVR (Toyoda et al. 2015). In addition, mammalian Phe hydroxylase catalyzes the rate-limiting step in Phe catabolism and is primarily present in the liver (Flydal and Martinez 2013). A high Phe level together with impaired conversion of Phe to Tyr has been observed in inflammatory conditions. The Tyr/Phe ratio, an indicator of Phe hydroxylase activity, has been shown to be inversely associated with the HIV load and positively associated with the $CD4^+$ T-cell count (Zangerle et al. 2010). In this study, an increased Tyr/Phe ratio was found to indicate attenuated inflammation in the CHC patients after SVR.

Circulating BCAA levels tend to increase in individuals with obesity in association with future insulin resistance or type 2 diabetes mellitus (Lynch and Adams 2014). The decreased levels of the BCAAs Leu and Ile in the G1 CHC patients after SVR, thus, indicated an attenuated risk of insulin resistance. However, among the three BCAAs, the level of Val was increased rather than decreased in the G1 patients after SVR. Val has been shown to promote maturation of monocyte-derived dendritic cells in patients with HCV-related liver cirrhosis. In addition, a nutritional Val deficiency causes the accumulation of hepatic lipid droplets (Kawaguchi et al. 2012). Val, thus, may suppress HCV replication by modulating both immune and nutrient functions (Kawaguchi et al. 2012). As the G1 CHC patients had a significantly higher pre-therapy viral load than the G2 patients, the increased Val level might suggest the elimination of viral-related suppression of Val after viral clearance, which was particularly observed among the G1 patients. In addition to Kyn, Trp is metabolized into several downstream physiologically active substances, including serotonin. Different stressors can selectively induce the expression of genes involved in Trp, Tyr or Phe biosynthesis (Boulet et al. 2015). After SVR, the serotonin/Trp and Tyr/Phe ratios were increased in the G1 and G2 patients, respectively, suggesting the presence of a significant physiological difference between these two genotypes of HCV infection. The brain–gut axis is a bidirectional system for communication between the central nervous system and the gastrointestinal tract. Serotonin functions as a key neurotransmitter at both terminals of this network (O'Mahony et al. 2015). Thus, the gut microbiota might have an uninvestigated role in the metabolic alterations in G1 patients after SVR (Rooks and Garrett 2016). Moreover, sarcosine

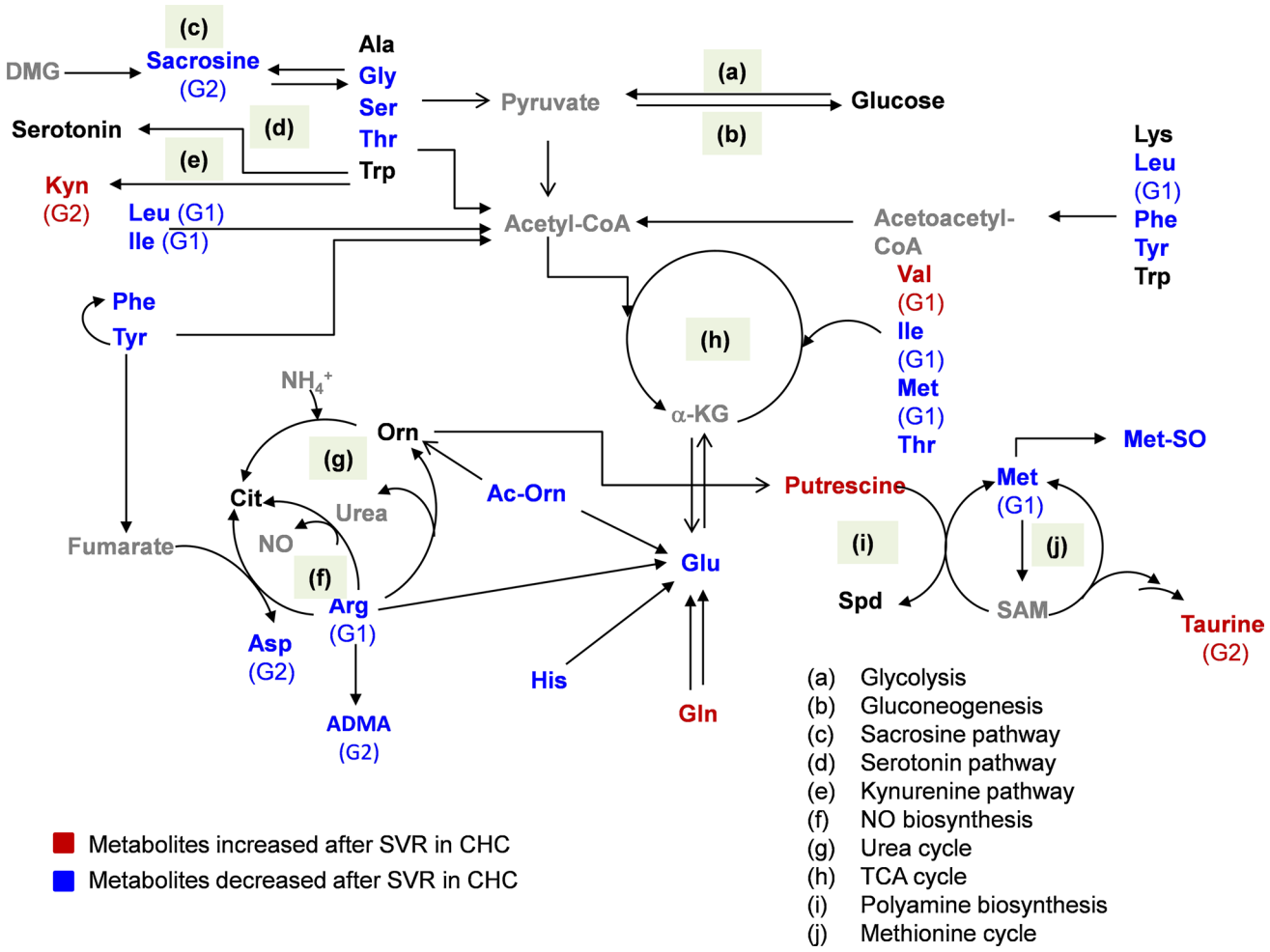


Fig. 3 Proposed schemes to illustrate the pan-genotype and genotype-specific amino acid (AA) and amine alterations in chronic hepatitis C (CHC) after sustained virological response (SVR), as determined by paired metabolomics analyses. A scheme for AA-centered metabolic pathway alterations with incorporation of the tricarboxylic acid (TCA), NO, urea, methionine, polyamine and kynurenine cycles. *DMG* dimethylglycine, *Ala* alanine, *Asp* aspartate, *Gly* glycine, *Ser* serine, *Thr* threonine, *Trp* tryptophan, *Kyn* kynurenine, *Leu* leucine, *Ile* isoleucine, *Acetyl-CoA* acetyl coenzyme A, *Acetoacetyl-CoA* acetoacetyl coenzyme A, *Lys* lysine, *Phe* phenylalanine, *Tyr* tyrosine, *Met* methionine, *Met-SO* methionine sulfoxide, *SAM* S-adenosylmethionine, *Orn* ornithine, *Ac-Orn* acetylornithine, *Cit* citrulline, *Gln* glutamine, *Glu* glutamate, *Arg* arginine, *ADMA* asymmetric dimethylarginine, *His* histidine, *Spd* spermidine. *Words in red* up-regulated metabolites, *words in blue* down-regulated metabolites. *Words in black* unchanged metabolites. *Words in grey* unchecked metabolites. *G1* metabolites frequently alerted in genotype 1 CHC patients, *G2* metabolites frequently alerted in genotype 2 CHC patients

is an intermediate in Gly synthesis. Its level is substantially increased during prostate cancer progression (Sreekumar et al. 2009). A decreased sarcosine level after SVR, which was commonly observed among the G2 patients, might indicate a genotype-specific reduction in the risk of prostate cancer, while an increased Kyn level might suggest an increased risk of cancers other than prostate cancer. Further study must be conducted to elucidate the basis of these associations.

Taken together, these results indicate that after viral clearance, the recovery of pan-genotypic AA alterations in the CHC patients was demonstrated by attenuation of the hypermetabolic status and slowing of the TCA cycle. Associations among the accelerated urea, NO, Met, polyamine cycles following improvement in liver function might attenuate the cardiometabolic risk, but the increased immune tolerance indicated by the augmented Kyn cycle might elicit neoplasm formation. The genotype-specific AA metabolic recovery was reflected in the differing trends of neurotransmitter formation between the G1 and G2 patients and the post-therapeutic increase in the valine level of the G1 patients after SVR. Moreover, with the exception of prostate cancer, the general risk of oncogenesis might be increased particularly in G2 CHC, at least within 24 weeks post-therapy.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

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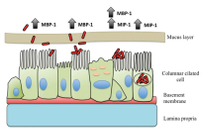

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RESEARCH PAPER

The evolving relationship between adiponectin and insulin sensitivity in hepatitis C patients during viral clearance

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ABSTRACT

Background: The evolution of the relationship between adiponectin and insulin sensitivity in hepatitis C virus (HCV) patients during viral clearance is unclear and warrants investigation. **Methods**: A prospective study including 747 consecutive chronic hepatitis C (CHC) patients, of whom 546 had completed a course of anti-HCV therapy and underwent pre-, peri- and post-therapy surveys for anthropomorphic, viral, metabolic and hepatic profiles and adiponectin levels, was conducted in a tertiary care center. **Results**: Multivariate analyses indicated associations of sex, triglyceride levels and hepatic steatosis with adiponectin levels and of triglyceride levels and interferon λ3 (IFNL3) genotype with homeostasis model assessment-estimated insulin resistance (HOMA-IR) levels before anti-HCV therapy. In patients with a sustained virological response (SVR; n = 455), at 24 weeks post-therapy, sex, BMI, aspartate aminotransferase to platelet ratio index (APRI), HOMA-IR and steatosis were associated with adiponectin levels, and IFNL3 genotype was associated with HOMA-IR levels. GEE analysis demonstrated that SVR affected longitudinal trends in adiponectin levels. Compared with pre-therapy levels, adiponectin and APRI levels decreased 24 weeks post-therapy in SVR patients, regardless of baseline insulin resistance (IR). However, HOMA-IR levels decreased in SVR patients with baseline IR but increased in those without baseline IR. Compared with controls, immunohistochemical studies showed that pre-therapy CHC patients had higher hepatic adiponectin expression associated with hepatic fibrosis. **Conclusions**: During HCV infection, adiponectin may affect insulin sensitivity through triglycerides. After viral clearance, adiponectin levels were directly associated with insulin sensitivity and decreased upon improved hepatic fibrosis; with a link to the IFNL3 genotype, insulin sensitivity improved only in patients with baseline IR.

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adiponectin; HCV; HOMA-IR; insulin sensitivity; triglycerides

Introduction

Hepatitis C virus (HCV), a human pathogen responsible for acute and chronic liver disease, has variants classified into 7 major genotypes and infects an estimated 130–170 million individuals worldwide.[1] HCV causes cardiometabolic alterations including hepatic steatosis, dyslipidemia, insulin resistance (IR), diabetes, obesity and cardiovascular events in addition to liver cirrhosis and hepatocellular carcinoma (HCC).[2-3] Much of the HCV life cycle is closely associated with lipid metabolism in the host.[2-3] Additionally, HCV down-regulates glucose transporters and inhibits insulin receptor substrate function to alter host glucose metabolism.[2] Although most HCV infections are currently curable using potent direct-acting anti-viral agents, not all HCV-associated metabolic and oncogenic complications are reversible after viral clearance,[2,3] especially among those with baseline diabetes and cirrhosis.[4]

As an important endocrine organ, adipose tissue regulates metabolism through adipokines.[5] Adiponectin, a 30-kDa adipokine, is highly expressed in adipocytes and is also expressed in hepatocytes.[6] However, increased visceral adipose tissue stores reduce the abundance of circulating adiponectin.[5] Several IR-associated hormones such as insulin and catecholamines might dysregulate adiponectin expression.[7] Post-translational adiponectin modifications result in the secretion of oligomers of 90-kDa trimers, which are found in the circulation as low molecular weight (LMW) and high molecular weight (HMW) adiponectins. HMW adiponectin is more closely correlated with insulin sensitivity than LMW adiponectin.[8] Adiponectin mediates

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its effects on target cells via at least 2 adiponectin receptors, adiponectin receptor I (AdipoR1) and receptor II (AdipoR2). AdipoR1 is abundantly expressed in skeletal muscle and the liver, whereas AdipoR2 is primarily expressed in the liver.[9] Adiponectin and its receptors might protect hepatocytes from triglyceride accumulation by increasing β-oxidation, decreasing the de novo synthesis of fatty acids, and promoting the uptake and inhibiting the production of glucose in the liver.[10-11] Thus, hepatitis steatosis is usually associated with low levels of adiponectin.[11] In addition, statin-inducted hypolipidemia is associated with hyperadiponectinemia.[12] Moreover, adiponectin has anti-inflammatory, anti-atherosclerotic and anti-apoptotic properties.[13] Paradoxically, circulating adiponectin has been positively correlated with heart failure, coronary artery disease and all-cause mortality.[14-16] Because both HCV infection and adiponectin are critically involved in metabolism, their precise relationship might aid to probe the therapeutic targets for HCV-associated cardiometabolic complications but remains inconclusive. For example, compared with controls, serum adiponectin levels have been reported to be higher,[17-19] lower[20] or not different[21] in chronic hepatitis C (CHC) patients. All studies[17, 22-23] but one[24] failed to correlate HCV viral load with adiponectin levels. Low adiponectin levels in CHC patients have been linked to poor anti-HCV immune and therapeutic responses as well as hepatic steatosis, metabolic syndrome and IR.[20,23,25] However, how adiponectin levels change after a sustained virological response (SVR) following anti-HCV therapy remains conflicting.[22,25-27] Moreover, an inconsistent association between hyperadiponectinemia and HCV-associated fibrosis, HCC and liver-unrelated mortality has been noted.[28-30] The situation regarding intrahepatic adiponectin and its receptors is even more complicated.[31] In addition to various HCV genotypes and the pleiotropic function of adiponectin, these tremendous obscurities are primarily due to individual bias, which is difficult to completely eliminate from case-controlled, retrospective or prospective studies with small sample sizes or with limited adjusting confounders.

Accordingly, we sought to elucidate the impact of HCV infection on adiponectin levels and associated metabolic alterations after adjusting for crucial confounders in a prospective study of CHC patients before, during and after anti-HCV therapy.

Results

Baseline characteristics

The baseline characteristics of the CHC patients are listed in Table 1. Of 747 patients, 407 (54.5%) and 295 (39.5%) were infected with genotype 1 (G1) and G2 HCV, respectively. The SVR patients had lower levels of HCV RNA and homeostatic model assessment for insulin resistance (HOMA-IR) and lower rates of G1 HCV infection and cirrhosis but higher rates of G2 infection and the interferon λ3 (IFNL3) CC genotype than non-

Table 1. Baseline characteristics of all the enrolled chronic hepatitis C patients.

	Total, n = 747 (treated and untreated)	SVR (+), n = 455	SVR (−), n = 91	*p* values
Male, n (%) [#]	404 (54.1)	257 (57.8)	47 (52)	0.394
Age (yr)	55.04+/−12.08	53.04+/−12.93	57.5+/−12.47	0.160
BMI	24.93+/−3.81	24.79+/−3.68	25.84+/−4.28	0.057
HCV RNA (Log_{10} IU/ml)	5.97+/−1.12	5.84+/−1.18	6.46+/−0.74	<0.001[*]
HCV genotype (G), n (%)[#]				
G1	407 (54.5)	214 (47.1)	72 (79.1)	<0.001[*]
G2	295 (39.5)	214 (47.1)	17 (18.7)	<0.001[*]
G3	17 (2.3)	12 (2.6)	0 (0)	
G6	12 (1.6)	6 (1.3)	2 (2.2)	
G1+G2	7 (1.0)	6 (1.3)	0 (0)	
G1+G3	1 (0.1)	1 (0.2)	0 (0)	
Unidentified	8 (1.0)	2 (0.8)	0 (0)	
HOMA-IR	3.24+/−5.33	3.02+/−6.56	5.08+/−8.47	0.043[*]
Hepatic steatosis, n (%)[#]	347 (46.5)	218 (48)	40 (43.75)	0.373
Liver cirrhosis, n (%) [#]	176 (23.5)	100 (22)	40 (44.1)	0.001[*]
ALT (U/L)	93.41+/−100.63	104.7+/−96.0	85.6+/−80.0	0.116
APRI	1.66+/−2.11	1.479+/−1.92	1.758+/−1.986	0.21
Platelets count ($10^3/\mu L$)	176.77+/−65.08	182.2+/−58.5	155.6+/−57.7	0.001[*]
TC (mg/dL)	171.74+/−34.30	168.05+/−32.08	176.29+/−27.90	0.735
TGs (mg/dL)	105.34+/−55.44	98.53+/−44.58	116.71+/−70.32	0.123
Adiponectin (μg/mL)	9.56^3+/−7.09	10.1+/−7.48	8.04+/−5.23	0.097
HMW Adiponectin (μg /mL)	4.74 +/−4.16	6.01+/−4.04	4.85 +/−2.56	0.641
eGFR	90.54+/−36.365	82.75+/−34.89	84.39+/−35.82	0.983
IFNL3-rs12979860				
CC, n (%) [#]	634 (84.9)	392 (88.2)	61 (67)	0.003[*]

Notes. [#]:chi-square test; SVR: sustained virological response; BMI: body mass index; G: genotype; Log: logarithmic;
[*]: $p < 0.05$; G: genotype; HOMA-IR: homeostasis model assessment-estimated insulin resistance; ALT: alanine aminotransferase; APRI: aspartate aminotransferase to platelet ratio index; TC: total cholesterol; TGs: triglycerides; HMW: high-molecular weight; eGFR:estimated glomerular filtration rate; IFNL3: interferon-λ3.

SVR patients. Before anti-HCV therapy, male sex, triglyceride (TG) levels and hepatic steatosis were negatively associated with adiponectin levels (Table 2 and Fig. 1). TG levels and IFNL3 genotype were associated with HOMA-IR levels (Table 2 and Fig. 1). Subgroup analyses showed that the IFNL3 CC genotype [95% confidence interval (CI) of β: −13.53∼−5.24, estimated β:-9.38, $p<0.001$] was the only factor associated with HOMA-IR levels among those with baseline IR (n = 321); whereas body mass index (BMI) (95% CI of β: 0.021∼0.093, estimated β: 0.057, $p = 0.002$), and TG (95% CI of β: 0.001∼0.006, estimated β: 0.004, $p = 0.01$) levels were associated with HOMA-IR levels among those without baseline IR (n = 426). HMW and total adiponectin levels were highly correlated (Pearson's correlation coefficient: 0.903, $p<0.001$).

Factors associated with the longitudinal trend of adiponectin levels

The factors affecting the longitudinal trend in adiponectin levels are listed in Table S1. Sex, hepatic steatosis, SVR, age, BMI, and HOMA-IR, platelet, total cholesterol (TC), TG and estimated glomerular filtration rate (eGFR) levels were associated with the longitudinal trends of adiponectin levels. The effects of categorical variables including sex, hepatic steatosis and SVR on adiponectin levels were further analyzed as shown in Fig. 2. Throughout therapy, (1) male patients had lower adiponectin levels than female patients (Fig. 2A); (2) patients with hepatic steatosis had lower adiponectin levels than patients without hepatic steatosis (Fig. 2B); (3) only SVR patients had a trend of decreased adiponectin levels, whereas the non-SVR patients showed fluctuating adiponectin levels (Fig. 2C).

Factors associated with adiponectin and HOMA-IR levels in SVR patients at 24 weeks post-therapy

Among the SVR patients at 24 weeks post-therapy, male sex, levels of BMI and HOMA-IR, and hepatic steatosis were negatively associated with adiponectin levels, whereas aspartate transaminase to platelet ratio index (APRI) levels were positively associated with adiponectin levels (Table 3 and Fig. 1). For post-therapy HOMA-IR levels, the IFNL3 genotype was the only independent factor. Subgroup analyses showed that the IFNL3 genotype was particularly important for post-therapy HOMA-IR levels among those with baseline IR (95% CI of B: −7.76∼−3.23, estimated B:-5.49, $p < 0.001$).

Table 2. Univariate and multivariate analyses of factors associated with pre-therapy HOMA-IR and adiponectin levels in all 744 enrolled chronic hepatitis C patients.

	HOMA-IR		Adiponectin (μg/mL)	
Variants	Univariate analysis: 95% CI of estimated β (p values)	Multivariate analysis: 95% CI of estimated β [estimated β](p values)	Univariate analysis: 95% CI of estimated β (p values)	Multivariate analysis: 95% CI of estimated β [estimated β](p values)
Sex (Male)	−0.366∼1.168 (0.305)		−6.1∼−2.53 (< 0.001*)	−5.39∼−1.8 [−3.6](< 0.001*)
Age	0.001∼0.063 (0.046*)	−0.061∼0.106 [0.041](0.587)	32.8∼194.7 (0.006*)	−0.008∼0.153 [0.072](0.078)
BMI	0.16∼0.369 (< 0.001*)	−0.251∼0.276 [0.097] (0.923)	−0.71∼−0.23 (< 0.001*)	−0.467∼0.037 [−0.215](0.094)
HCV genotype	−0.75∼1.07(0.14)		−0.69∼0.91 (0.789)	
HCV RNA (Log_{10} IU/ml)	−0.016∼0.733 (0.061)		−0.83∼0.74 (0.801)	
ALT (U/L)	−0.02∼0.006 (0.352)		−0.01∼0.07.1 (0.569)	
APRI	−0.69∼0.313 (0.211)		−0.23∼0.84 (0.264)	
Platelets count ($10^3/\mu$L)	−0.014∼−0.002 (0.015*)	−0.026∼0.015 [−0.005] (0.613)	−0.035∼−0.048 (0.01*)	−0.030∼0.015 [0.01](0.077)
TC (mg/dL)	−0.01∼0.011(0.92)		−0.007∼0.052 (0.138)	
TGs (mg/dL)	0.008∼0.021 (< 0.001*)	0.001∼0.042 [0.022] (0.036*)	−0.056∼−0.019 (< 0.001*)	−0.042∼−0.003 [0.023] (0.021*)
HOMA-IR	NA	NA	−0.33∼−0.018 (0.029*)	−0.279∼0.02 [−0.129] (0.093)
Adiponectin (μg/mL)	−0.242∼−0.015 (0.029*)	−0.234∼0.051 [−0.09] (0.183)	NA	NA
Hepatic steatosis	0.298∼1.702 (0.005*)	−1.66∼3.25 [1.064] (0.337)	−0.53∼−1.63 (< 0.001*)	−4.31∼−0.151 [−0.21](0.035*)
Liver cirrhosis	0.219∼1.889 (0.013*)	−0.11∼6.54 [2.64] (0.065)	−1.9∼2.24 (0.909)	
eGFR	−0.012∼0.006 (0.495)		−0.025∼0.034 (0.758)	
IFNL3-rs12979860 (CC)	−3.28∼−0.609 (0.004*)	−4.82∼−1.06[−2.94] (0.002*)	−2.47∼1.42 (0.723)	

Notes. CI: confidence interval; OR: odds ratio.
*: $p < 0.05$; NA, not accessible; HCV: hepatitis C virus; SVR: sustained virological response; BMI: body mass index; Log: logarithmic; *: $p < 0.05$; HOMA-IR: homeostasis model assessment-estimated insulin resistance; ALT: alanine aminotransferase; APRI: aspartate aminotransferase to platelet ratio index; hsCRP: high sensitivity C- reactive protein; WBC: white blood cells; C3: complement component 3; C4: complement component 4; TC: total cholesterol; TGs: triglycerides; NA: not accessible; eGFR:estimated glomerular filtration rate; IFNL3: interferon-λ3.

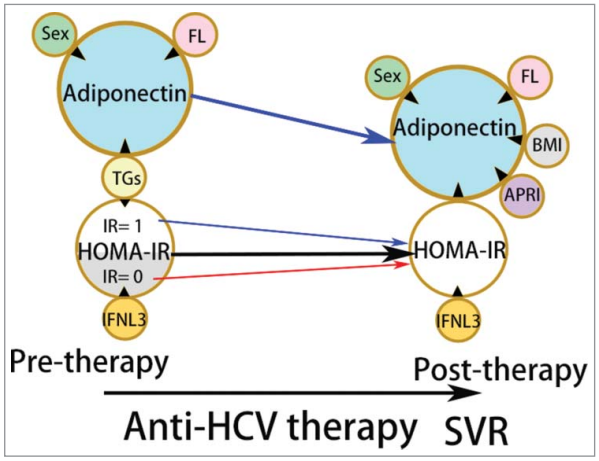

Figure 1. The cross-sectional adiponectin and homeostasis model assessment-estimated insulin resistance (HOMA-IR)-centered associations between dependent and independent factors before (pre-therapy) and 24 weeks after anti-hepatitis (C)virus (anti-HCV) therapy (post-therapy). Tips of black arrowheads: dependent factors; bases of black arrowheads: independent factors; FL: fatty liver, i.e., hepatic steatosis; TGs: triglycerides; IR: insulin resistance; IFNL3: interferon, $\lambda 3$; BMI: body mass index; APRI: aspartate aminotransferase to platelet ratio index; pre-therapy: levels of variables before anti-HCV therapy; SVR: sustained virological response. Red arrows indicate post-therapeutic increases in HOMA-IR (baseline IR = 0) levels, while blue arrows indicate post-therapeutic decreases in HOMA-IR (baseline IR = 1) and adiponectin levels.

Changes in adiponectin and HOMA-IR levels in SVR patients at 24 weeks post-therapy

Compared with pre-therapy levels, paired t-tests demonstrated that APRI (1.479+/−1.92 vs. 0.418+/−0.297, $p < 0.001$) and adiponectin (10.1+/−7.48 vs. 8.13+/−5.92 μg/mL, $p < 0.001$) levels decreased in SVR patients at 24 weeks after therapy, regardless of the HCV genotype (APRI: G1, $p < 0.001$, G2, $p < 001$; adiponectin: G1, $p = 0.001$, G2, $p < 0.001$). None of the aforementioned variables changed significantly in non-SVR patients (Table S2). Interestingly, when we stratified the SVR patients by baseline IR, although adiponectin decreased after SVR regardless of baseline IR (Fig. 2D), HOMA-IR levels decreased in patients with baseline IR but increased in patients without baseline IR (Fig. 2E).

To elucidate the factors that independently affect SVR, the impacts of sex, age, HCV and IFNL3 genotype, BMI, HCV viral load, levels of HOMA-IR, APRI, TGs, TC, adiponectin and eGFR, liver cirrhosis and fatty liver in SVR were surveyed by multivariate analyses. Among the surveyed factors, only BMI [95% CI of odds ratio (OR): 0.763∼0.997], HCV (95% CI of OR: 2.72∼5.31) and IFNL3 (95% CI of OR: 1.29∼7.19) genotypes as well as liver cirrhosis (95% CI of OR: 0.061∼0.742) independently affected SVR.

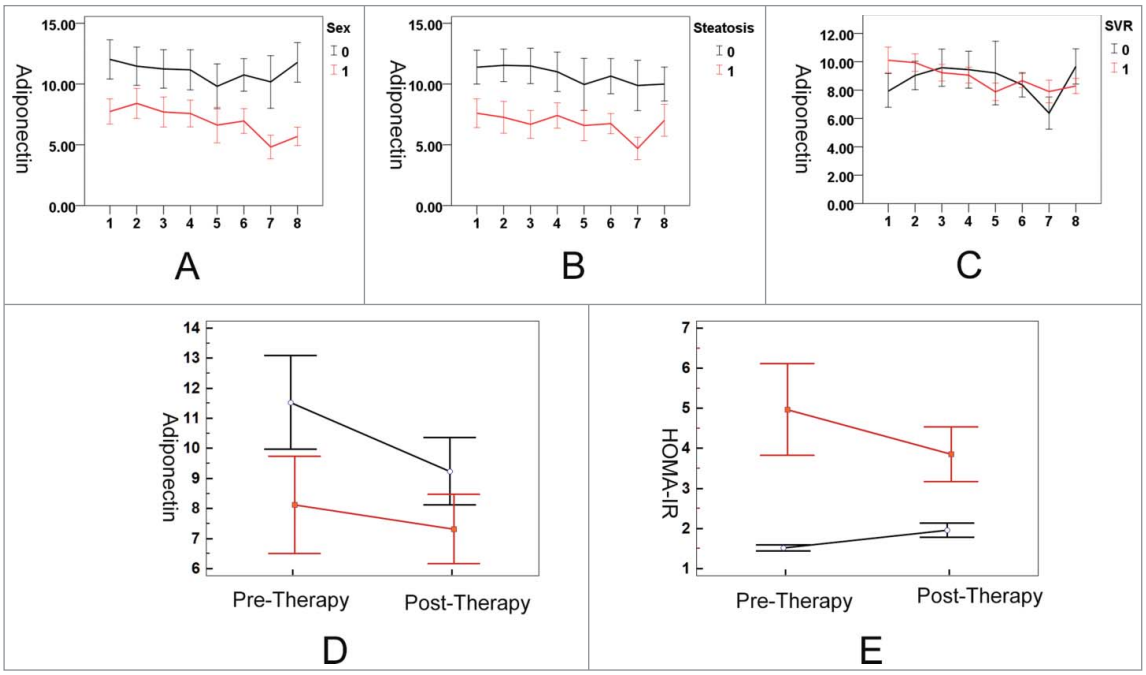

Figure 2. The longitudinal trends of adiponectin levels (μg/mL) and homeostasis model assessment-estimated insulin resistance (HOMA-IR). The trends were stratified by sex (A), steatosis (B) and SVR (C). Blood drawing time points: 1, 2 weeks before therapy; 2, after 4 weeks of therapy; 3, after 12 weeks of therapy; 4, after 24 weeks of therapy; 5, after 36 weeks of therapy; 6, after 48 weeks of therapy; 7, after 60 weeks of therapy; and 8, after 72 weeks of therapy. 1: yes (or male for A); 0: no. (or female for A) D-E, Alterations of levels of adiponectin (μg/mL) (D) and HOMA-IR (E) in SVR patients. Red lines: SVR patients with baseline IR; black lines: SVR patients without baseline IR. Pre-therapy: levels of variables before anti-hepatitis C virus therapy; post-therapy: levels of variables at 24 weeks post-therapy.

Table 3. Univariate and multivariate analyses of factors associated with post-therapy HOMA-IR and adiponectin levels in the 455 chronic hepatitis C patients with SVR.

	HOMA-IR		Adiponectin (μg/mL)	
Variants	Univariate analysis: 95% CI of estimated β (p values)	Multivariate analysis: 95% CI of estimated β [estimated β](p values)	Univariate analysis: 95% CI of estimated β (p values)	Multivariate analysis: 95% CI of estimated β [estimated β](p values)
Sex (Male)	−0.79∼0.403 (0.523)		−6.3∼−3.2 (< 0.001*)	−5.66∼−2.6 [−4.1](< 0.001*)
Age	0.0∼0.050 (0.053)		−0.052∼0.092 (0.584)	
BMI	0.127∼0.302 (< 0.001*)	−0.071∼0.234 [0.081](0.294)	−0.79∼−0.34 (< 0.001*)	−0.54∼−0.096 [−0.28](0.011*)
ALT (U/L)	0.0∼0.042 (0.048*)	−0.034∼0.033 [−0.001](0.969)	−0.10∼0.005 (0.077)	
APRI	−6.0∼20.1 (0.045)		1.73∼5.35 (< 0.001*)	1.40∼4.76 [2.6](< 0.001*)
Platelets count ($10^3/\mu$L)	−009∼0.002 (0.263)		−0.024∼0.005 (0.195)	
TC (mg/dL)	−0.007∼0.01 (0.679)		−0.037∼0.008 (0.215)	
TGs (mg/dL)	0.003∼0.012 (< 0.001*)	−0.07∼0.1 [0.02](0.666)	−0.028∼−0.006 (0.003*)	−0.017∼0.004 [−0.006] (0.269)
HOMA-IR	NA	NA	−0.61∼−0.11 (0.005*)	−0.47∼−0.017 [−0.024] (0.041*)
Adiponectin (μg/mL)	−0.149∼−0.033 (0.002*)	−0.017∼0.019 [−0.049](0.158)	NA	NA
Hepatic steatosis	0.411∼1.648 (0.01*)	−0.475∼0.539[0.532](0.299)	−5.18∼−2.0 (< 0.001*)	−3.7∼−0.66 [−2.43](0.002*)
Liver cirrhosis	−0.227∼1.277 (0.171)		−1.40∼2.64 (0.547)	
eGFR	−0.018∼0.033 (0.188)		−0.020∼0.033 (0.631)	
IFNL3-rs12979860 (CC)	−2.6∼−0.63 (0.001*)	−3.0∼−0.81[−1.92](0.001*)	−2.16∼2.07 (0.966)	

Notes. CI: confidence interval; OR: odds ratio.
*: $p < 0.05$; NA, not accessible; HCV: hepatitis C virus; SVR: sustained virological response; BMI: body mass index; Log: logarithmic; *: $p < 0.05$; HOMA-IR: homeostasis model assessment-estimated insulin resistance;NA: not accessible; ALT: alanine aminotransferase; APRI: aspartate aminotransferase to platelet ratio index; TC: total cholesterol; TGs: triglycerides; eGFR:estimated glomerular filtration rate; IFNL3: interferon-λ3.

CHC patients exhibited higher pre-therapy hepatic adiponectin levels than controls

Before anti-HCV therapy, the CHC patients displayed higher hepatic adiponectin (Fig. 3A) levels than the controls (Fig. 3B) (29.88+/−13.21% vs. 11.02+/−5.64%, $p = 0.011$). In the controls, most adiponectin-positive cells were endothelial cells. By contrast, in CHC patients, both endothelial cells and some hepatocytes were

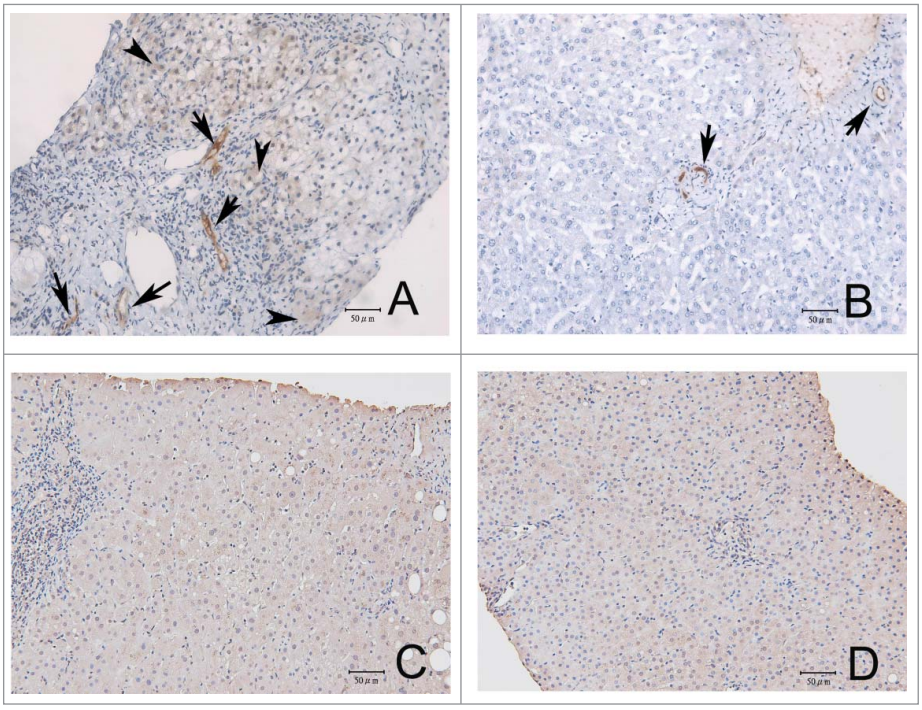

Figure 3. Immunohistochemical studies of adiponectin (A and B, 200X) and adiponectin receptor II (C and D, 200X) in representative liver's sections from chronic hepatitis C patients before anti-hepatitis C virus therapy (A and C) and in controls (B and D). Arrows: adiponectin-positive biliary and endothelial cells; arrow heads: adiponectin-positive hepatocytes.

adiponectin-positive. Adiponectin-positive hepatocytes were surrounded by hepatic inflammation and fibrosis foci. No difference in hepatic AdipoR2 expression (Fig. 3C and D) was noted between the CHC patients and controls (39.28%+/−8.36 vs. 42.65+/−10.12%, $p = 0.898$). Almost all AdipoR2-positive cells were hepatocytes, regardless of HCV infection. No visible hepatic expression of AdipoR1 could be demonstrated regardless of HCV infection (data not shown).

Discussion

To the best of our knowledge, this prospective study is the first to comprehensively analyze the evolving relationship between adiponectin levels and insulin sensitivity in CHC patients during viral clearance. The most compelling results are as follows: (1) Before anti-HCV therapy, male sex, TG levels and hepatic steatosis were negatively associated with adiponectin levels. The IFNL3 genotype and TG levels were associated with HOMA-IR levels. The subgroup analyses showed that the IFNL3 CC genotype was associated with HOMA-IR levels particularly in CHC patients with baseline IR; BMI and TG levels were associated with HOMA-IR, particularly in those without baseline IR. (2) Sex, hepatic steatosis and SVR were independent factors for the longitudinal trend in adiponectin levels. (3) In SVR patients at 24 weeks post-therapy, sex, levels of BMI, APRI, HOMA-IR, and hepatic steatosis were associated with adiponectin levels, whereas the IFN-L3 genotype was associated with HOMA-IR levels, particularly among those with baseline IR. Neither pre-therapy HOMA-IR nor adiponectin levels independently affected SVR. (4) Compared with pre-therapy levels, adiponectin and APRI levels decreased in SVR patients 24 weeks after therapy, regardless of viral genotype. The subgroup analyses showed that HOMA-IR levels decreased in SVR patients with baseline IR but increased in those without baseline IR. (5) Compared with controls, CHC patients exhibited significantly higher pre-therapy hepatic adiponectin expression levels, which were associated with hepatic fibrosis.

Hypoadiponectinemia was reported to be a positive predictor for SVR in G4 HCV infection,[32] but whether pre-therapy HOMA-IR levels determine SVR remains controversial.[33-34] In the current study, neither HOMA-IR nor adiponectin levels independently affected the therapeutic response in patients mainly infected with G1 or G2 HCV. Differences in HCV genotypes and baseline glucose metabolism among the patients in these studies might account for these discrepancies. By contrast, all factors associated with pre-therapy adiponectin levels in the current study have been consistently reported, regardless of HCV infection.[2, 5, 11] Moreover, the negative effects of male sex and hepatic steatosis on adiponectin levels were constant and existed during pre-, peri- and post-therapy. These effects seem to be independent of HCV infection. In addition, before anti-HCV therapy, adiponectin levels were not associated with HCV RNA levels, consistent with the results of most studies.[2, 17, 22-23] Combined, these results indicate that the impact of HCV infection on adiponectin levels, if any, does not occur directly through viral RNA modulation but through alterations subsequent to HCV infection, probably in metabolic or hepatic aspects.[2] The high correlation noted between HMW adiponectin and adiponectin (total) confirmed the representativeness of adiponectin in surveying insulin sensitivity.[8] Although adiponectin is regarded as an insulin-sensitizing adipokine that metabolically mimics insulin,[10] the connection between pre-therapy adiponectin and HOMA-IR levels was not a direct association but rather was mediated through TGs (Fig. 1). Consistently, a previous study of untreated CHC patients showed that HCV-associated IR is likely an adipokine-independent effect.[21] Furthermore, the association between HCV clearance and IR improvement was considered independent of adiponectin levels in CHC patients.[35] Adiponectin regulates the hepatic secretion of very low density lipoprotein,[36] which accounts for 85% of TGs and is secreted from the liver to carry TGs into the blood stream. Thus, TGs seem to be a feasible linker between adiponectin and insulin sensitivity in the CHC patients.

Of note, SVR patients showed a gradual decrease in adiponectin throughout therapy, with a significant decrease 24 weeks post-therapy compared with pre-therapy levels, regardless of viral genotype. Whether viral clearance leads to increased or decreased adiponectin levels in CHC patients remains unclear and may differ among various HCV genotypes.[2, 22, 25-27] This lack of clarity may arise from the heterogeneous hepatic pathologies and metabolic conditions of these patients, as fibrosis and steatosis are associated with hyperadiponectinemia and hypoadiponectinemia, respectively.[18, 37] Thus, after SVR, the decrease in adiponectin in G4 CHC patients[22, 26] might reflect the reversal of hepatic fibrosis, whereas the increase in adiponectin in G3 CHC patients[25] might indicate an improvement in hepatic steatosis, which is most evident in G3 CHC and regarded as "viral steatosis."[2] By contrast, steatosis appears to be secondary to IR and is regarded as "metabolic steatosis" in G1, G2 or G4 CHC,[2] which may explain why patients in the current study (mainly G1 and G2 patients) had adiponectin alteration patterns similar to those noted in G4 CHC.[22, 26] In addition, adiponectin regulates immune responses in HCV infection.[20] The decreased adiponectin levels after SVR might indicate that eliciting an immune response is unnecessary to expel HCV. After HCV clearance, namely, without viral interference, the levels of adiponectin were

directly and negatively associated with levels of HOMA-IR as reported.[5] Compared with pre-therapy levels, sex, hepatic steatosis and levels of HOMA-IR remained unchanged (Table S2). After being counterbalanced by BMI (Table 3, $\beta = -0.28$), the decreased adiponectin levels seemed to follow decreased levels of APRI (Table 3, $\beta = 2.6$). Concordantly, IHC studies showed higher hepatic adiponectin expression in CHC patients than in controls, and most adiponectin-positive hepatocytes were surrounded by hepatic fibrosis with inflammatory cell infiltration. The major driving force for decreasing adiponectin after SVR was thus directed to, at least partly, attenuated hepatic fibrosis in many aspects. However, the current study did not support the connection between adiponectin alterations and adiponectin resistance,[19] as IHC studies failed to demonstrated a significant difference in hepatic adiponectin receptor expression between CHC patients and the controls. In addition, after SVR, the HCV-associated hypolipidemia was reversed (Table S2).[2] As mentioned previously, hypolipidemia is positively associated with adiponectin levels.[12] Consistent with the link between TGs and adiponectin levels during HCV infection, SVR-associated hyperlipidemia may also contribute to the decreased adiponectin levels after viral clearance, probably through negative transcriptional regulation. Moreover, because of the pleiotropic functions of adiponectin mentioned above,[5-10, 12-15] the alteration pattern of adiponectin might serve as a feasible reference to monitor co-morbidities in CHC patients after SVR.[14-15, 28-30]

A trend toward an inverse correlation between the change in adiponectin and in IR, although not statistically significant, has been noted in CHC patients.[35] Thus, the opposing trends of HOMA-IR levels between those with and without baseline IR after SVR were particularly notable. Because adiponectin enhances insulin sensitivity and counteracts IR in animal studies,[10, 38] the decreased adiponectin after SVR should cause the increase in HOMA-IR levels as observed in patients without baseline IR. By contrast, a comprehensive review failed to demonstrate modulation of adiponectin in human insulin sensitivity.[39] In CHC patients with baseline IR, the concurrent decreases in adiponectin and HOMA-IR levels after viral clearance suggest a paradoxical homeostasis in glucose metabolism. Adiponectin is stably present in plasma with little evidence of being an acutely regulated protein. The major physiologic role of adiponectin is to adapt to long-term metabolic dysregulation,[38-39] which might explain why adiponectin increased after SVR, when metabolism was altered, regardless of baseline IR. However, the baseline IR may be a consequence of HCV infection, which impairs host glucose metabolism.[2] These effects likely explain why viral clearance led to decreased HOMA-IR levels (i.e., improved IR) in CHC patients with baseline IR, even when adiponectin levels decreased. More specifically, pre-therapy anthropometric (i.e., BMI) and metabolic factors (i.e., TGs) were associated with pre-therapy HOMA-IR levels among those without baseline IR. By contrast, among those with baseline IR, the IFNL3 genotype, a genetic factor associated with anti-HCV therapeutic responses,[2, 40-42] was more important than anthropometric and metabolic factors. This finding indicates that the physiologic regulation between adiponectin and insulin sensitivity was primarily preserved in patients without baseline IR as determined by the associations among the anthropometric and metabolic factors, which are crucial for homeostasis. Whether IR is associated with the IFNL3 genotype in CHC patients remains unclear.[40] The results of the current study indicate that the impact of the IFNL3 genotype on HOMA-IR levels would not be evident unless analyzed in a large-scale study including many patients with baseline IR or even diabetes.

Because adipose tissue is the major source of the main adipocytokines,[5-8] one of the major limitations of this study is the lack of pathological study of adipose tissue, which is the origin of adiponectin.[5-8] Second, the precise role of hepatic fibrosis in altered adiponectin levels may not be revealed without comparing quantitative measurements of hepatic fibrosis before and after anti-HCV therapy. Third, the potential role of transcriptional regulation of adiponectin by the altered metabolism after HCV clearance, particular hyperlipidemia,[2, 12] could not be evaluated in the current study. Fourth, although our previous study precluded a pro-diabetic function of resistin in CHC,[1] adipokines other than adiponectin or resistin may play roles in the paradoxical glucose metabolism of CHC patients with baseline IR. For example, the HCV core protein increases reactive oxygen species, which activate nuclear factor kappa-light-chain-enhancer of activated B cells,[43] subsequently increasing cytokines including tumor necrosis factor α (TNFα). TNFα modulates adipocytes and induces a decrease in the production of adiponectin and its receptor[44] but is not investigated in the current study. Future studies of adiponectins in CHC patients with adipose tissue pathology surveys, quantitative scoring of hepatic fibrosis, in vitro studies of the transcriptional regulation of adiponectin levels and comprehensive assessment of adipokine profiles may be required to confirm our findings and elucidate the associated molecular basis.

Taken together, sex and hepatic steatosis consistently affected adiponectin levels, regardless of HCV infection. During HCV infection, adiponectin might indirectly affect insulin sensitivity through TGs. After SVR, adiponectin levels were directly associated with insulin sensitivity and decreased, likely subsequent to attenuated hepatic fibrosis. However, the HOMA-IR levels increased in patients without baseline IR but decreased in patients

with baseline IR, which was associated with the IFNL3 genotype. This alteration pattern of adiponectin levels may serve as a feasible reference to monitor associated co-morbidities in CHC patients after SVR. Moreover, the observation of the paradoxical alterations of adiponectin and insulin sensitivity in SVR patients with baseline IR might provide a basis for the investigation of therapeutic targets for HCV-associated cardiometabolic complications, especially irreversible ones.

Patients and methods/materials

Patients

The study group comprised subjects 18 y or older with CHC, defined as the presence of documented HCV antibodies and detectable HCV RNA for >24 weeks. Subjects with human immunodeficiency virus, hepatitis B infection, hemochromatosis, primary biliary cholangitis, primary sclerosing cholangitis, autoimmune hepatitis or malignancy and recipients of solid organ transplants were excluded.

Methods/materials

A total of 747 patients with CHC were recruited consecutively at a tertiary referral center, Chang Gung Memorial Hospital, Taoyuan, Taiwan, between July 2010 and October 2015. Of these patients, 546 received anti-HCV therapy with weight-based pegylated interferon-α-2b and ribavirin for either 24 or 48 weeks.[1, 35-37] HCV RNA levels, genotypes, and single-nucleotide polymorphisms (SNPs) of IFNL3 were assessed as described previously.[1, 35-37] For all included 747 patients, several baseline factors were evaluated, including sex, age, body mass index (BMI), HCV RNA and genotype, presence of hepatic steatosis and cirrhosis, eGFR, APRI, TC, TGs, *HOMA-IR* [fasting insulin (μU/mL) $\times$ fasting glucose (mmol/L)/22.5], alanine aminotransferase (ALT), adiponectin (i.e., total adiponectin) and HMW adiponectin (R&D Systems, MN, USA) levels. For the 546 patients who completed anti-HCV therapy, the aforementioned factors were evaluated 2 weeks before therapy; after 4, 12 and 24 weeks of therapy; at the end of therapy; and 12 and 24 weeks after the end of therapy. Abdominal ultrasound studies were performed to assess the presence of hepatic steatosis and cirrhosis. IR was defined as an HOMA-IR score $\geq$ 2.5. An SVR was defined as undetectable levels of HCV RNA 24 weeks after the completion of therapy.

Liver biopsy was performed in CHC patients before anti-HCV therapy (n = 20). Control liver samples were acquired from the livers of sex- and age-matched participants taken from the tissue bank of the hospital (n = 20). IHC studies of adiponectin (Novus Biologicals), AdipoR1 (Enzo Life Sciences, NY, USA) and AdipoR2 (Phoenix Pharmaceuticals, CA, USA) were performed using paraffinized liver samples according to the manufacturers' protocols. Protein expression intensity was determined as described previously.[1]

Statistics

All statistical analyses were performed using Statistical Package for Social Science (SPSS package version 21, SPSS Inc., Chicago, IL, USA) software. Univariate and multivariate linear regression models were used to assess relationships between various dependent and independent variables. Generalized estimating equation (GEE) repeated measures tests were applied to determine the relationships between the dependent and independent variable levels longitudinally. Paired t-tests were used to compare variables before and at 24 weeks after anti-HCV therapy within individuals. Statistical significance was defined at the 5% level based on 2-tailed tests of the null hypothesis.

Informed consent

Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the local institutional review board.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Notes on contributors

MLC designed and completed the study, drafted the article and critically revised it for intellectual content. CJK collected and analyzed the data and wrote the manuscript. LHP interpreted the data and wrote the manuscript. CMH and CTC collected and analyzed the data and wrote the manuscript. All authors approved the final version of the article, including the authorship list.

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105年度專題研究計畫成果彙整表

計畫主持人：張明鈴	**計畫編號：**105-2629-B-182-001-
計畫名稱：探索性別對三大本土慢性肝病的影響: 一基於人與動物模式的聯合研究	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			3	篇	Three papers entitled " "Association between Leptin and Complement in Hepatitis C Patients with Viral Clearance: Homeostasis of Metabolism and Immunity" and "Recovery of pan-genotypic and genotype-specific amino acid alterations in chronic hepatitis C after viral clearance: transition at the crossroad of metabolism and immunity." and "The evolving relationship between adiponectin and insulin sensitivity in hepatitis C patients during viral clearance" supported by the current grant had been published.
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	

		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
參與計畫人力	本國籍	大專生			0	人次	
		碩士生			0		
		博士生			0		
		博士後研究員			0		
		專任助理			1		學士級專任助理:陳儀芳
	非本國籍	大專生			0		
		碩士生			0		
		博士生			0		
		博士後研究員			0		
		專任助理			0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）		無					

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：■已發表　□未發表之文稿　□撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   Based on the current grant, we had published three papers regarding the roles of adiponectin and leptin and the amino acid levels alteration in the viral clearance of HCV.

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：□否　□是
   說明：（以150字為限）